UAB
Universitat Autònoma de Barcelona

crag CENTRE DE RECERCA EN AGRIGENÒMICA
CSIC IRTA UAB UB

Calcium-dependent protein kinases in the stress signaling cascades of rice plants

Mireia Bundó Barberà
Barcelona, 2015

Universitat Autònoma de Barcelona

CRAG CENTRE DE RECERCA EN AGRIGENÒMICA
CSIC IRTA UAB

UNIVERSITAT AUTÒNOMA DE BARCELONA

Facultat de Biociències

Dept. Biologia Animal, Biologia Vegetal i Ecologia

Calcium-dependent protein kinases in the stress signaling cascades of rice plants

Mireia Bundó Barberà

Barcelona, September 2015

UNIVERSITAT AUTÒNOMA DE BARCELONA
Facultat de Biociències
Dept. Biologia Animal, Biologia Vegetal i Ecologia
Doctorat en Biologia i Biotecnologia Vegetal

PhD thesis

Calcium-dependent protein kinases in the stress signaling cascades of rice plants

Dissertation presented by Mireia Bundó Barberà for the degree of Doctor in Biology and Plant Biotechnology by Universitat Autònoma de Barcelona.

This work was performed in Centre for Research in Agricultural Genomics (CRAG), Cerdanyola del Vallès (Barcelona)

Dr. María Coca	**Dr. Mercè Llugany**	**Mireia Bundó Barberà**
Thesis director	Tutor	Author

Barcelona, September 2015

A la meva família

Acknowledgements

Un cop finalitzada la meva tesi, ha arribat el moment d'agrair públicament a aquelles persones que m'han ajudat durant aquest període.

En primer lugar, evidentemente, quiero agradecer a mi directora de tesis, la Dra. María Coca, la confianza depositada en mí desde el primer momento. Gracias María por darme la oportunidad de realizar esta tesis doctoral aún sin tener beca. Sé que para ti ha supuesto un esfuerzo económico y te lo agradezco enormemente. También quiero darte las gracias por todos los conocimientos que he adquirido a tu lado durante estos 5 años y por hacerme querer un poco más a las proteínas! Gracias por tu esfuerzo especialmente este último año. Te deseo lo mejor a partir de ahora y que triunfes en tus nuevos proyectos.

Por supuesto también quiero agradecer a las dos otras "jefas" del grupo de arroz, la Dra. Blanca San Segundo y la Dra. Belén López, sus consejos, lecciones, favores, y experiencia que tanto me han ayudado durante mi doctorado.

Gracias a las doctoras Sonia Campo, Silvia Manrique y Lidia Campos-Soriano, que trabajaron con las CPKs mucho antes de que yo llegara. Sus recursos y conocimientos me han ayudado muchísimo en mi tesis.

També vull donar les gràcies a totes aquelles persones que formen part dels serveis del CRAG que m'han facilitat i ajudat en la realització dels meus experiments. Gràcies al servei d'hivernacles, sense la vostra feina diària el centre de plantes s'enfonsaria! Vull agrair especialment a la Pilar la seva comprensió amb els arrossaires i les nostres converses de descans a peu de CRAG. I a la Mina, por tu cariño desmesurado cada día! Tus abrazos son mágicos. També vull agrair especialment la paciència de la Montse Amenós, encarregada del servei de Microscòpia, per explicar-me el funcionament dels diferents programes incomptables vegades i per les hores davant del confocal! Gracies al servei de Genòmica, Oriol i Sara, de seqüenciació, Mercè i Mireia, al d'imatges, Àngel, i al de proteòmica, Sami.

Vull donar les gràcies també a altres persones que m'han ajudat en un moment o altre, compartint els seus coneixements. A la Gisela i la Delphine, gràcies per ensenyar-me a transformar arròs. Ha estat un tècnica crucial en la meva etapa al CRAG. Gràcies també a la Dra. Lola Ludevid per estar sempre disposada a ajudar i pels seus consells. També a tots els companys del programa d'estrès per aconsellar-me en els diferents seminaris. No me puedo olvidar del Dr. Jose Luís La Paz. Gracias Jose por las interminables conversaciones científicas y no científicas en las que tanto me has enseñado. Gracias por todos tus consejos, por la paciencia resolviendo problemas de real time y por tu compañía en las horas de coche! Te deseo lo mejor en tu nueva aventura.

Si nos fos pels meus companys de laboratori, el meu doctoral hagués estat una experiència totalment diferent. Us agraeixo a TOTS els que heu passat pel "Lab Rosa" i després pel "Lab 1.06" que m'hagueu fet tan feliç durant aquests anys. Pat, si no fos per

tu jo no hagués arribat a aquest laboratori. Gràcies. Gràcies per això i per haver estat una gran companya durant tota la meva tesi. Sempre volent-me ajudar encara que jo no ho demanés. Ets extremadament generosa amb el teu temps i amb els teus coneixements. Gràcies també per tots el moments que hem passat fora del lab, en aquest continent i en d'altres ☺ Espero i desitjo que triomfis als USA i que siguis feliç fent el que tan t'agrada. Lidi...Gracias en el sentido más amplio de la palabra. Gracias por tu generosidad estos años, me has enseñado tantas cosas, siempre has tenido un momento para sentarte conmigo y aconsejarme, te lo agradezco muchísimo. Pero no solo por eso, gracias por las noches de fiesta y locura que hemos compartido! Eso también se lo agradezco a Ste! Os deseo lo mejor, Sofía tiene suerte de teneros! Perla del Túria! Gràcies a tu també, per les experiències que em passat juntes i per tantes abraçades! Gràcies per fer-te responsable del "Rosa-Lab productions". Espero que aviat acabis la tesi i facis allò que et faci feliç. Aarón, muchas gracias a ti también. Gracias por hacerme REÍR tanto y por escucharme cuando lo he necesitado. En mis mejores anécdotas del lab siempre apareces tú ☺ Suerte! Mi bro! Quién me iba a decir a mí que tendría un hermanito a media tesis!? Pues así fue, y gracias a eso he tenido un gran compañero en el amarillo! Gracias por tu apoyo y tu caballerosidad! Siempre preocupándote por mí...Gracias. A vosotros también os deseo lo mejor. Soni, ya sabes que eres mi gurú! Este grupo no sería lo mismo sin ti, ha sido un placer trabajar contigo, aunque haya sido poco tiempo! He aprendido un montón de cosas a tu lado, gracias. Espero que sigas con tu alegría siempre! A les noves generacions, Marcel, Raquel y Rosany, gracies per renovar els aires del lab! Marcel gracies pel teu sentit de l'humor constant! Et desitjo el millor en aquesta nova etapa que comences! No t'oblidis de l'agüela! Raquel, gracies a tu també per portar bon ambient al lab. Ets una currante, estic segura que t'anirà tot molt bé! Ànims! Gracias Rosany por esos momentos de confusión lingüística que tanto nos hacen reir! Te deseo lo mejor. També vull agrair a altres persones que han passat pel lab, Klem, Pepiño, Izar, Carlos, Mauricio, Giacomo, que m'han ajudat d'una manera o altra durant aquests anys.

Gracias al resto de compañeros del CRAG por compartir conmigo buenos momentos dentro y fuera del laboratorio. Nico y Jorge, gracias por las noches de fiesta y por las tardes en la estación, fueron momentos geniales! Gracies Xiki per tots els moments que hem compartit, tan els bons com els del rincón del lamento! Ares, gracies per ser tan bona companya tant al laboratori com als descansos! I a la meva Bo! Tu també has treballat al CRAG però ja saps que això és insignificant comparat amb els anys d'amistat! Gràcies per estar-hi sempre, ets d'aquelles persones en les que sempre puc confiar.

Vull aprofitar també per agrair a la gent que m'ha format en les pràctiques que vaig fer abans de començar la tesi. Marta i Vanessa gracies per la vostra generositat. Xavi gràcies per tot el que em vas ensenyar de l'arròs, que tan bé m'ha anat durant la tesi. I gràcies també a la resta d'agros oscuros i luminosos per fer-me gaudir tant la meva estada a Oryzon.

Aquesta tesi va dedicada a la meva família. Els la dedico a ells perquè tot i que a nivell científic no poden ajudar-me molt, sense el seu suport no hagués pogut fer res. Gràcies als meus pares per transmetre'm els valors de la responsabilitat, l'esforç i la tenacitat. Aquesta tesi també es vostra. A la Sílvia, el Tete i la Mariló, gràcies pel vostre suport i per creure sempre en mi; i als meus nebots que m'alegren els dies! La família però, té un sentit ampli per mi. Martí, Anna, Aida i Dani, vosaltres també sou la meva família. Us dono les gràcies pel suport i per la paciència que heu tingut cada cop que parlava de la tesi! I com no, gràcies a tu Guiu. Gràcies per cuidar-me, estimar-me, ajudar-me i fer-me feliç. Sense tu no sé com ho hagués fet aquests últims anys. Aquesta tesi també te la dedico a tu.

Index of contents

Summary....................i
Resumen....................iii
Resum....................v
Abbreviations....................vii

GENERAL INTRODUCTION....................11

1. Rice....................3
  1.1- Morphological description....................3
  1.2- Rice cultivation....................3
    1.2.1- Origin and dissemination of rice....................3
    1.2.2-Rice ecosystems....................6
    1.2.3- Importance of rice cultivation....................7
  1.3- Rice culture problems....................9
    1.3.1- Abiotic Factors....................10
    1.3.2- Biotic Factors....................11
  1.4 – Rice as a model plant for monocots....................16
2. Stress signaling in plants....................17
  2.1 - Calcium....................17
  2.2- Reactive oxygen species....................18
    2.2.1- ROS production....................18
    2.2.2-ROS detoxification....................19
  2.3- Protein phosphorylation....................20
    2.3.1-Plant receptor protein kinases....................20
    2.3.1 – Mitogen-activated protein kinases....................21
    2.3.2 – Calcium-dependent protein kinases....................21
3. Defense response against pathogens....................24
  3.1- Innate immunity in plants....................24
  3.2- Molecular mechanisms implicated in the plant defense response....................25

3.2.1- Oxidative burst 25
3.2.2- Hormones involved in the plant defense response 27
3.2.3-Transcriptional regulation 30
3.2.4-Pathogenesis proteins 32
3.3- Systemic Acquired Resistance...32
3.4- Priming 33
4. Drought responses in plants 34
4.1- Plant responses to altered water status 34
4.2- Molecular mechanisms implicated in drought response 35
4.2.1-ROS signaling 35
4.2.2-Hormones 36
4.2.3-Transcription regulation 37
4.2.4- Drought tolerance Proteins 38
4.3- Systemic acquired acclimatation 40
5. OsCPKs in the rice stress signaling network 41

OBJECTIVES..43

CHAPTER I - Characterization of *OsCPK*s gene expression in the rice defense response and in rice varieties 47
Abstract..49
Introduction 50
Results...51
Discussion 56
Experimental Procedures...60
References 62

CHAPTER II - Functional characterization of *OsCPK4* in the rice defense response 65
Abstract..69
Introduction 70
Results...72

Discussion 85
Experimental procedures..89
Acknowledgments..93
References 94

CHAPTER III - Functional characterization of *OsCPK10* in the rice defense response and drought stress...101
Abstract 103
Introduction 104
Results...106
Discussion 119
Experimental procedures...124
References 128

GENERAL DISCUSSION 137

CONCLUSIONS 145

BIBLIOGRAPHY 149

Index of figures

General Introduction

Figure I.1: Images of different parts of rice 4

Figure I.2: Rice domestication 6

Figure I.3: Rice ecosystems 8

Figure I.4: Graphics on rice cultivation and world's population 9

Figure I.5: Rice major diseases 15

Figure I.6: CPK structure and activation 22

Figure I.7: Schematic representations of the plant immune system models 26

Figure I.8: Plant responses to low ψ_w stress 35

Chapter I - Characterization of *OsCPK*s gene expression in the rice defense response and in rice varieties

Figure CI.1: Expression levels of selected *OsCPKs* genes in response to *M.oryzae* elicitor treatment 52

Figure CI.2: Expression levels of selected *OsCPKs* genes in response to *M.oryzae* infection 54

Figure CI.3: Circadian clock regulation of *OsCPK13* and *OsCPK5* gene expression 54

Figure CI.4: Expression levels of *OsCPKs* selected genes in different cultivated rice varieties and wild rice species 56

Chapter II - Functional characterization of *OsCPK4* in the rice defense response

Figure CII.1: *OsCPK4* expression and protein accumulation in response to fungal infection 73

Figure CII.2: OsCPK4 accumulation and activity in transgenic rice leaves 74

Figure CII.3: *OsCPK4* overexpressing plants are more resistant to *Magnaporthe oryzae* infection 76

Figure CII.4: Microscopic analysis of *Magnaporthe oryzae* infection process on rice leaves 77

Figure CII.5: *OsCPK4* overexpressing plants are not more resistant to *Fusarium verticillioides* and *Erwinia chrysanthemi* 79

Figure CII.6: Rapid and strong ROS formation in *OsCPK4* overexpressing leaves during defense response 80

Figure CII.7: Callose deposition in *OsCPK4* overexpressing rice leaves 81

Figure CII.8: Defense gene expression in *OsCPK4* overexpressor plants in response to *Magnaporthe oryzae* infection 82

Figure CII.9: Increased content of total SA, free SA and glucoside conjugate (SAG) in *OsCPK4* overexpressor plants 83

Figure CII.10: Plant performance of *OsCPK4*-overexpressing rice plants 84

Figure CII.11: Model for OsCPK4-mediated defense responses 86

Chapter III - Functional characterization of *OsCPK10* in the rice defense response and drought stress

---

Figure CIII.1: Expression of *OsCPK10* gene in response to biotic and abiotic stress rice plants 107

Figure CIII.2: Plasma membrane localization of OsCPK10 111

Figure CIII.3: OsCPK10HA accumulation in transgenic rice plants 112

Figure CIII.4: OsCPK10HA accumulation in T_0 transgenic plants 113

Figure CIII.5: Performance of *OsCPK10HA* rice plants 114

Figure CIII.6: Resistance of *OsCPK10HA* plants to *Magnaporthe oryzae* infection 115

Figure CIII.7: Drought tolerance of *OsCPK10HA* rice plants 117

Figure CIII.8: Higher H_2O_2 detoxifying capacity of *OsCPK10HA* rice plants in response to drought stress 120

Index of tables

General Introduction

Table I.1: PR proteins classification 32

Chapter I - Characterization of *OsCPK*s gene expression in the rice defense response and in rice varieties

Table CI.1: Significative Fold Change values for *OsCPKs* in response to *M. oryzae* elicitor treatment searched in the microarray data of Campo *et al.* 2013 51

Table CI.2: *Oryza* cultivars and species used in this work, accession numbers and geographical region of its cultivation. 57

Table CI.3: Primer sequences of genes used for gene expression analysis. 61

Chapter II - Functional characterization of *OsCPK4* in the rice defense response

Table CII.1: Primer sequences of genes used for gene expression analysis 90

Chapter III - Functional characterization of *OsCPK10* in the rice defense response and drought stress

Table CIII.1: *cis*-related motifs identified in the 1375 bp upstream region of *OsCPK10* gene 108

Table CIII.2: Primer sequences of genes used for gene expression analysis 125

Summary

Calcium-dependent protein kinases (CDPKs or CPKs) are signaling components in many aspects of plant biology, from developmental processes to stress responses. This thesis addresses the identification and functional characterization of the *OsCPK* isoforms mediating the rice defense response to pathogens, as well as their contribution to other signaling pathways. These studies are important to understand the interactions between different stress signaling pathways and the development process in rice plants. A better understanding of these crosstalks among signaling pathways, as well as the identification of the signaling components mediating them, are relevant to develop strategies to improve plant stress tolerance. All these studies apply to rice plants, an economical and social important crop worldwide.

The work of this thesis is divided in three chapters. The **first chapter** focuses on the identification of *OsCPK* genes involved in the defense response to the rice blast fungal pathogen *Magnaporthe oryzae* by global expression analysis. These studies complemented with specific expression profile analyses which identified *OsCPK4* and *OsCPK10* as upregulated genes in response to *M. oryzae* fungal infection, although the *OsCPK5* and *OsCPK13* genes were also responding to fungal elicitors.

The **second chapter** addresses the functional characterization of the *OsCPK4* gene in the rice defense response to the *M. oryzae* pathogen. The overexpression of the *OsCPK4* gene conferred enhanced resistance to *M. oryzae* infection in rice plants by potentiating their defense responses, including the production of reactive oxygen species, callose deposition, and defense gene expression, associated to an increased accumulation of conjugated salicylic acid in leaves without compromising rice yield. Given that *OsCPK4* overexpression was known to confer also salt and drought tolerance in rice, these results demonstrate that OsCPK4 acts as a convergence component that positively modulates both biotic and abiotic signaling pathways in rice.

The functional characterization of the *OsCPK10* gene is presented in the **third chapter**. These studies demonstrate that OsCPK10 is also a positive modulator of both the defense response to *M. oryzae* infection and the drought stress response in rice plants. The constitutive accumulation of OsCPK10 conferred the rice plants with an improved tolerance to oxidative stress by increasing their antioxidant capacity. This improved capacity to scavenge the produced toxic hydrogen peroxide upon desiccation is due in part to an increased accumulation of the Catalase A, which leads to a reduction in lipid peroxidation and preservation of the cellular membrane integrity, and as a result drought stress tolerance.

All together, the results of this thesis identify the OsCPK4 and the OsCPK10 proteins as convergence components that positively modulate both biotic and abiotic signaling pathways, suggesting they are good molecular targets to improve tolerance to different stresses in rice plants.

Resumen

Las proteínas quinasa dependientes de calcio (CDPKs o CPKs) son componentes de señalización presentes en muchos aspectos de la biología de las plantas, desde los procesos de desarrollo hasta las respuestas a estrés. Esta tesis aborda la identificación y la caracterización funcional de las isoformas *OsCPK* que median la respuesta de defensa del arroz frente a patógenos, así como su contribución en otras vías de señalización. Estos estudios son necesarios para entender las interacciones entre distintas vías de señalización de estrés y desarrollo. Un mejor conocimiento de estas interacciones entre vías de señalización, y la identificación de los componentes que los regulan, son importantes para el diseño de estrategias de mejora de la tolerancia a estrés en plantas. Todos estos estudios se aplican a las plantas de arroz, un importante cultivo tanto económico como social a nivel mundial.

El trabajo de esta tesis se divide en tres capítulos. El **primer capítulo** se centra en la identificación de genes *OsCPK* implicados en la respuesta de defensa frente al hongo del quemado del arroz, *Magnaporthe oryzae*, mediante un análisis de expresión global. Estos estudios se complementan con análisis específicos de los perfiles de expresión en los que se identifican a *OsCPK4* y *OsCPK10* como genes inducidos en respuesta a la infección por *M. oryzae*, aunque los genes *OsCPK13* y *OsCPK5* también responden a elicitores fúngicos.

El **segundo capítulo** aborda la caracterización funcional del gen *OsCPK4* en la respuesta de defensa del arroz frente a *M. oryzae*. La sobreexpresión de *OsCPK4* confiere una mayor resistencia a la infección por *M. oryzae* en las plantas de arroz, potenciando sus respuestas de defensa, que incluyen la producción de especies reactivas del oxígeno, deposiciones de callosa, y la expresión de genes de defensa. Todo ello asociado a una mayor acumulación de ácido salicílico conjugado en hojas, sin comprometer el desarrollo de las plantas. Dado que se sabe que la sobrexpresión de *OsCPK4* confiere además tolerancia a la salinidad y a la sequía, estos resultados demuestran que la OsCPK4 actúa como punto de convergencia, modulando positivamente ambas vías de señalización de estrés biótico y abiótico en plantas de arroz.

La caracterización funcional del gen *OsCPK10* se presenta en el **tercer capítulo**. Estos estudios demuestran que *OsCPK10* también es un modulador positivo de la respuesta de defensa frente a la infección por *M. oryzae* y de la respuesta a estrés por sequía en plantas de arroz. La acumulación constitutiva de la proteína OsCPK10 confiere a las plantas de arroz una mejor tolerancia al estrés oxidativo debido a una mayor capacidad antioxidante. Esta mayor capacidad de eliminar el peróxido de hidrógeno tóxico producido durante la desecación es debida en parte a una mayor acumulación de Catalasa A, lo que conlleva la reducción de peroxidación lipídica y la preservación de la integridad de la membrana celular, dando como resultado la tolerancia a estrés por sequía.

En conjunto, los resultados de esta tesis identifican las proteínas OsCPK4 y OsCPK10 como componentes de convergencia que modulan positivamente ambas vías de señalización a estrés biótico y abiótico, sugiriendo que son buenas dianas moleculares para la mejora de la tolerancia a distintos estreses en las plantas de arroz.

Resum

Les proteïnes quinasa dependents de calci (CDPKs o CPKs) són components de senyalització presents en molts aspectes de la biologia de les plantes, des dels processos de desenvolupament fins a les respostes a estrès. Aquesta tesi aborda la identificació i la caracterització funcional de les isoformes *OsCPK* que intervenen en la resposta de defensa de l'arròs contra els patògens, així com la seva contribució a d´altres vies de senyalització. Aquests estudis són necessaris per entendre les interaccions entre diferents vies de senyalització d'estrès i desenvolupament. Un millor coneixement d'aquestes interaccions entre vies de senyalització, i la identificació dels components que les regulen, són importants per al disseny d'estratègies de millora de la tolerància a estrès en plantes. Tots aquests estudis s'apliquen a l'arròs, un important cultiu tant econòmic com social a nivell mundial.

El treball d'aquesta tesi es divideix en tres capítols. El **primer capítol** es centra en la identificació de gens *OsCPK* implicats en la resposta de defensa contra el fong del cremat de l'arròs, *Magnaporthe oryzae*, mitjançant una anàlisi d'expressió global. Aquests estudis es complementen amb anàlisis específiques dels perfils d'expressió en els quals s'identifiquen *OsCPK4* i *OsCPK10* com a gens induïts en resposta a la infecció per *M. oryzae*, encara que els gens *OsCPK13* i *OsCPK5* també responen a elicitors fúngics.

El **segon capítol** aborda la caracterització funcional del gen *OsCPK4* en la resposta de defensa de l'arròs contra *M. oryzae*. La sobreexpressió d´*OsCPK4* confereix una millor resistència a la infecció de *M. oryzae* en les plantes d'arròs potenciant les seves respostes de defensa, que inclouen la producció d'espècies reactives de l'oxigen, deposicions de calosa, i l'expressió de gens de defensa. Totes s'associen a una acumulació superior d'àcid salicílic conjugat en fulles, sense comprometre el desenvolupament de les plantes. Atès que se sap que la sobreexpressió d´O*sCPK4* confereix més tolerància a la salinitat i a la sequera, aquests resultats demostren que la OsCPK4 actua com a punt de convergència modulant positivament ambdues vies de senyalització d'estrès biòtic i abiòtic en plantes d'arròs.

La caracterització funcional del gen *OsCPK10* es presenta en el **tercer capítol**. Aquests estudis demostren que *OsCPK10* també és un modulador positiu de la resposta de defensa contra *M. oryzae* i de la resposta a estrès per sequera en plantes d'arròs. L'acumulació constitutiva de la proteïna OsCPK10 confereix a les plantes d'arròs una millor tolerància a l'estrès oxidatiu causada per un increment de la capacitat antioxidant. Aquest augment de la capacitat d'eliminar el peròxid d'hidrogen tòxic produït durant la dessecació és degut en part a una acumulació més gran de Catalasa A, cosa que comporta la reducció de peroxidació lipídica i la preservació de la integritat de la membrana cel·lular, donant com a resultat la tolerància a estrès per sequera.

En conjunt, els resultats d'aquesta tesi identifiquen les proteïnes OsCPK4 i OsCPK10 com a components de convergència que modulen positivament ambdues vies de

senyalització a estrès biòtic i abiòtic, suggerint que són bones dianes moleculars per a la millora de la tolerància a diferents estressos en les plantes d’arròs.

Abbreviations

ABA	Abscissic acid
ABA-GE	ABA glucosyl ester
ABRE	ABA responsive-element
AP2/ERF	APETALA2/Ethylene responsive factor
APX	Ascorbate peroxidase
bHLH	Basic helix-loop-helix
BR	Brassinoesteroid
BTH	Benzothiadiazole S-methyl ester
CAT	Catalase
CDPK or CPK	Calcium-dependent protein kinase
cv.	cultivar
dpi	Days post-infection
DRE	Dehydratation-responsive element
DREB	Dehydratation-responsive element binding protein
erd	Early responsive to dehydratation
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
GA	Gibberellic acid
GFP	Green fluorescence protein
GPX	Glutathione peroxidase
hpi	Hours post-inoculation
HR	Hypersensitive response
JA	Jasmonates
LEA	Late embryogenesis abundant protein
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MDA	Malondialdehyde
MV	Methyl viologen
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
pv.	pathovar
RBOH	Respiratory burst oxidase homolog
RLK	Receptor-like protein kinase
ROS	Reactive oxygen species
SA	Salicylic acid
SAA	Systemic acquired acclimatation
SAG	Salicylic acid β-glucoside
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
SPS	Sucrose phosphate synthase

TF	Transcription factor
WT	Wild type
Xoo	*Xanthomonas oryzae* pv. *Oryzae*

GENERAL INTRODUCTION

1. Rice

1.1- Morphological description

Rice is a monocotyledonous plant that belongs to the Poaceae family (commonly known as grasses). Like all cereals, rice is a herbaceous plant with a fasciculate root system (Figure I.1A). Its stems are erect, cylindrical, glabrous and hollow, with very marked nodes and internodes. Rice leaves are lanceolate, with parallel nervation, attached to the stem through the sheath and distributed alternately on the stem (Figure I1A).

Every tiller produces a lax panicle inflorescence consisting in a main axis divided into other secondary branches and sometimes tertiary (Figure I.1B-C). The secondary (or tertiary) branches wear the spikelets (Figure I.1D). Every individual spikelet is formed by two very small external glumes and the flowers, which are located along the rachis. The flower is hermaphroditic and consists of two bracts called glumella: a lower and an upper, or lemma and palea, respectively. The flowers contain six stamens and a pistil with feathery stigma (Figure I.1, 1-4).

The grain of rice, as in all cereals, is a caryopsis type fruit. The lemma and palea are the shell. The embryo is at the ventral side of the grain, by the lemma. The remaining part of the grain is occupied by the starchy endosperm which is separated from the embryo by the scutellum. The scutellum is a transfusion tissue which corresponds to the transformed cotyledon. The radicle and the plumule of the embryo are protected by coleorhiza and coleoptile respectively (Figure I.1, 5-8) (Gran Enciclopedia Catalana, www.enciclopedia.cat/EC-GEC-0080754.xml).

1.2- Rice cultivation

1.2.1- Origin and dissemination of rice

The **genus *Oryza*** is believed to come from South and Southeast Asia, when it was part of the great continent Gondwana 100 million years ago. This genus includes 21 wild species, and all of them have 12 chromosomes (Vaughan *et al.*, 2003). The genus is divided into four specific complexes: O. sativa, O. officinalis, O. ridelyi and O. granulata.

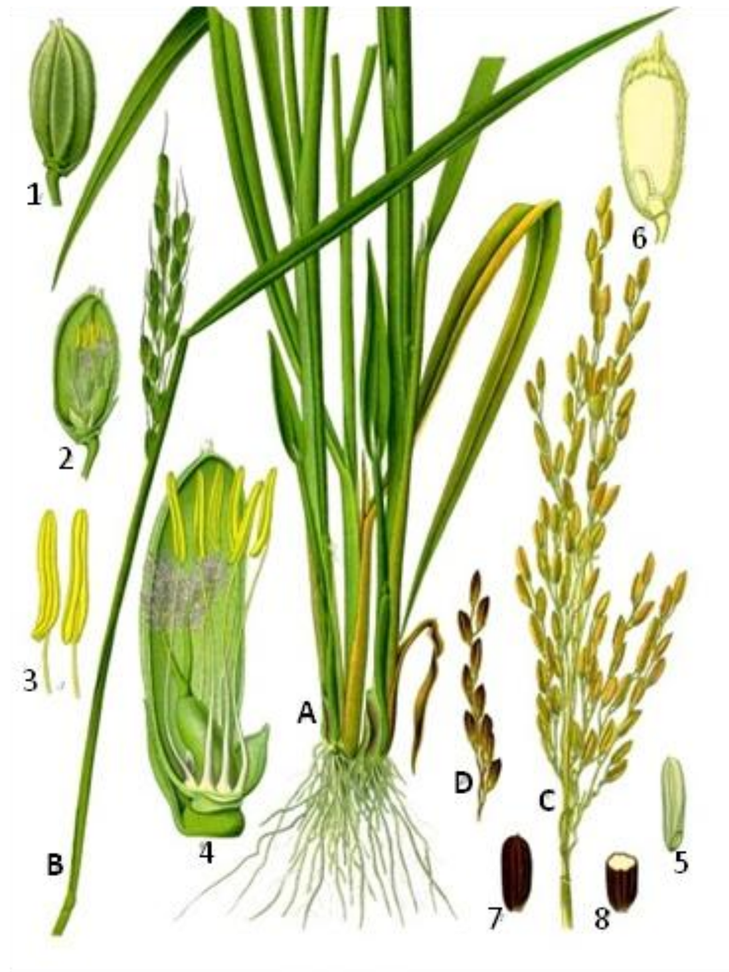

Figure I.1: Images of different parts of rice. A, flowering rice plant, bottom part. B, Flowering panicle. C, panicle grain. D, spikelet grain. 1 is a flower of A; 2 is the longitudinal section of 1; 3 stamens; 4 is a flower increased sharply after removing the lemma; 5 is the endosperm with embryo; 6 represents the seed in longitudinal section; 7 is the fruit of D; 8 is the cross section of 7. A, B, C, and D slightly smaller; 1-7 enlarged. Images obtained from Franz Eugen Köhler, Köhler's Medizinal-Pflanzen, 1897.

The complex *O. sativa* contains two domesticated species, *O. sativa* (from Asia) and *O. glaberrima* (from Africa), and five or six wild species *O. rufipogon*, *O. nivara* (also considered an ecotype of *O. rufipogon*), *O. barthii*, *O. longistaminata*, *O. meridionalis* and *O. glumaepatula*, all of them are diploid species (Figure I.2A).

Archaeological evidence points to the valley of the Yangtze River as a source of rice, and it is estimated that might have started 11,500 years ago. The first domesticated rice plants are believed to have been moved to North Korea and Japan, and Southeast Asia. The valley of the Ganges in India is postulated as another independent Rice domestication site (Kovack *et al*., 2007).

Molecular phylogenetic studies have confirmed that the closest species of *O. sativa* is *O. rufipogon*. The transformation of *O. rufipogon* to *O. sativa* during the domestication is a consequence of specific characters selected by humans (Figure I.2B). Compared to its parental species, cultivated rice has a typical grain flattening, reduced seed dormancy, loss of pigmentation in the seed coat and reduced outcrossing rate. Modern rice varieties also have more secondary branches in the panicle, an increase in the number and weight of grain and a modified response to photoperiod (Sang and Ge, 2007; Kovack *et al*., 2007; Sweeney and McCouch. 2007).

Two genetically distinct subspecies exist in *O. sativa*, known as *indica* and *japonica*. Traditionally, the separation of the two varieties was made based on morphological characters and also taking into account the numerous reproductive barriers between them. Garris *et al.*, in 2005, identified five subpopulations using molecular markers: *indica*, *aus*, *tropical japonica* (also known as *javanica*), *temperate japonica* and *aromatic*. Among them, *indica* and *aus* belong to the *indica* variety, while *tropical japonica*, *temperate japonica* and *aromatic* belong to the japonica.

The genetic diversity center of ***O. glaberrima*** is thought to be the delta of the Niger river in West Africa. Molecular data shows the close genetic relationship with *O. barthii* (Second 1982; Semon *et al*., 2005), so it is postulated as its parental species. Asian rice was introduced in the area of *O. glaberrima* after the initial domestication and now the two species are planted beside one another in West Africa. Recently, breeders have crossed *O. sativa* and *O. glaberrima*, combining the characteristic stress tolerance of *O. glaberrima* with the potential production of *O. sativa* (Jones *et al.*, 1997; Gridley *et al.*, 2002). Known as NERICA (New Rice for Africa), these varieties have become popular among farmers in West Africa.

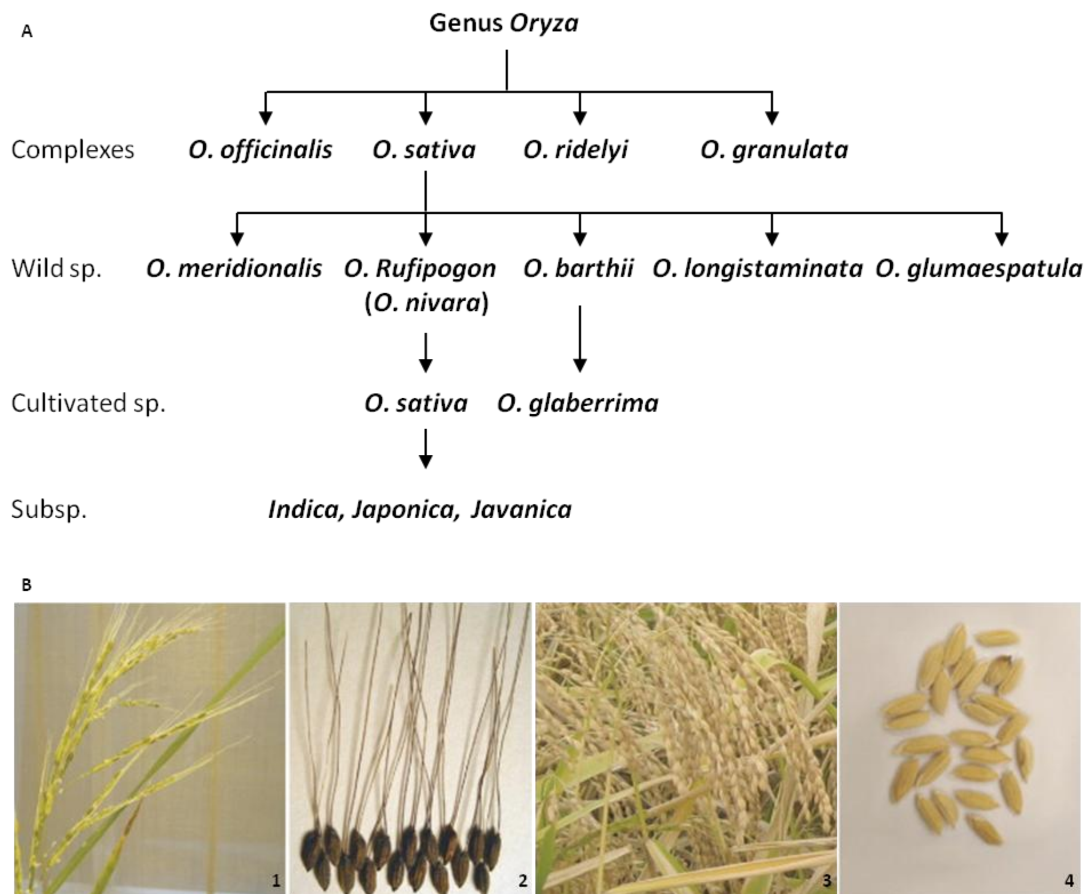

Figure I.2: Rice domestication. A, Genus *Oryza* scheme representation showing the different wild and cultivated species. B, Representative pictures of the principal differences between *O. rufipogon* and *O. sativa*. 1, panicle of *O. rufipogon*. 2, seeds of *O. rufipogon*. 3, *O. sativa* panicles. 4, *O. sativa* seeds. Images from Kovack *et al*., 2007.

1.2.2-Rice ecosystems

Rice is grown in a wide range of environments from tropical to temperate, and from sea level to elevations. Furthermore, rice grows under different water regimes, unsubmerged upland rice, moderately submerged lowland rice (irrigated or rainfed), and submerged rice. Except for the upland case, the others are characterized by wet rice cultivation. Rice is not a water plant in the botanical sense, as it can be seen in its root system, but thrives in waterlogged soils where no other cereal crop survives (Moormann & Van Breemen, 1978).

Irrigated systems (Figure I.3A, B) cover more than half of the world's rice lands and it produces about 75 % of world supply of rice. This growing system is a highly water-demanding production system. The rice rainfed lowland ecosystem is characterized by

its lack of control over water, and therefore it has problems with floods and drought. About a quarter of the world's rice lands are rainfed. The upland rice ecosystem (Figure I.3C, D) varies from low undulating valleys and steep slopes with high runoff and lateral water movement. Less than 13 % of world rice area is upland rice. The remaining are classified as flooded rice paddy ecosystems (almost 8%) (Figure I.3E, F), subjected to uncontrolled flooding, and submerged up to five months, with brackish water intermittent flooding caused by tidal fluctuations. Flooding is not the only problem in these areas, as they may also suffer drought and soil salinity (Halwart and Gupta, 2004).

1.2.3- Importance of rice cultivation

Rice is the staple food for nearly half of the seven billion people in the world. Although it is grown in 113 countries worldwide (Figure I.4A), over 90% is consumed in Asia. In South and Southeast Asia more than 600 million people live in poverty, and rice is their only livelihood form. During the last decade, rice has quickly become the source of food also in sub-Saharan Africa, and as a result, the region has had to increase imports of rice. Rice is the second most-produced cereal in the world, behind maize (Figure I.4B). In the past 40 years, rice consumption per capita has doubled worldwide. On the other hand, in the middle of this century, two billion more people must be fed and it is estimated that they will exceed ten billion at the end of the century. If rice consumption per capita follows the same trend, the total consumption will grow at the rate of population growth (Figure I.4C) (Mohanty, 2013).

Rice farming is the main activity and source of income for millions of households in Asia, Africa and South America. Its culture not only provides 27% of food energy in developing countries but also various kinds of livestock are supported with the byproducts of harvesting. The amount of work and wealth generated by the production, maintenance, harvesting and marketing of rice should not be underestimated, creating millions of jobs (Mohanty, 2013; Solh, 2005).

Though neither a staple food nor a major crop in Europe, rice has an important sociocultural significance and ecological importance in several Mediterranean countries of Europe (http://ricepedia.org/rice-around-the-world/europe).

Figure I.3: Rice ecosystems. Rice rainfed lowland fields in Ninh Binh (Vietnam) (A) and in Mekong river banks (Cambodia) (B). Rice terraces in Longsheng Mountains, Guilin (China) (C) and in the mountains of Sapa (Vietnam) (D). Flooded rice fields in the Mekong Delta (Cambodia) (E) and Ebre delta (Spain) (F).

In the European Union, rice culture occupies approximately 475,000 hectares with a production of 3.2 million tonnes of paddy (1.8 million of white rice). Italy is the first producer with 52 % of the total area. Spain is the second producer in Western Europe with 20% of total area. Regarding production of rice, percentages are 50 and 30%, respectively, due to higher rice agronomic performance in Spain (Fig. I.4D). The whole

Union is in deficit in the production of rice while Italy and Spain have surplus of rice and are the primary exporters within Europe (Ministerio de agricultura, alimentación y medio ambiente, http://www.magrama.gob.es/).

The work of this thesis focuses on rice due to the economic relevance of this crop worldwide, and particularly in Spain.

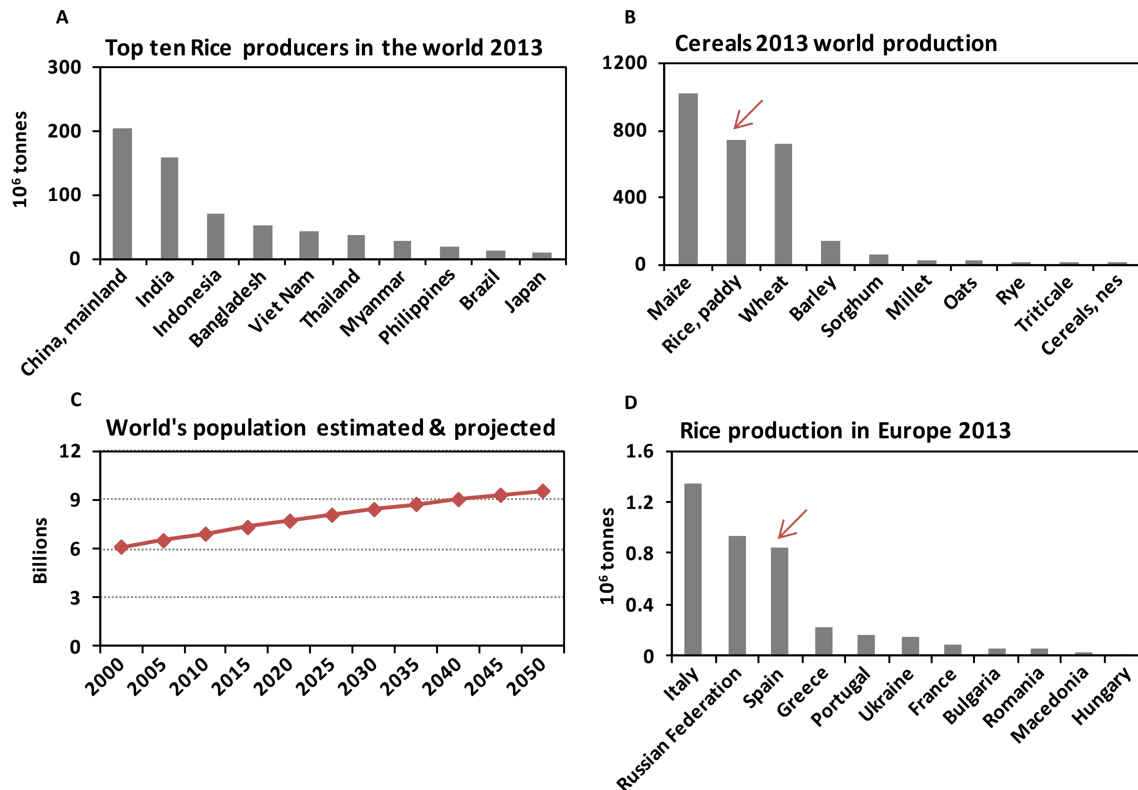

Figure I.4: **Graphics on rice cultivation and world's population**. A, Top ten rice producers in the world in 2013. B, Top ten most produced cereals in the world in 2013. Arrow indicates the second position of rice, behind maize. C, World's population estimated and projected by 2050. D, Rice production in Europe in 2013. Arrow indicates the third position of Spain, behind Italy and the Russian Federation. Data obtained from FAOSTAT (faostat3.fao.org).

1.3- Rice culture problems

Rice cultivation is constantly subjected to adverse environmental situations that negatively affect their development and production. Traditionally they have been classified in **abiotic factors**, those not living and physical components, and **biotic factors**, the living components.

1.3.1- Abiotic Factors

The need to increase rice production due to the growth of the world population, in addition to the expected environmental changes in the coming years (temperature increase, sea level rise, changes in rainfall patterns...) make the study of responses to abiotic stresses really necessary. The major abiotic stresses that affect rice culture worldwide are high salinity, drought and cold (Nguyen, 2005).

Salinity is a major environmental stress and poses a substantial constrain to crop production. High concentrations of NaCl cause disruption of intracellular ion homeostasis, membrane dysfunction and inhibition of metabolic activity. As a result, the seedling growth is affected, the panicle emergence is delayed and the grain yield decreases through reduced pollen viability.

Most affected areas are coastal areas with periodic invasions of the sea water and semi arid or arid lands with ineffective drainage which accumulate salts when irrigation water evaporates. This situation is predicted to worsen with the expected rise of temperature from global warming. Salinity has also increased in different irrigated areas as a result of prolonged rice production, including southern Spain (Aguilar *et al*., 1997; Mackill, 2010; Hasegawa *et al*., 2000).

Drought is one of the major constraints limiting crop production, and most of the popular rice varieties are drought-sensitive (Serraj *et al*., 2011). Rice consumes twice as much water as it takes to grow wheat. In a drought situation, the plant closes its stomata to reduce water loss, which leads to a reduction of CO_2 assimilation. Moreover, the increase in reactive oxygen species (ROS) production causes the oxidative damage of the chloroplasts. Both processes negatively affect photosynthesis and therefore plant growth and productivity (Aroca, 2012).

Drought stress is a growing problem worldwide, affecting 50% of world production of rice every year, and it is expected to increase also with climate change and growing water scarcity (Mackill, 2010).

This expected increase in **temperature** can also affect the growth and development of rice, especially pollination. Moreover, due to its tropical and subtropical origin, rice is

also sensitive to cold stress, more than other cereals, especially when the temperature decrease occurs at the reproductive stage, causing a reduction in yield and grain quality. Cold stress affects chlorophyll content, reducing the photosynthetic activity, and leading to ROS production. Thus, as in salinity and drought stresses, ROS accumulation impairs the metabolism via cellular oxidative damage. (Nguyen and Ferrero 2006; Zhang *et al*., 2014).

1.3.2- Biotic Factors

It is estimated that diseases, insects and weeds are responsible for 37% of annual rice crop loss, according to the IRRI in 2012 (Sparks, Nelson & Castilla, 2012). Therefore, improving rice health can contribute substantially to the decrease of global need for food and poverty. Rice diseases result in yield reductions of 10-15% in tropical Asia, of which 5% or more losses are caused by sheath blight and blast disease (Zeigler and Savary 2010; Gianessi 2014).

Sheath blight

Sheath blight is a fungal disease caused by *Rhizoctonia solani*. It is distributed in temperate, subtropical and tropical countries, in all producing areas. The fungus lives in the soil and floats when fields are flooded.

The pathogen interrupts the water and nutrients flow in the plant, which provokes leaf and young tillers senescence with the subsequent yield reduction. The first symptoms observable are oval or ellipsoidal greenish gray lesions, usually 1-3 cm long, on the leaf sheath, initially just above the soil or water level in the case of conventionally flooded rice. Lesions on the leaves usually have irregular shape, often with gray-white centers and brown margins as they grow older (Figure I.5A).

Rice sheath blight is an increasing concern for rice production. The disease cause yield losses of as high as 50% in USA when susceptible cultivars were planted, of 20% in Japan, or 6% in tropical Asia. It has been difficult to breed varieties with a high genetic resistance to sheath blight, so the disease has to be managed through the use of chemical fungicides (Rice knowledge bank, www.knowledgebank.irri.org; Gianessi, 2014).

Bakanae

Bakanae ("foolish seedling" in Japanese) is an important fungal pathogen of many crops. It is caused by the seedborne fungus *Fusarium* spp. *(Gibberella fujikuroi* species complex). *F. fujikuroi*, *F. proliferatum* and *F. verticillioides* are commonly found associated with bakanae of rice (Ou, 1985; Wulff *et al*., 2010). The infection focus is usually infected seeds but the fungus is also present in the soil and it can be spread from an infected plant thought wind or water.

The pathogen infects plants through the roots or crowns and then grows systemically within the plant. Infected seedlings exhibit abnormal elongation which is attributed to gibberellins (a plant growth hormone) produced by the fungus. The plants become thin, with yellowish green and pale green leaves. Early infection can cause seedlings death at early tillering stage. Later infection results in plants that develop few tillers and have dry leaves. If the plants survive to maturity stage, they develop partially filled grains, sterile, or empty grains. Moreover, the fungus complex is able to produce different micotoxins that can be toxic for animals and humans (Figure I.5B) (Rice knowledge bank, www.knowledgebank.irri.org; Desjardins *et al*., 1997)

Crop losses caused by the disease may reach up to 20% in epidemic cases. The fungus is present both in tropical and temperate areas but, in the last decade, it has become an increasing problem in Europe, especially in Italy (Amatulli *et al*., 2010). Chemical fungicides are used in seeds before planting and also some rice varieties have been breed for bakanae resistance (Ahangar *et al*., 2012; Zheng *et al*., 1993).

Bacterial blight

The bacterial blight is caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) and it is one of the most destructive diseases of rice in Asia. The bacteria affects rice plants in the seedling stage, causing that leaves turn gray and rolled at the beginning, yellow and withered later, and finally dry up and die. When plants are infected at booting stage, bacterial blight does not affect yield but results in poor quality grain and a high proportion of broken kernels (Figure I.5C).

This disease occurs in both temperate and tropical environments and it develops in areas that have weeds and stubbles of infected plants, particularly in irrigated and

rainfed lowland areas. In general, temperatures at 25−34°C and high humidity favors this disease (Rice knowledge bank, www.knowledgebank.irri.org)

It has historically been considered one of the great epidemics. When susceptible varieties in environments that promote bacterial blight are grown, crop losses may reach 70 %. The most efficient strategy to combat this disease is the use of resistant varieties. These varieties normally have an introgression of a *Xoo* resistance gene (*Xa* genes) (Rice knowledge bank, www.knowledgebank.irri.org)

Other bacterial pathogens of rice are *Dickeya zeae* (previously known as *Erwinia chrysanthemi* pv. *zeae*), the causal agent of bacterial foot rot (Pu *et al*., 2012) and *Burkholderia glumae* which causes bacterial panicle blight of rice. *B. glumae* is now considered as an emerging major pathogen of rice (Ham *et al*., 2011).

Blast disease

Blast disease or Piriculariosis is caused by the ascomycete fungi *Magnaporthe oryzae* and it is able to attack all the parts of the rice plant. Rice can have blast in all growth stages, however, leaf blast incidence tends to decrease as plants mature and develop adult plant resistance to the disease. Blast disease is one of the most destructive diseases of rice due to its extensive distribution worldwide and degree of damage under favorable conditions. It occurs in areas with low soil moisture, frequent and prolonged periods of rain shower, and cool temperature in the daytime. In upland rice, large day-night temperature differences that cause dew formation on leaves and overall cooler temperatures favor the development of the disease. Rice varieties resistant to blast frequently lose their resistance within a few years because of strain variability of the fungal population.

Initial leaf symptoms appear as white to gray-green lesions or spots, with dark green borders. Older lesions on the leaves are elliptical or resemble diamond shape and whitish to gray centers with red to brownish or necrotic border. Lesions can enlarge and coalesce, growing together, to kill the entire leave. Infection in the sheath can also kill the entire leaf. The node blast turns the stem blackish and easily breakable. In the panicle, the infected parts manifest with grayish brown or injury and falling panicle in

more severe infections (Figure I.5D) (Rice knowledge bank, www.knowledgebank.irri.org).

Its life cycle consists in a hemibiotrophic infection cycle with a predominantly asexual reproduction mode. The sexual phase exists but not frequently found in infected rice fields. During its asexual cycle, it produces tricellular conidia that spread by falling dew drops to the cuticle of a new rice surface. The adherence of the conidia to the hydrophobic surface of the leaf allows the formation of germ tubes, which culminates in the formation of appressoria. Once formed, it matures accumulating high concentrations of glycerol, as a compatible solute, that generates the appresorium turgidity together with the resistance exercised by the melanin layer between the cell wall and cellular membrane. Turgidity results in mechanical strength, forcing the fungus entrance through leaf cuticle by the penetration peg (Figure I.5E) (Howart and Valent, 1996; Wilson and Talbot, 2009).

Once inside, the fungus hyphae ramify through the plant tissue resulting in typical diamond-shaped lesions. In these lesions, the fungus sporulates in high humidity conditions allowing the disease spreading to neighboring plants. Invasive growth of *M. oryzae* involves a prolonged biotrophic stage in which the fungus grows inside plant cells surrounded by the plasma membrane of the cell invaginated. This early-infection structure has a nutritional function deriving nutrients from the plant cell. After this phase, when lesions appear, the fungus becomes necrotrophic. That is why *M. oryzae* has been classified as a hemibiotrophic pathogen (Figure I.5E) (Wilson and Talbot, 2009).

M. oryzae has emerged as one of the major model organisms for plant-pathogen interactions. The fungus can grow *in vitro* and infection structures can be generated on artificial surfaces like Teflon. Efficient transformation protocols and mutant collections are available. Moreover, the complete sequence of its genome was published in 2005 (Dean *et al*., 2012; Perez - Nadales *et al*., 2014).

Studies of this thesis are directed toward the search for strategies to improve blast disease resistance and drought tolerance, two major objectives of rice breeding.

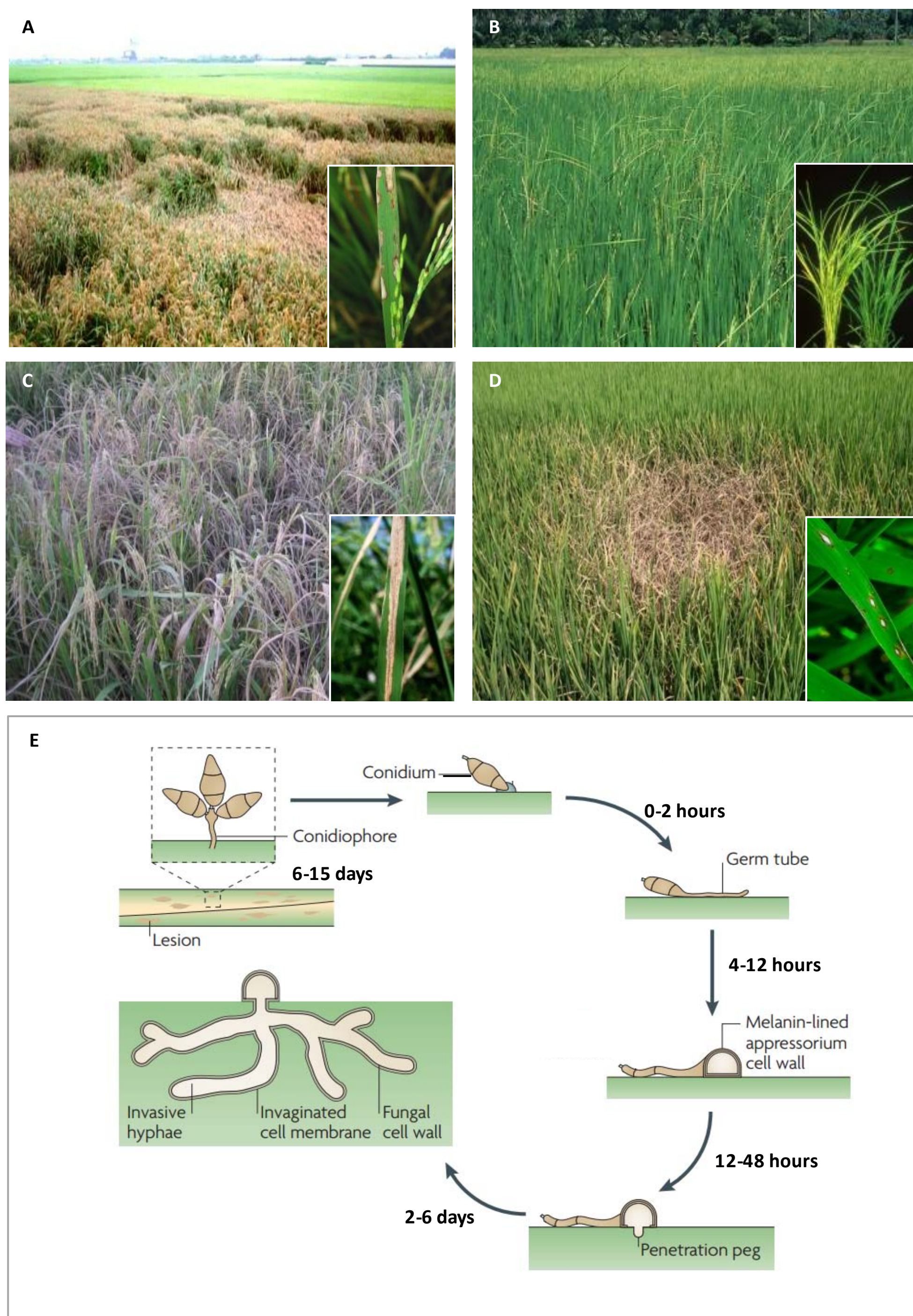

Figure I.5: Rice major diseases. A-D, images of rice fields affected by sheath blight (A), bakanae (B) bacterial blight (C) and blast disease (D). Details of the leaves sympthoms are shown in the corresponding inset boxes. Pictures obtained from Gianesi, 2014 (A), http://visualsunlimited.photoshelter.com/ (B) Rice knowledge management portal (rkmp.co.in) (C), UC Rice blog, California Rice production (ucanr.edu) (D), and Rice knowledge bank (www.knowledgebank.irri.org) (inset boxes). E, Life cycle of the rice blast fungus *Magnaporthe oryzae*. The arrows show the timing of the different phases. Modified from Wilson and Talbot, 2009.

1.4 – Rice as a model plant for monocots

Rice is not only an important food crop but also has become the model species for the study of monocotyledonous plants. Rice has a relatively small genome of about 430 Mbp, which is the smallest among all cereal crops. Sorghum, maize, barley, and wheat have significantly larger genomes of about 1,000, 3,000, 5,000, and 16,000 Mbp, respectively. The small genome size of rice results in higher gene density relative to other cereals (Izawa & Shimamoto, 1996). Also due to the synteny among cereal genomes, rice is used as the base for comparative mapping in cereals

Many different tools have been developed during the last years for rice functional genomics studies. The combined international efforts facilitated the elucidation of the rice genome (japonica and indica subspecies (Goff *et al*., 2002; Yu *et al*., 2002). All the sequences are available at the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu), and the Knowledge-based Oryza Molecular biological Encyclopedia (KOME) (http://cdna01.dna.affrc.go.jp/cDNA). A large expressed sequence tag (EST) database is available at the Rice EST DataBase (REDB) (http://redb.ncpgr.cn/). Transposon and T-DNA-tagged rice collections exist (Taiwan Rice Insertional Mutant database (TRIM), http://trim.sinica.edu.tw; Tos17, http://tos.nias.affrc.go.jp; CIRAD/Genoplante oryza tag lines, (http://oryzatagline.cirad.fr), and the microarray technology for studying mRNA expression profiles is also available (Rice Oligonucleotide Array Database, www.ricearray.org; RiceXPro, ricexpro.dna.affrc.go.jp; The Bio-Analytic Resource for Plant Biology, http://bar.utoronto.ca/welcome.htm). Rice transformation protocols are available, being relatively easy the production of transgenic rice plants as compared with other major cereals (Shimamoto, 2002).

Many of these tools have been used for the experimental work carried out in this thesis.

2. Stress signaling in plants

Plants are sessile organisms that cannot escape from adverse environmental conditions. The fast adaptation to these environmental changes is essential to successfully complete their life cycle. Plants perceive the external stress, get stimulated and then generate the appropriate cellular responses leading to the plant adaptive mechanism. These cellular responses work by transmitting the stimuli from the sensors, located on the cell surface or the cytoplasm, to the transcriptional machinery with the help of various signal transduction pathways. The signaling pathways are the indispensible links between perception of the stress and the generation of the appropriate physiological and biochemical response.

2.1 - Calcium

Signals perceived by cells are transmitted by secondary messengers, such as Ca^{2+} ions, cyclic nucleotide monophosphates, inositol polyphosphates, nitric oxide, and other small molecules. During the last three decades, numerous studies have shown that Ca^{2+} is the main messenger in plants compared to any other known messenger. Nearly all plant signals (developmental, hormonal, and stresses) induce changes in intracellular Ca^{2+} levels, primarily in the cytosol but also in the nucleus and other organelles (Reddy *et al*., 2011).

At higher concentrations, Ca^{2+} can chelate negatively charged molecules in the cell, and hence can cause toxicity. Plants have developed elaborated mechanisms to maintain a low Ca^{2+} concentration in the cytosol, including Ca^{2+} channels, pumps and exchangers. They maintain Ca^{2+} homeostasis, and also allow the generation of rapid signal-specific changes in cellular Ca^{2+} in response to different stimulus (Reddy *et al*., 2011; Chinnusamy *et al*., 2004).

Plant response to signals is encoded by different Ca^{2+} signatures (magnitude, duration, transient or multiple peaks). Different calcium sensors are able to decode the calcium fluctuations and trigger specific cellular responses. Calmodulins (CaMs), calmodulin-like proteins (CaMLs), calcineurin B-like proteins (CBLs), and calcium-dependent protein kinases (CDPKs or CPKs) suffer conformational changes upon binding Ca^{2+},

activating their respective Ca^{2+} responders, CaM-dependent protein kinases (CaMKs), Ca^{2+} and CaM-dependent protein kinases (CCaMKs) and CBL-interacting protein kinases (CIPKs) which phosphorylate specific downstream proteins (Gao *et al*., 2014; Boudsocq and Sheen, 2013; Valmonte *et al*., 2014; Romeis and Herde, 2014). These Ca^{2+} sensors are encoded by complex gene families and form intricate signaling networks in plants that enable specific, robust and flexible information processing (Dodd *et al*., 2010; Batistic and Kudla 2012).

2.2- Reactive oxygen species

Reactive oxygen species act as major signaling molecules in diverse processes in plants, and its production is triggered during both biotic and abiotic stresses (Choudhury *et al*., 2013). Spatial and temporal fluctuations of ROS levels are interpreted as signals required for growth, development, tolerance to abiotic stress factors, and response to pathogens or cell death.

ROS are perceived by the cell through three different mechanisms: unidentified receptor proteins, redox-sensitive transcription factors (such as AtNPR1 or heat shock factors) and direct inhibition of phosphatases by ROS (Barna *et al*., 2012).

2.2.1- ROS production

ROS result from excitation or incomplete reduction of molecular oxygen, being the toxic by-products of normal cellular metabolism in aerobic organisms (Sharma *et al*., 2012). Plants use molecular dioxygen as a terminal electron acceptor, producing highly reactive ROS. The first ROS formed in the O_2 reduction is the superoxide ($O_2^{\cdot-}$) or hydroperoxide ($HO_2^{\cdot}$) radicals. The second step is the H_2O_2 formation, which is a relatively stable molecule. H_2O_2 can migrate quite a distance from the site of its production and cross biological membranes through specialized aquoporins called peroxiporins (Gechev *et al*., 2006). The superoxide protonated form, $HO_2^{\cdot-}$, can also cross membranes and initiate lipid oxidation. $O_2^{\cdot-}$ and H_2O_2 can interact and form the highly reactive hydroxyl radical ($HO^{\cdot}$). $HO^{\cdot}$ can react with and damage anything with which it comes in contact. Therefore, cells do not possess enzymatic mechanisms for its detoxification and rely on mechanisms that prevent its formation. Singlet oxygen ($^1O_2$) is a non radical ROS produced by spin reversal of one electron of the ground state

triplet oxygen ($^3O_2$). $^1O_2$ can transfer its energy to other molecules and damage them, like peroxidation of polyunsaturated fatty acids (Gechev *et al*., 2006).

Numerous biological processes generate ROS, as photosynthesis, respiration, glycolate oxidase, oxalate oxidase, xanthine oxidase, amine oxidase, excited chlorophyll, fatty acid oxidation and peroxidases (Baxter *et al*., 2014). Chloroplasts are major sites of ROS production in plants. The ability of oxygen to accept electrons prevents overreduction of the electron transport chains, thus minimizing the chance of $^1O_2$ production. Peroxisomes and glyoxysomes are other major sites of ROS generation during photorespiration and fatty acid oxidation, respectively. Mitochondrial respiration is another process leading to $O_2^{\cdot -}$ and H_2O_2 formation. NADPH oxidases (respiratory burst oxidase homologues or RBOHs) and cell-wall associated peroxidases are the main $O_2^{\cdot -}$ and H_2O_2 producing apoplastic enzymes (Gechev *et al*., 2006).

Plant RBOHs contain six conserved transmembrane domains, with a cytosolic N-terminal domain which contains two Ca^{2+}-binding EF-hand motifs and phosphorylation target sites that are important for their activity. They accumulate superoxide in the apoplast, which dismutates to H_2O_2 spontaneously or catalytically by superoxide dismutase (SOD) and then it can play a key role is signaling processes. RBOHs homologs can be regulated depending on different signaling components including protein phosphorylation, Ca^{2+}, calcium-dependent protein kinases and phospholipase DαI (PL DαI) (Baxter *et al*., 2014).

2.2.2-ROS detoxification

To use ROS as signaling molecules, non-toxic levels must be maintained. This is achieved by the balance between ROS production and ROS scavenging processes. Plants have evolved an elaborate enzymatic and non-enzymatic antioxidant system to control ROS levels and prevent their toxicity. They are located in different parts of the cell: cytoplasm, chloroplast, mitochondria, peroxisomes, vacuole, apoplast, etc. SODs are the only plant enzymes capable of scavenging $O_2^{\cdot -}$, whereas H_2O_2 can be catabolized directly by catalases or with the help of various reductants as ascorbate peroxidases (APXs), peroxiredoxins, glutathione peroxidases (GPXs) and guaiacol

peroxidases. The most abundant non-enzymatic antioxidants are ascorbate, glutathione, tocopherol and carotenoids (Gechev *et al*., 2006).

2.3- Protein phosphorylation

Protein phosphorylation is the master posttranslational modification that regulates cell activity. Phosphorylation of a protein involves the enzymatically mediated addition of a phosphate group (PO_4) to its amino acid side chains and this reaction is reversible (dephosphorylation), thus modulating protein activity.

Phosphorylation occurs thanks to the action of the protein kinases, which phosphorylate proteins by transferring a phosphate group from ATP or GTP to their target protein.

Protein phosphorylation has a crucial role in intracellular signal transduction, from the plant receptors protein kinases (RLKs from receptor-like protein kinase) in the cell surface to downstream kinases as mitogen-activated protein kinases (MAPKs) or calcium-dependent protein kinases, which establish phosphorylation cascades that transmit and amplify the signal through the cell.

2.3.1-Plant receptor protein kinases

RLKs are the main plasma membrane receptors that receive the multiple different external and internal stimuli. They form a large gene family in plants. RLK family includes more than 600 members in Arabidopsis and 1100 in rice (Shiu and Bleecker, 2003; Gish and Clark, 2011). This diversity indicates the wide range of signals that they can perceive.

When these proteins interact with its specific ligand, they are able to phosphorylate their target proteins in the cytoplasm through its Ser/Thr kinase cytosolic domain. RLKs regulate the environmental stress response and play essential roles in the resulting adaptive mechanisms. In this sense, transcription of different *RLK* genes has been found to be controlled by various environmental cues. Moreover, a significant number of *RLK* genes are induced by both biotic and abiotic stresses, indicating that they may mediate cross-talk between both responses (Chae *et al*., 2009).

Extracellular signals, such as hormones, small peptides, small chemical molecules, and physical stimuli could be the potential ligands of RLKs. Its interaction triggers different downstream intracellular events as kinase cascades (MAPK), Ca^{2+} fluxes, ROS signaling, metabolic adjustments and membrane dynamics. How RLKs activate these different events is still unknown (Osakabe *et al*., 2013; Tena *et al*., 2011).

2.3.1 – Mitogen-activated protein kinases

MAPK cascades are highly conserved signaling modules downstream of receptors that transduce extracellular stimuli into intracellular responses in eukaryots. MAPK activation is one of the earliest signaling events after the stress sensing. Its activation is realized by their upstream kinases, MAPK kinases (MAPKK), through the phosphorylation of a Thr and a Tyr residue in the Thr-Glu-Tyr (TEY) or in the Thr-Asp-Tyr (TDY) (which is unique in plant MAPKs) activation motifs. MAPKKs, in turn, are phosphorylated by their upstream kinases, MAPKK kinases (MAPKKK), in two Ser/Thr residues of the Ser/Thr-$X_{3\text{-}5}$-Ser/Thr MAPKK motifs (Meng and Zhang, 2013).

Plants have expanded families of MAPKs in comparison to yeast and animals. There are 20 MAPKs in Arabidopsis (Ichimura *et al*., 2002) and 17 in rice (Reyna and Yang 2005). They are divided into four or six groups (depending on the author) based on their sequence similarities. This multigene family participates in multiple functions such as development, immune defense system, hormones signaling and responses to abiotic stress (Meng and Zhang, 2013; Moustafa *et al*., 2014).

2.3.2 – Calcium-dependent protein kinases

CPKs are the only protein kinases that are able to sense calcium signals and translate them into protein phosphorylation signals, triggering then signaling cascades.

CPKs contain four major domains: an N-terminal variable domain; a Ser-Thr protein kinase domain; a junction autoinhibitory domain; and finally a calmodulin C-terminal domain, with four EF-hands Ca^{2+} binding sites (Figure I.6A). These four EF-hands Ca^{2+} binding sites are organized in two lobes, which differ in their Ca^{2+} affinities. In the resting state, the junction autoinhibitory domain and the N-terminal lobe of the calmodulin domain form two α-helices that block the kinase catalytic center. In this conformation, the N-terminal variable domain may be hidden, so it has no access to

the substrate. Upon the intracellular Ca^{2+} increase, and the subsequent binding to the C-terminal EF-hand lobe of the calmodulin domain, the two α-helices structure breaks down, releasing the kinase catalytic center and probably also the N-terminal variable domain (Figure I.6B) (Wernimont *et al*., 2010; Liese and Romeis 2013; Schulz *et al*., 2013). Apart from Ca^{2+} activation, CPK activity can be further modulated by autophosphorylation, 14-3-3 protein interaction, and phospholipids (Cheng *et al*., 2002; Harper *et al*., 2004; Klimecka and Muszynska, 2007; Boudsocq and Sheen, 2010; Boudsocq and Sheen 2013).

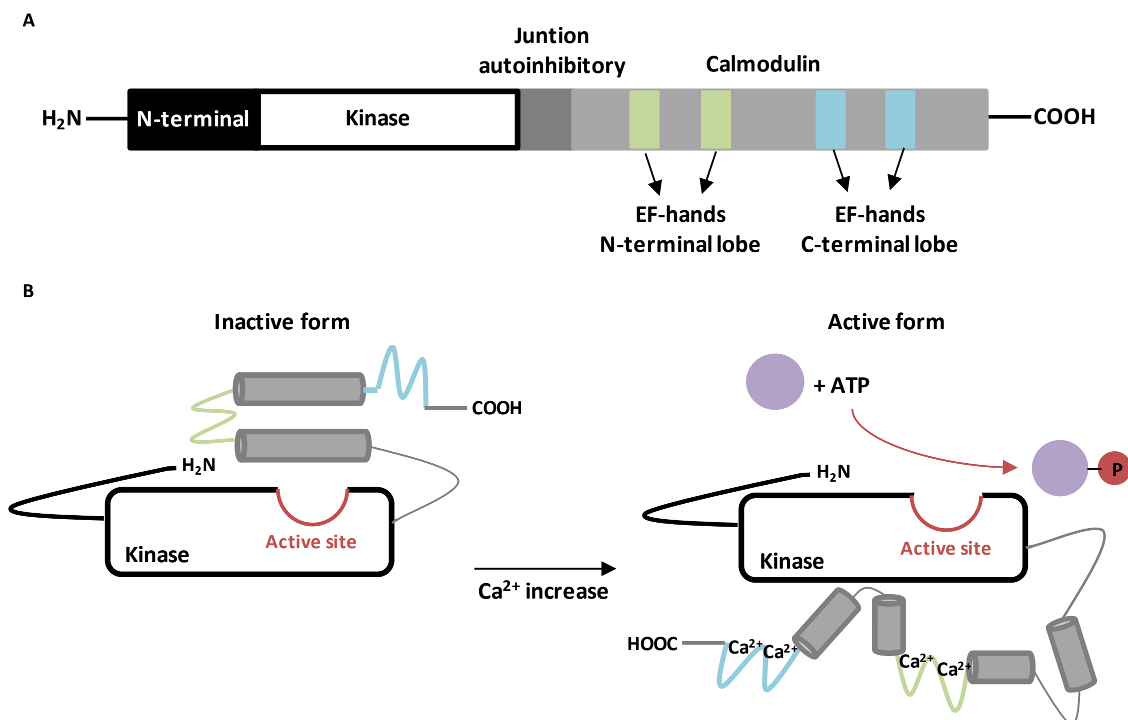

Figure I.6: CPK structure and activation. A, Domain structure of CPKs, including N-terminal domain, kinase domain, junction autoinhibitory (in dark grey) domain and calmodulin domain (in light grey) with the four EF-hand motifs grouped in the N-terminal and C-terminal lobes. B, Model for CPK activation (modified from Schulz *et al*., 2013). After the intracellular calcium concentration increases, calcium binds to the EF motifs inducing a conformational change in which both α-helices break into segments rotating the calmodulin domain. As a result, N-terminal domain and kinase active site (red) are accessible for the substrate (purple) to be phosphorylated.

CPKs are encoded by multigene families with 34 members in Arabidopsis (Cheng *et al*., 2002), 17 in wheat (Li *et al*., 2008), 41 in maize (Kong *et al*., 2013) and 31 in rice (Ray *et al*., in 2007). They are also present in protists, oomycets, and green algae (Valmonte, 2013). Arabidopsis CPKs are divided into four major evolutionary subgroups (I-IV)

(Cheng *et al*., 2002). Upon the inclusion of rice and wheat CPKs, group II and III were separated into subgroups (IIa, IIb, IIIa, IIIb) (Asano *et al*., 2005; Li *et al*., 2008). During plants evolution (from green algae to higher plants) several whole genome duplications have been occurred. Consistent with that, green algae have the shortest number of CPKs whereas angiosperms have the largest number of CPKs among land plants.

CPKs are highly conserved proteins, the main differences being restricted to the N-terminal domain (Boudsocq and Sheen, 2013). This highly variable domain determines thesubcellular targeting, stability, and substrate specificity. They contain an *N*-myristoylation site at their N-terminal domain, which is necessary for membrane targeting. This irreversible translational acylation requires a second post-translational signal to maintain the membrane association, such as reversible palmitoylation. Most of the CPKs are membrane anchored, 20 in Arabidopsis (Boudsocq and Sheen, 2013) and 18 CPKs with predicted *N*-myristoylation site in rice (Asano *et al*., 2005). Diverse subcellular localizations have been described for CPKs including plasma membrane, chloroplast, mitochondria, nucleus, endoplasmic reticulum, oil bodies, and peroxisomes (Boudsocq and Sheen, 2013). Additionally, PEST (proline, glutamine, serine and threonine rich regions) sequences have been located at the N-terminal domain of some CPKs. These sequences are frequenly found in proteins undergoing rapid proteolytic degradation (Klimecka and Muszynska, 2007). Moreover, the N-terminal domain is thought to be responsible for the CPK specificity of function. Several CPKs participate in more than one cellular signaling process, and one of the possible explanations could be a different acylation/phosphorylation pattern in its N-terminal domain depending on the stimulus (Schulz *et al*., 2013). Substrate specificity could also rely on the different Ca^{2+} affinities that can affect substrate accessibility.

*CPK*s seem to respond transcriptionally to different developmental and stress stimuli (Ray *et al*., 2007; Ye *et al*., 2009; Wan *et al*., 2007; Das and Pandey 2010; Kong *et al*., 2013; Zuo, 2013; Li *et al*., 2008; Asano *et al*., 2005; Cheng *et al*., 2002). There is no correlation between functional response and phylogenetic grouping or with particular organ- or cell-type specific expression. In green algae, CPKs function primarily in signaling cascades involved in osmotic pressure and cytoplasmatic movements. These

functions diversified with the land plant evolution in response to osmotic, developmental, nutritional and immunological challenges imposed by the new and constantly evolving terrestrial environment (Valmonte *et al*., 2013). Expression analyses show the variability of processes in which CPKs could be implicated, suggesting a functional diversification for this family. Functional characterization has been performed in few CPKs (Saijo *et al*., 2000; Kobayashi et al., 2007; Coca *et al*., 2010; Asano *et al*., 2011; Campo *et al*., 2014; Ho *et al*., 2013; Wei *et al*., 2014), and these studies are required to understand in which specific process participates every CPK, if there is redundancy of functions or if a single CPK can be involved in different processes.

This thesis is focused on the study of CPKs as signaling components of the rice defense response to *M. oryzae* infection and drought stress.

3. Defense response against pathogens

3.1- Innate immunity in plants

In the last years, the model proposed by Jones and Dangl in 2006 has been widely accepted and used by most researchers in plant pathology. This model, called **zigzag model**, provides a basic dynamic representation of key behaviors of the plant immune system considering the co - evolution of host *R* genes and pathogen effectors genes.

This model suggests that the plant immune system is divided in two branches. In the first one, pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), resulting in **PAMP-triggered immunity** (**PTI**), also known as basal disease resistance. In the second phase, successful pathogens release effectors that contribute to the pathogen virulence. These effectors can interfere with PTI, causing the effector-triggered susceptibility (ETS). In phase 3, a specific effector is recognized by a specific NB-LRR protein (NB for nucleotide binding, LRR for leucine rich repeat domains) encoded by an *R* gene, resulting in **effector-triggered immunity** (**ETI,** the formerly known as gene-for-gene resistance). ETI is an accelerated and amplified PTI response, which results in pathogen resistance and, usually, a hypersensitive cell death response (HR) at the infection site. In the fourth phase, pathogens can overcome

ETI by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection in turn, results in new *R* genes specificities, so that ETI can be triggered again (Figure I.7A) (Jones & Dangl, 2006; Boller and He, 2009).

However, Pritchard & Birch have questioned recently the zigzag model (Pritchard & Birch, 2014). The main limitation is that it is not a quantitative or predictive framework for the direct study of plant-pathogen interactions. Even it is a good model for the evolutionary history of the plant immune system. The authors argue the different concepts that are not taken into account in the model, as endogenous elicitors or damage-associated molecular patterns (DAMPs) (Boller and Felix, 2009), symbiosis and necrotrophy, the environmental context (biotic and abiotic stresses suffered simultaneously which may lead to a negative impact on the interaction or positive as in the case of priming), the time scale of the different phases and the lack of quantifiable responses. Thus, they propose a **dynamic and quantitative model of the plant immune system**, where the key features are the same as the zigzag model but it contains 15 reactions with 19 kinetic parameters. In the absence of host immune response, the pathogen reaches an arbitrary level of one unit, and no callose deposition occurs. If only PTI is active, callose deposition occurs and the pathogen fails to reach as high a level. If the pathogen is able to introduce an effector to suppress callose deposition, the steady-state level of the pathogen is increased and the amount of callose deposition reduced. Finally, a host having both PTI and ETI systems active supports the presence of the pathogen even if it introduces a PTI-suppressing effector (Figure I.7B).

3.2- Molecular mechanisms implicated in the plant defense response

3.2.1- Oxidative burst

Pathogen recognition by plant causes a rapid and transient production of ROS, which is known as ‘oxidative burst’. ROS produced is typically apoplastic and biphasic, with a first unspecific and transitory phase that usually takes place within minutes of the interaction with the pathogen, and a second sustained phase that occurs hours after

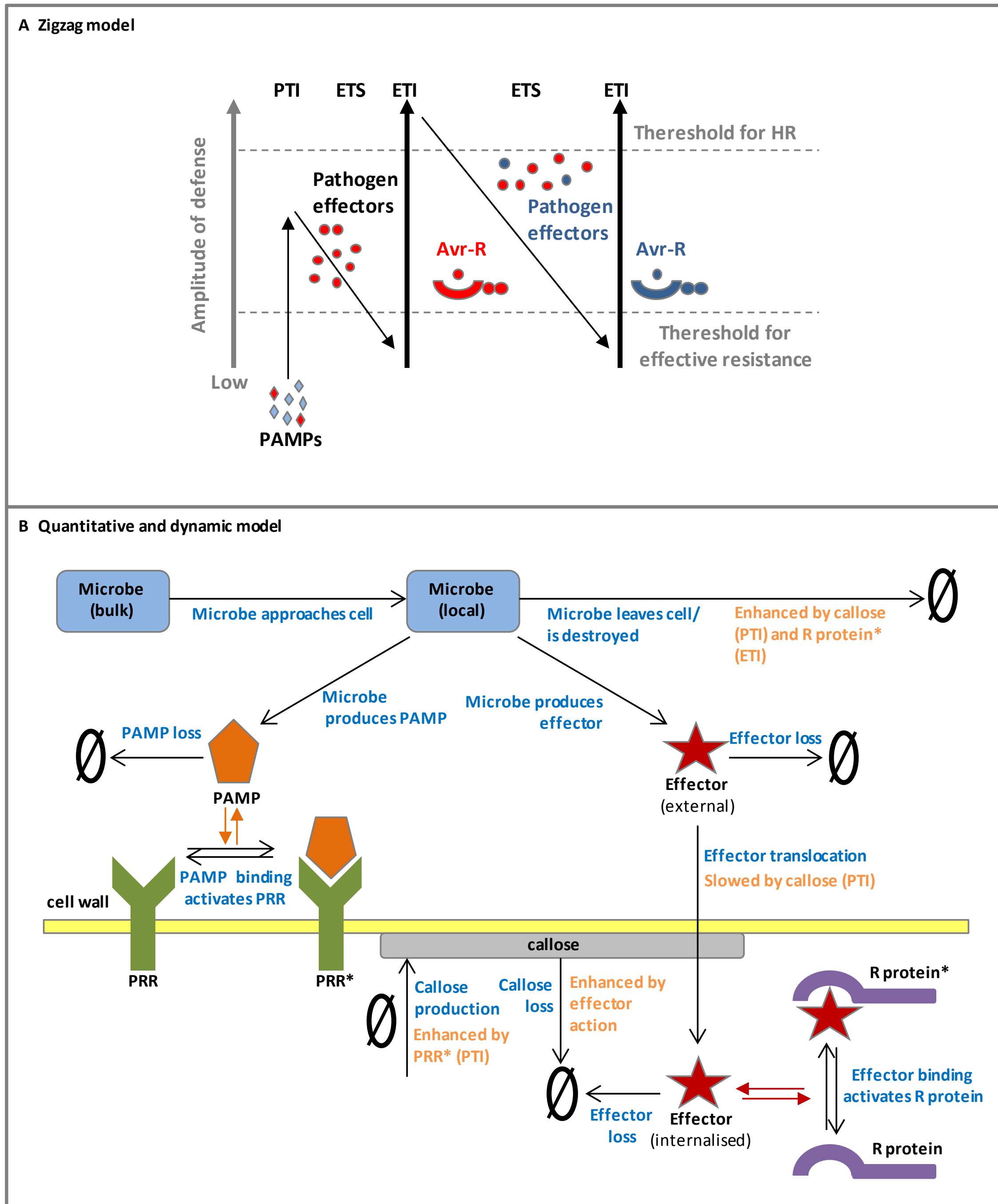

Figure I.7: Schematic representations of the plant immune system models. A, zizgzag model described by Jones and Dangl in 2006. B, quantitative and dynamic model described by Pritchard and Birch in 2014.

pathogen attack. This second phase is usually associated with the establishment of the defenses and the hypersensitive response (Torres, 2010). ROS production is detected during activation of the PTI and ETI associated to many defense functions. ROS could have a direct effect on the pathogens, killing them or interfering in their growth. They

can also contribute to the establishment of physical barriers, such as cell wall cross linking or callose depositions (Torres, 2010); and to the generation of chemical barriers, such as accumulation of phytoalexins and secondary metabolites. Finally, ROS are the main cause of the **hypersensitive response** at the focus of infection to limit the pathogen spread, and to propagate the defense signal. The apoplastic ROS produced by RBOHs and cell wall peroxidases were proposed to synergize in a signal amplification loop with salicylic acid (SA) to drive the HR and the establishment of systemic acquire resistance (SAR) (Torres, 2010; Barna *et al.*, 2012). Moreover, SA accumulation can also downregulate the ROS scavenging systems, promoting ROS accumulation (Torres, 2010).

The role of HR and antioxidants is not the same depending on the pathogen lifestyle. The biotrophic pathogens need plant cells remain alive to get nutrients, so the oxidative burst blocks its infection process. Therefore, reduced ROS content is favorable to biotrophic pathogens, and a major antioxidant activity contributes to the plant susceptibility. On the other hand, necrotrophic pathogens damage plant tissues to degrade and feed from them. In this case, accumulation of ROS promotes pathogen growth and disease development (Barna *et al.*, 2012)

3.2.2- Hormones involved in the plant defense response

Plant hormones play a central role regulating defense signaling responses and systemic signaling. At primary levels, plant hormones are responsible for the integration and processing of developmental and environmental cues. They also prime the host cell for both biotic and abiotic stress responses. The three classical defense-related hormones are salicylic acid, jasmonates (JA) and ethylene (ET) (Knepper and Day 2010). But, during the last decade, researchers also found that growth-controlling hormones, such as auxin, gibberellic acids (GAs), brassinosteroids (BRs), and abscissic acid (ABA) are actively involved in plant immunity (De Vleesschauwer *et al.*, 2013; Yang *et al.*, 2013).

Salicylic acid

SA is an important hormone that mediates plant defense responses against biotrophic and hemibiotrophic pathogens. SA accumulation upon pathogen recognition is

essential for local and systemic acquired resistance (Boatwright and Pajerowska-Mukhtar, 2013).

Rice plants normally maintain high levels of free SA in leaves, and low levels in roots. Moreover, rice plants do not show increased accumulation of SA after pathogen attack, contrary to what happens in *Arabidopsis* (Silverman *et al*., 1995: Boatwright and Pajerowska-Mukhtar 2013). However, they can respond to the exogenously applied SA. This high free-SA content appears to function as an antioxidant that protects plants from oxidative damage caused by aging, pathogen attack, or abiotic stress (Yang *et al*., 2004; Horváth *et al*., 2007; Pandey and Srivastava, 2013).

NPR1 (Non-expressor of PR-1) is a transcriptional cofactor that functions as a key regulator of the SA signaling pathway (later it is explained in detail). The *Arabidopsis* NH1-dependent (NPR1 homolog 1) SA pathway is conserved in rice, and also promotes pathogen resistance (Chern *et al*., 2005). A second branch of SA-signaling independent of NH1 has been described in rice. This pathway relies on *OsWRKY45* (Shimono *et al*., 2007). This transcription factor (TF) is induced in response to SA and BTH (benzothiadiazole S-methyl ester, a functional analogue of SA) treatments, and its overexpression promotes blast resistance. *OsWRKY13* was shown to modulate the expression of SA biosynthetic and responsive genes, and its overexpression also promotes blast resistance (Qiu *et al*., 2007).

Jasmonates

Jasmonates are crucial lipid-derived regulators that play essential roles in plant defense and development. Particularly they are known to function in herbivores and necrotrophic pathogen defense (Browse, 2009). Interestingly, jasmonates also participates in the (hemi)biotrophs defense of rice plants (Riemann *et al*., 2013). Exogenously applied JA induced many *PR* (pathogenesis-related) genes and increased resistance to rice blast (Yang *et al*., 2013). The F-box protein Coronatine insensitive 1 (COI1) is the principal JA receptor in *Arabidopsis* and rice but, in the case of rice, OsCOI1-mediated JA pathway is indispensable for the disease resistance conferred by OsNH1 (Yang *et al*., 2013). In rice, JA might function as an endogenous priming agent

that amplifies pathogen-induced defense reactions independently of the pathogen lifestyle (De Vleesschauwer et al., 2013).

Ethylene

Ethylene regulates various growth and developmental processes in plants, including seed germination, seedling growth, organ development, fruit ripening and organ senescence and abscission. This hormone is also involved in responses to stress. ET could act as a positive or negative modulator of disease resistance. It is widely accepted that ET collaborate with JA in the defense response against necrotrophic pathogens. ET plays a negative role in rice immunity to *Xoo* but it has been described that promotes blast disease resistance (Helliwell *et al*., 2013, Singh *et al*., 2004).

Developmental hormones

Auxins regulate almost all the developmental processes. It has been reported that many pathogens produce indole acetic acid (IAA, the most abundant auxin) during infection, which supports the idea that auxins stimulates disease susceptibility in Arabidopsis and rice. **Gibberellins** promote plant growth by regulating the degradation of a class of nuclear growth-repressing proteins called DELLA (For their DELLA conserved motif, formed by aspartate, glutamate, two leucines and alanine). It was shown that DELLAs positively regulates disease resistance in rice, partially through its crosstalk with the JA signaling pathway. In contrast, in Arabidopsis, DELLAs promote susceptibility to biotrophs and enhance resistance to necrotrophs. **Brassinosteroids** regulate many developmental and physiological processes, such as cell elongation, vascular differentiation, root growth, light responses, stress tolerance and senescence (Kim and Wang, 2010). BRI1 (BR insensitive 1), a leucine-rich repeat receptor-like kinase (RLK), functions as the receptor of BR that is located at the plasma membrane. Binding of BRs to BRI1 activates the BRI1-XA21 chimeric receptor kinase to induce XA21-mediated defense response in rice cells. Moreover, BRI1 can also interact with BAK1 (BRI1-associated receptor kinase 1), which regulates rice resistance to blast and bacterial blight (He *et al*., 2000; Nakashita *et al*., 2003). **Abcisic acid** regulates many physiological processes but it has been widely studied for its role in abiotic stress. Recently, ABA was found to be an important regulator of biotic stress as well. It likely acts as a negative regulator of plant defense in Arabidopsis. In rice, ABA suppresses

host resistance against *M. oryzae*, and exogenous applications reduced ET production in rice (Koga *et al*., 2004; Bailey *et al*., 2009). Moreover, ABA represses the induction of *OsNH1* and *OsWRKY45*, both important regulators of the SA signaling pathway. However, it was found that ABA positively regulates resistance against brown spot caused by *Cochliobolus miyabeanus* (De Vleesschauwer *et al*., 2010). And also enhances the timing and intensity of callose deposition against invading necrotrophic pathogens (Flors *et al*., 2008).

3.2.3-Transcriptional regulation

Activation of immune response is achieved by the action of a multitude of transcriptional regulators that reprogram the transcriptome to favor defense responses over normal cellular requirements. Transcriptional regulators consist not only of DNA binding TFs, but also of cofactors that do not physically associate with DNA (Moore *et al*., 2011). Transcriptional modulation activated by the defense response involves the induction of a large amount of genes encoding different proteins. These response genes are mainly related to PR proteins accumulation, oxidative stress and secondary metabolites synthesis.

NPR1, or its homolog in rice NH1, are master co-activators of most SA-induced genes during the defense response, and NPR1 was the first redox sensor described for SA-regulated genes (Mou *et al*., 2003). SA stimulates NPR1 interaction with TGA transcription factors, which enhances its binding to TGA boxes of *PR1* gene promoter, forming a trans-activating complex for RNA polymerase II recruitment. Inactive NPR1 is located in the cytosol in a tetramer form. SA promotes its redox modification to separate the different NPR1 monomers, which allows the monomers translocation to the nucleus where its interaction with TGAs takes place (Cao *et al*., 1994; Herrera-Vásquez *et al*., 2015).

TFs act as transcriptional activators or repressors. Their involvement in plant defense has been elucidated through transcriptome profiling of plant responses to pathogen infections (Venu *et al*., 2007; Li *et al*., 2006; Bagnaresi *et al*., 2012). In these studies, different TF families are overrepresented: WRKY, APETALA2/Ethylene responsive

factor (AP2/ERF), basic-domain leucine zipper (bZIP), basic helix-loop-helix (bHLH) and NAC.

WRKYs: They comprise a large family of 72 members in Arabidopsis and 102 in rice. They have one or two WRKY domains consisting of 60 amino acids. The general cis-element bound by WRKY TFs is called W-box, which has a consensus sequence of TTGACT/C. WRKYs are involved in: PAMP signaling downstream of MAPK cascades, interaction with R proteins, and antiviral defense (Ryu *et al*., 2006; Seo and Choi 2015).

AP2/ERF: This is one of the largest families of TFs (146 members in Arabidopsis, and 158 in rice). They have one or two AP2 domains and they are divided into three groups: AP2, ERF and RAV. ERF groups can be subdivided in dehydratation-responsive element binding proteins (DREBs) and Ethylene responsive factors (ERFs). AP2/ERF TFs can act as positive and negative regulators of the defense response (Nakano *et al*., 2006; Liu *et al*., 2012; Seo and Choi 2015).

bZIP: They have a bZIP domain, consisting of 60-80 amino acids, a DNA-binding basic region, and a leucine zipper domain for homo- or hetero-dimerization. The best-known bZIP TFs involved in plant defense belong to the TGA family. Seven of ten TGAs in Arabidopsis interact with NPR1 and play roles in basal resistance and/or regulation of *PR* genes (Zhou *et al*., 2000; Seo and Choi 2015).

bHLH: They all have a conserved bHLH domain of 60 amino acids approximately, that comprises a basic region for DNA binding and a helix-loop-helix region for protein-protein interaction. Among them, the MYC family is known to be involved in plant defense through JA signaling (Boter *et al*., 2004; Seo and Choi 2015).

NAC: They share a NAC domain consisting about 150 amino acids at their N-terminus, which has DNA-binding ability. They also have a transcriptional regulatory domain in the C-terminal region. NAC TFs play diverse roles in response to biotic and abiotic stresses and in growth and development (Nuruzzaman *et al*., 2010; Seo and Choi 2015).

3.2.4-Pathogenesis proteins

The PR proteins accumulation is one of the crucial events of the plant defense response, and they are usually used as markers of defense induction. (Jwa *et al*., 2006).

PR genes are defined as genes encoding for host proteins that accumulate after pathological or related stimuli (Van Loon and Van Strien, 1999). In 2006, Van Loon *et al*. classified the PR proteins into seventeen distinct families. This classification is still used and accepted (Table I.1).

Table I.1: PR proteins classification of Van Loon *et al*., 2006. Examples of rice members obtained from Jwa *et al*., 2006; Zhu *et al*., 2005; Muthukrishnan *et al*., 2001; Ouyang *et al*., 2007.

Family	Properties	Rice members
PR1	Unknoun	PR1a, PR1b
PR2	β-1,3-glucanase	Gns1, Gns2-6
PR3	Chitinase type I, II, IV, V, VI, VII	CHIT2, OsPR3
PR4	Chitinase type I, II	WIP4
PR5	Thaumatin-like	OsPR5
PR6	Proteinase-inhibitor	OsBBPI, OsPIN
PR7	Endoproteinase	
PR8	Chitinase type III	Glycyl hydrolase
PR9	Peroxidase	POX 22.3, POX 8.1, POX 5.1
PR10	Ribonuclease-like	PBZ1, JIOsPR10
PR11	Chitinase type I	
PR12	Defensin	
PR13	Thionin	OsThi1
PR14	Lipid-transfer protein (LTP)	
PR15	Oxalate oxidase	Oxalate oxidase
PR16	Oxalate-oxidase-like	
PR17	Unknown	

3.3- Systemic Acquired Resistance

Even in the absence of a circulatory system, plants are able to defense themselves against infection locally and systemically. The local infection defense response induces the production of signals which lead to systemic expression of the antimicrobial *PR* genes in the non-inoculated distal tissue. This process called systemic acquired resistance protects the plant from secondary infection of a broad spectrum of different pathogens (fungi, oomycetes, viruses, and bacteria). SAR can also be induced by

exogenous application of SA or its synthetic analogs 2,6-dichloroisonicotinic acid (INA) and BTH. SAR resistance can last for weeks to months, and possibly even the whole growing season (Durrant and Dong 2004).

Several mobile signals have been proposed, such as salicylic acid, methyl salicylic acid (MeSA), the lipid transfer protein DIP1, azelaic acid (AzA), glycerol-3-phosphate (G3P) and abietane diterpenoid dehydroabietinal (DA) (Fu and Dong, 2013)

The onset of SAR is associated with massive transcriptional reprogramming which is dependent on NPR1 and its associated TFs such as TGAs and WRKYs. This leads to the antimicrobial *PR* genes expression, which are secreted to the extracellular space, limiting a second infection in the plant (Fu and Dong, 2013; Durrant and Dong 2004).

3.4- Priming

Priming consists in a faster and stronger induction of basal resistance mechanisms upon subsequent pathogen attack (Conrath *et al*., 2006). It provides disease resistance with relatively minor reductions in plant fitness, therefore priming constitutes a beneficial strategy for plant survival in adverse environments. This state can be maintained long time after the initial stimulus.

There are some natural examples of priming states in plants. The classical one is SAR, which is a systemic priming of SA-inducible defense mechanism. The volatile organic compounds (VOCs) emitted by herbivore-infested plant can prime JA-dependent defenses in neighboring plants. But also beneficial organisms can induce JA-dependent defenses against up-coming pathogen attacks, a process known as induced-systemic resistance (ISR) (Pastor *et al*., 2009).

4. Drought responses in plants

4.1- Plant responses to altered water status

Drought is a period of below normal precipitation that limits plant productivity by reducing plant turgor and cell enlargement, closing the stomata, stopping the photosynthetic process and many other basic metabolic processes. The continued dehydratation situation causes disorganization of the protoplast and death (Boyer, 1982; Kramer and Boyer, 1995). The direct consequence of a drought period is the decrease in the availability of soil water, which is defined as a decrease in water potential (**ψ_w**, Boyer and Kramer, 1995). Mathematically, ψ_w is the chemical potential of water divided by the partial molar volume. A decrased ψ_w means that plant has more difficulties to take the water. This is the stress signal that triggers a range of responses to different drought degrees (Verslues *et al*., 2006). The terminology used for low ψ_w plant responses was proposed by Levit in 1972 (Figure I.8).

Low ψ_w avoidance

This is the plant's first response in most cases. It consists in maintaining the water content by limiting the water loss or increasing the water uptake, through stomatal closure (short term response) and increasing the root/shoot growth ratio (long term response). These mechanisms can be sufficient in the case of mild water stress, with the inconvenience of the lost of photosynthesis caused by reduced stomatal CO_2 uptake or the resource consumption for the root growth.

Low ψ_w stress tolerance

If the stress becomes more severe, other mechanisms are needed to maintain plant function. First, plant tries to **avoid the dehydratation** by accumulating compatible solutes (proline, glycine betaine, trehalose), known as osmotic adjustment, and by the hardening of cell wall. These two responses allow a lower ψ_w by decreasing the osmotic potential (ψ_s) and increasing the elastic modulus of the cell wall (ε) that maintains relative water content of the plant.

As low ψ_w stress becomes more severe, the mechanisms to tolerate reduced water content show up (**dehydratation tolerance**). These mechanisms pretend to avoid the cellular damage caused by dehydratation. Among them are included the synthesis of protective proteins, mainly dehydrins and other late embryogenesis abundant (LEA) proteins, the control of ROS levels, and the protection of the ROS damage.

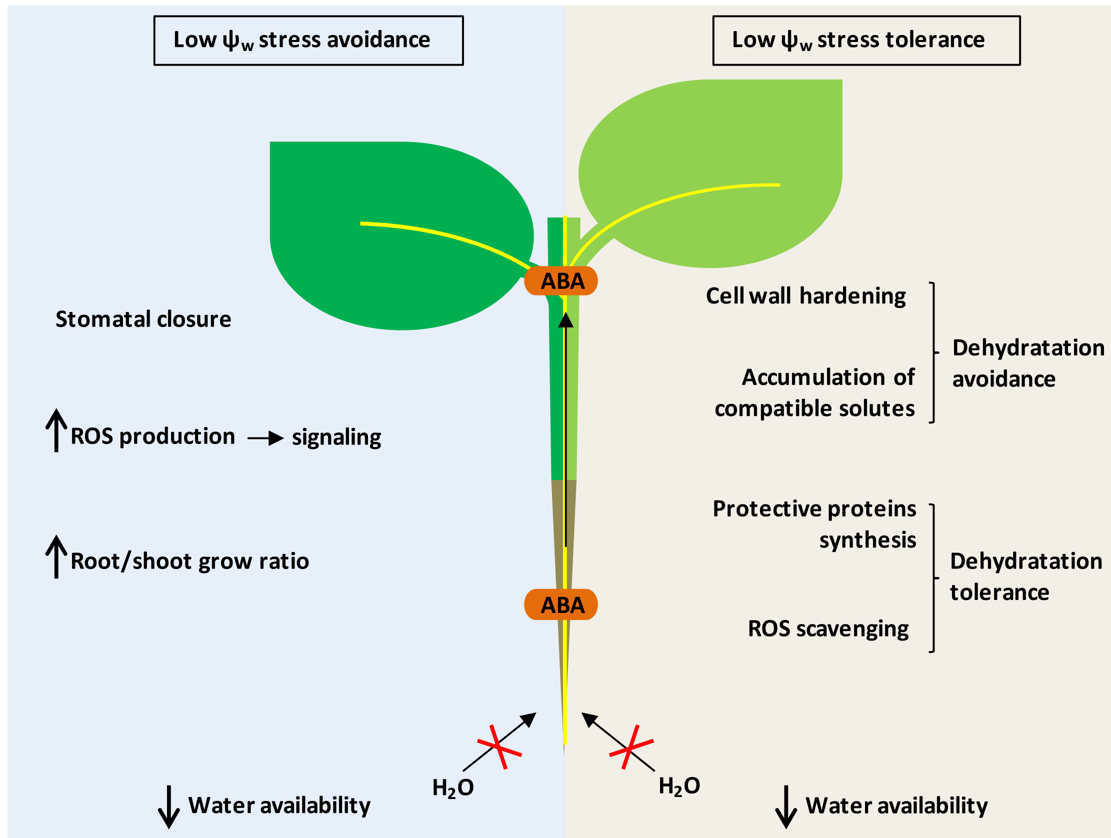

Figure I.8: **Plant responses to low ψ_w stress.** The left panel shows the stress avoidance responses to low ψ_w, and the right panel shows the stress tolerance responses to low ψ_w.

4.2- Molecular mechanisms implicated in drought response

4.2.1-ROS signaling

One of the first responses of the plant to the lack of water is the stomata closure to prevent the water loss. As a result, the CO_2 uptake decreases, triggering then a photosynthesis reduction, and leading to the accumulation of ROS. ROS can be produced by different processes upon drought stress, including the increase of photorespiration activity, the over-reduction of the photosynthetic electron transport

chain, and the higher leakage of electrons to oxygen by the Mehler reaction (Choudhury *et al*., 2013; Cruz de Carvalho, 2008).

Depending on its concentration, ROS can have two different functions in the plant. At low levels, they act as stress-signaling components, modulating calcium mobilization, protein phosphorylation and gene expression. It has been shown that H_2O_2 can regulate the expression of genes that participates in cellular repair and protection mechanisms or in the H_2O_2 stress response signal transduction pathway (Kar, 2011; Desikan *et al*., 2000).

When ROS reach phytotoxic levels, they can cause oxidative damage of membranes and other components, and eventually cell death. In order to prevent this situation, antioxidants regulate ROS levels by detoxification, which is directly correlated with the protection from abiotic stresses (Choudhury *et al*., 2013; Cruz de Carvalho, 2008). Some authors have described a direct correlation between the induction of the scavenging system and the degree of drought tolerance (Guo *et al*., 2006; Chugh *et al*, 2011; Nakabayashi *et al*., 2014; Kumar *et al*., 2014), suggesting that the enhancement of the oxidative stress tolerance have a positive effect on drought stress tolerance.

4.2.2-Hormones

ABA is known to be a key player in drought stress response. It is accumulated in response to low ψ_w and it is involved in all the responses as stomatal closure, root growth, accumulation of compatible solutes, synthesis of dehydrins and ROS control (Verslues *et al*., 2006).

ABA can be synthesized from different pathways. It can be produced in a pathway originated from isopenthenyl pirofosfate. Other biosynthesis pathway starts from the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which occurs in chloroplasts and is catalyzed by zeaxanthin epoxidase. After different reactions catalyzed by ABA4 and NCED (9-cis-epoxycarotenoid dioxygenase), xanthoxin is produced. Xanthoxin is the first cytoplasmic precursor and it is converted to ABA by ABA2 and AAO (ABA aldehyde oxidase). *NCED3* gene is responsible for the dramatically increase of ABA level in rice and Arabidopsis exposed to water stress (Ye *et al*., 2012; Peleg 2011).

ABA conjugation has also an important role in the rapid drought response. ABA glucosyl ester (ABA-GE) is the most widespread conjugate, which is catalyzed by ABA glucosyltransferase. The permeability of biomembranes for ABA-GE is very low, which makes ABA-GE well suitable for long-distance translocation and storage in vacuoles or in the apoplastic space. This ABA-GE will then can be transported to the endoplasmic reticulum where it is cleaved to release bioactive ABA (Ye *et al*., 2012; Peleg 2011).

In a mild stress situation, ABA accumulates in the root tissue and then release to the xylem vessels where ABA is transported to the acting site in the shoot. ABA is received by an ABC membrane transporter and then the PYR/PYL/RCAR receptor complex interacts with it, which inactivates the PP2Cs proteins, negative regulators of ABA signaling (Ye *et al*., 2012; Peleg 2011). The complex PYR/PYL/RCAR-ABA-PP2C release the PP2C downstream targets, SnRK2 protein kinases, to phosphorylate and activate ABA-depending transcription factors which enhance the expression of a number of genes involved in abiotic stress responses and tolerance (Ye *et al*., 2012; Todaka *et al*., 2012).

ABA treatments have been shown to result in transient increases in H_2O_2 production which induces tolerance to abiotic stress. ROS have been suggested to be the link between the metabolic status and ABA signaling that acts downstream of ABA (Baxter *et al*., 2014; Gechev *et al*., 2006; Osakabe *et al*., 2014)

SA and **JA** have also been described to participate in drought tolerance. Their levels increase in response to dehydratation. Exogenous applications promote compatible solutes accumulation and increased antioxidant activity (Horváth *et al*., 2007; Aimar *et al*., 2011).

4.2.3-Transcription regulation

Expression of thousands of genes is regulated by a variety of transcriptional cascades in response to abiotic stress (Zhou *et al*., 2007). Transcription factors involved in drought stress response include members of the AP2/ERF, NAC, bZIP, and MYC/MYB families (Shinozaki and Yamaguchi-Shinozaki 2007; Todaka *et al*., 2012; Agarwal *et al*., 2013).

AP2/ERF: the most important AP2/ERF TFs related to abiotic stress are DREBs. These proteins bind to dehydratation-responsive elements (DREs) to regulate ABA-independent stress-responsive genes. DREBs overexpression normally entails stress tolerance by activating LEA proteins, heat shock, detoxification, seed proteins and enzymes involved in metabolism. The rice genome contains at least ten *DREB1*-type genes, which usually respond to cold stress. *DREB2* genes respond to drought, high salinity and high temperature and there are 4 homologues, at least, in rice (Shinozaki and Yamaguchi-Shinozaki 2007)

NAC: NAC TFs regulate both the ABA-dependent and independent genes. In rice several NAC genes have been reported to be induced by drought, high salinity and cold stresses. *OsNAC6* is a transcriptional activator in both abiotic and biotic responses. *OsNAC5* and *SNAC1* promote drought tolerance (Nakashima *et al*., 2007; Takasaki *et al*., 2010; Hu *et al*., 2006).

bZIP: Two different bZIP TFs, AREB/ABF (ABA-responsive element binding/ ABA binding factor), can bind to ABRE (ABA responsive-element) *cis*-elements, thereby activating ABA-dependent gene expression. *TRAB1* (Transcription factor responsible for ABA regulation) expression is up-regulated after ABA application in rice and it is phosphorylated in response to ABA (Kagaya *et al*., 2002). *OsABF2*, *OsbZIP23* and *ABL1* genes are induced by drought and ABA, and enhance drought tolerance (Hossain *et al*., 2010; Xiang *et al*., 2008; Yang *et al*., 2001).

MYC and MYB: Some MYCs and MYBs TFs are synthesized following accumulation of endogenous ABA. *Osmyb4* and *OsMYB3R-2* are related to different abiotic stresses tolerance (Park *et al*., 2010; Dai *et al*., 2010).

4.2.4- Drought tolerance Proteins

These proteins are mainly subgrouped in water channels and transporters, enzymes for osmolyte biosynthesis, detoxification enzymes, and protective proteins.

Water channels and transporters: Transporters play an important role in drought and salt tolerance, allowing the movement of signaling molecules (e.g. ABA), ions and osmolytes (Jarzyniac and Jasinski, 2014). Aquaporins are instrinsic membrane

proteins that mediate water transport. They are regulated in response to environmental cues and particularly in the pathway controlling the ABA dependent stomatal conductance (Reguera *et al*., 2012).

Enzymes for osmolyte biosynthesis: For a cell to take up water from the soil or other medium, it must have lower ψ_w than the water source. One strategy to decrease ψ_w and thus import water to the cell is to decrease the osmotic potential (ψ_s) by accumulating solutes, which is called osmotic adjustment. These accumulated solutes should not interfere with cellular functions; therefore they are called compatible solutes, and proline, glycine betaine or trehalose are typical examples (Verslues *et al*., 2006). Different genes for osmolytes production have been used for genetic transformation, which confer desiccation tolerance to the transgenic plants (Agarwal *et al*., 2013). For instance, the *OsTPS1*, *OtsA* and *OtsB* genes encoding trehalose-6-phosphate synthase enzymes.

Detoxification enzymes: ROS produced in response to drought must be detoxified to avoid cellular damages. For this reason, once ROS have fulfilled their signaling function, the expression of ROS detoxification enzyme encoding genes is induced as a dehydration tolerance mechanism. The most important are the SOD, CATs, APXs, GPX and Glutathione reductasa (GR) enzymes (Agarwal *et al*., 2013; Reguera *et al*., 2012).

Protective proteins: The decrease in the cellular volume caused by desiccation promotes the crowding of cytoplasmic components and increases the chance for molecular interactions that can cause protein denaturation and membrane fusion. Several types of protective proteins are well-known to accumulate in response to the decrease in water content to prevent protein aggregation and denaturation. This group of proteins includes chaperones, heat-shock proteins and LEA proteins. LEA proteins are low molecular weight proteins that play crucial roles in cellular dehydration tolerance. Dehydrins are the subfamily of group 2 LEA proteins that predominately accumulates in vegetative tissues subjected to drought, salinity and cold. For this reason, these genes have been widely used as stress markers, such as *Rab21* (Responsive to Abcissic acid) or *OsDnhn1*. Moreover, overexpression of these

genes has been shown to improve drought and salinity tolerance (Kumar *et al*., 2014; Roychoudhuri *et al*., 2007).

4.3- Systemic acquired acclimatation

The systemic acquired acclimatation (SAA) consists in the long-distance communication among cells belonging to different tissues or organs of an abiotic stimulus from the local tissue in which was initiated. This is necessary to alert all remote and unstressed tissues of the plants of the abiotic threat existence to trigger the activation of acclimatation pathways in these tissues (Mittler and Blumwald, 2015).

ROS, Ca^{2+}, ABA and stomatal functions have been postulated to mediate the SAA. A burst of ROS production mediated by RBOHs proteins is initiated in response to abiotic stimuli. This burst was shown to trigger the production of ROS by neighboring cell initiating a long-distance signal termed the ROS wave (Mittler *et al*., 2011; Choudhury *et al*., 2013; Mittler and Blumwald, 2015). Every cell along the ROS wave path activated its own RBOHD proteins, generating a systemic autopropagating ROS wave that travels in the apoplast.

ROS wave is required for ABA accumulation that is in turn required for activation of stomatal functions (Mittler and Blumwald, 2015). Changes in environmental conditions, such as decrease in water potential, are sensed in leaf cells and lead to the accumulation of ABA and ROS. They, in turn, cause stomata closure or opening depending on the type of stress, which affects the microenvironment within the leaf and alter ROS and ABA levels that can serve as a long-distance signals.

5. OsCPKs in the rice stress signaling network

During the last years, the participation of OsCPKs in the stress responses of rice plants has been widely documented. As members of a large multigenic family, individual OsCPK isoforms have been associated to different signaling pathways (Ray *et al*., 2007; Wan *et al*., 2007; Ye *et al*., 2009). These studies are mainly at the transcriptional level, showing the induction of specific *OsCPK* genes in response to different stress inducers. Only few functional characterizations are found in the literature, and most of them relates to a single stress response. These include the OsCPK1 (Ho *et al*., 2013) and the OsCPK9 (Wei *et al*., 2014) which have been associated to drought stress; the OsCPK12 (Asano *et al*., 2012) and OsCPK21 (Asano *et al*., 2011) to salinity; and the OsCPK4 (Campo et al., 2014) and OsCPK13 (Saijo et al., 2000) to both salt and drought stress, these two stresses sharing the osmotic stress component. Hence, these reports support that OsCPKs are important players in the abiotic stress signaling processes in rice plants.

At the beginning of this thesis work, no OsCPK was to our knowledge reported yet as a positive modulator of pathogen defense responses. However, previous work in our research group demonstrated that a CPK isoform mediates Arabidopsis immunity, namely the AtCPK1 (Coca and San Segundo, 2010). And four more AtCPKs were reported by other group as players of Arabidopsis responses to PAMPs (Boudsocq et al., 2010). Similarly, the *NtCDPK2* gene was earlier reported as an essential player in a tobacco efector-mediated defense response (Romeis et al., 2001). These evidences support that specific OsCPKs could be mediators of the defense signaling pathways to pathogen infection in rice plants. Based on these evidences, this thesis work addresses the identification of those OsCPK isoforms playing a role in the rice defense response to pathogens, paying also attention to their contribution to other signaling pathways, mainly in drought stress response. Additionally, the contribution of the defense-related OsCPKs to plant development is also evaluated because some CPKs are known to participate in plant development processes (Ray et al., 2007; Schulz et al., 2013). Understanding the connections between signaling pathways is a relevant issue, since plants can be exposed simultaneously to different stresses under field conditions and have to complete their development. They need to integrate all the signaling pathways

to give the appropriate response to survive and reproduce upon adverse conditions (Suzuki *et al*., 2014). Interactions between the different signaling pathways could be antagonistic or synergistic, and they are thought to be responsible for the tradeoffs between resistance and yield, and between biotic and abiotic stress tolerance (Rejeb *et al*., 2014; Atkinson, 2015). The identification of components that participate in multiple stress signaling pathways that do not interfere with plant performance and confer multiple stress tolerance is an important scientific challenge. These studies could also have practical application in the development of agronomical superior rice cultivars.

OBJECTIVES

The general objective of this thesis is the identification and functional characterization of the OsCPK isoforms mediating the rice defense response to pathogens, and their contribution to other signaling pathways and to the plant development. This objective is divided into three more specific tasks corresponding to the three chapters of this work, which are the following:

1. Identification of *OsCPK* genes involved in the rice defense response and characterization of their expression in rice varieties. Results obtained in this introductory chapter have allowed the research of the chapters 2 and 3.

2. Functional characterization of *OsCPK4* in the rice defense response. This chapter corresponds to the article: “Enhancing blast disease resistance by overexpression of the calcium-dependent protein kinase OsCPK4 in rice”, submitted to Plant Biotechnology Journal.

3. Functional characterization of *OsCPK10* in response to fungal infection and to drought stress. This chapter is expected to be published.

CHAPTER I

Characterization of *OsCPKs* gene expression in the rice defense response and in rice varieties

Abstract

Plants possess a complex signaling network to translate the multiple environmental signals that they perceive into the appropriate adaptive response to complete their life cycle. Among the different signaling components, calcium-dependent protein kinases (CDPKs or CPKs) stand out as the only calcium sensors able to transduce the calcium signals into phosphorylation cascades, initiating then subsequent signaling processes. Although many CPKs have been associated to stress signaling responses in rice plants, only few of them have been reported to participate in the defense response against *Magnaporthe oryzae* infection. This pathogen is the causal agent of blast disease, the most devastating rice disease worldwide. In this work, *OsCPK4, OsCPK5, OsCPK10* and *OsCPK13* were identified as *M. oryzae* elicitor responsive genes by global expression analysis. The characterization of their expression profiles showed that *OsCPK4* and *OsCPK10* were also induced by the fungal infection. However, the characterization of the natural variability on their expression levels in rice cultivars and wild species could not be associated to known pathogen resistant-susceptible phenotypes. These studies identify the *OsCPK4* and *OsCPK10* as defense-related genes as candidates to modulate blast disease resistance in rice plants.

Introduction

Calcium-dependent protein kinases (CDPKs or CPKs) are important plant signal transducers (Harmon *et al*., 2001; Valmonte *et al*. 2014). They combine in a single polypeptide chain, both a calmodulin domain containing four EF-hands calcium binding motifs, and a Ser-Thr-kinase domain (Harmon *et al.,* 2001). This unique feature confers CPKs with the ability to sense calcium fluctuations and translate them into protein phosphorylation signals, which can trigger subsequent signaling cascades. CPKs comprise large multigene families with 34 members in Arabidopsis (Cheng *et al*., 2002) and 31 in rice (Ray *et al*., in 2007). A functional diversification has been proposed to explain this large number of CPKs, in which individual isoforms might participate in different signaling process. In the case of rice, three OsCPKs have been associated to plant developmental processes, for grain filling (OsCPK31, Manimaran *et al*., 2015) and light and seed development (OsCPK2 and OsCPK11, 1999). The vast majority of the characterized rice *OsCPK* genes mediate stress signaling processes. Many of them are related to salt and drought stress tolerance, as in the case of *OsCPK13* (Saijo *et al*., 2000), *OsCPK21* (Asano *et al*., 2011), *OsCPK12* (Asano et al., 2012), *OsCPK1* (Ho *et al*., 2013), and *OsCPK4* (Campo *et al*., 2014); the *OsCPK13* being also involved in cold tolerance (Abbasi *et al*., 2004; Komatsu *et al*., 2007; Saijo *et al*., 2000). Few *OsCPK*s are associated to rice biotic responses, including the *OsCPK18* which participates in the arbuscular micorrhyza perception (Campos-Soriano *et al.*, 2011), the *OsCPK12* which negatively regulates *M. oryzae* resistance (Asano *et al*., 2012), or the *OsCPK10* that promotes resistance to *M. oryzae* infection (Fu *et al*., 2013). In most of the functional characterizations, the transcriptional regulation of a *CPK* gene in response to a specific stress correlates with its functional involvement to the plant response to the stress inducer. With the purpose to identify the *OsCPK*s mediating defense responses in rice, we searched for *OsCPK* genes showing altered expression in response to *M. oryzae* infection. This fungus is the causal agent of the rice blast disease, one of the most devastating rice diseases worldwide (Wilson & Talbot, 2009). Searching a previous microarray analysis of rice leaves treated with *M. oryzae* elicitors (Campo *et al*., 2013), we identified several *OsCPK*s genes upregulated in response to fungal elicitors. Among them, the *OsCPK4*, *OsCPK10*, *OsCPK13* and *OsCPK5* genes were selected, and their

expression profiles in response to elicitor and fungal infection were characterized. Moreover, the natural variation of their expression levels was monitored in a collection of rice cultivated varieties and wild species from different locations in the world.

Results

OsCPK gene expression is induced by *M. oryzae* elicitors in rice plants

The global expression data of rice leaves from the cultivar Nipponbare in response to the *M. oryzae* elicitor treatment (300 µg/ml), obtained by microarray analysis (GeneChip® rice genome array of Affymetrix™), and previously described (Campo et al. 2013), was used for the search of *OsCPK* genes showing altered expression upon fungal infection. Nine different *OsCPK* genes were identified as upregulated genes (p-values < 0.05) at the two different analyzed treatment times (Table CI.1). The *OsCPK4* gene was the only one showing a maintained upregulation after 2h post-treatment and the highest induction level. The *OsCPK5* and *OsCPK10* genes showed the highest expression changes at 30 minutes. And, the *OsCPK13* gene was identified only after 2h treatment. These four genes were selected for further studies.

Table CI.1: Significative Fold Change values for *OsCPKs* in response to *M. oryzae* elicitor treatment searched in the microarray data of Campo *et al.* 2013 (p-value ≤ 0.05).

		30 min		2 h	
Gene	**Locus**	**Fold Change**	**p-value**	**Fold Change**	**p-value**
OsCPK3	LOC_Os01g61590	1.28	0.047		
OsCPK4	LOC_Os02g03410	1.23	0.027	1.94	0.000
OsCPK5	LOC_Os02g46090	1.56	0.000		
OsCPK10	LOC_Os03g57450	1.32	0.031		
OsCPK13	LOC_Os04g49510			1.55	0.010
OsCPK18	LOC_Os07g22710	1.16	0.043		
OsCPK22	LOC_Os09g33910			1.16	
OsCPK24	LOC_Os11g07040	1.20	0.022		
OsCPK27	LOC_Os12g30150			1.27	

The expression dynamics of these four genes were monitored by qRT-PCR analyses at different times in response to elicitor treatment of rice leaves. The results of this study are shown in Figure CI.1. The four genes showed the same profile with an expression peak at 30 minutes post treatment followed by an induction decrease that ended 6 hours later, when the expression levels were once again indistinguishable from control levels. Notice that the induction was already detected at very early, right after treatment (time 0 in the plots), revealing very rapid induction of these four *OsCPK* genes. These results confirmed the transcriptional regulation of these four *OsCPK* genes by fungal elicitor treatment, suggesting their involvement in the early defense response of rice leaves.

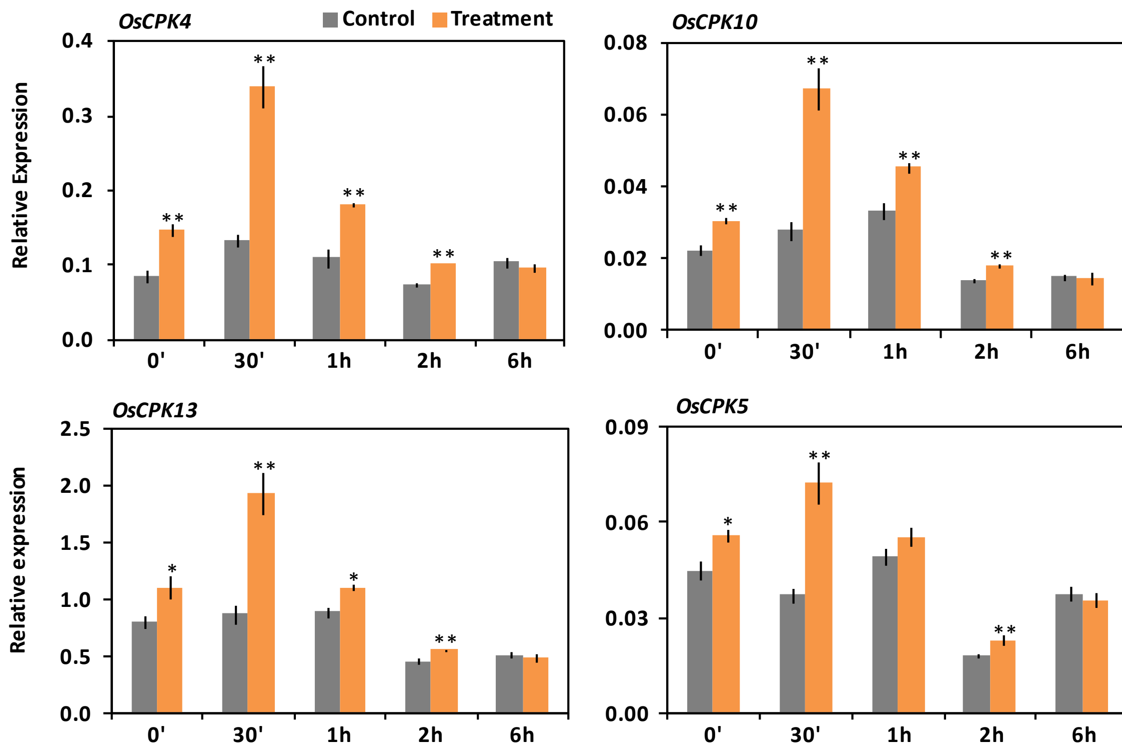

Figure CI.1: Expression levels of selected *OsCPKs* genes in response to *M.oryzae* elicitor treatment. A pool of 3 leaves of 3 week-old rice plants (cv. Nipponbare) were sprayed with a 300 µg/ml *M.oryzae* elicitor solution, and collected at the indicated period of time for total RNA extraction. Expression levels of the selected *OsCPK* genes were determined by qRT-PCR normalized to *OsUbi5*. Results are representative of two independent experiments. Asterisks represent significant differences (one-way ANOVA analysis, *P≤0.05, **P≤0.01).

Selected *OsCPK* gene expression is induced by *M. oryzae* infection in rice plants

The expression dynamics of the selected *OsCPK* genes in response to *M. oryzae* fungal infection was also evaluated. For that, three week-old Nipponbare rice leaves were locally infected with live fungus, using a 10^5 *M. oryzae* spore/ml solution. The expression levels of the selected *OsCPK* genes were monitored by qRT-PCR analyses, and results are shown in Figure CI.2. A rapid and strong induction of *OsCPK4* gene was detected in response to *M. oryzae* infection, which started with the fungal appresorium formation at 6 hours post inoculation (hpi), increases until 12 hpi (approximately an 8 fold-increase), and decreased once fungal penetration had already occurred at 24 hpi (Campos-Soriano and San Segundo, 2009). Same expression profile was observed for *OsCPK10*, although the induction was not as pronounced as for *OsCPK4,* reaching only twice the control expression level at 12 hpi. However, the *OsCPK13* showed a different expression profile, decreasing its expression at the early infection stages (3 hpi), slightly increasing at 12 hpi, and decreasing again at 24 h. Although, the upregulation of *OsCPK13* was also detected at 12 hpi with only 1.5 fold change as compared to control plants. Similarly, the detected expression change of *OsCPK5* was small and only observed at 6 hpi. Altogether, these results showed two different expression profiles for the selected *OsCPK* genes. *OsCPK4* and *OsCPK10* are clear early response genes to *M. oryzae* infection, whereas *OsCPK13* and *OsCPK5* seem not to be induced by infection at analyzed times.

Notice that important variation in the expression levels of *OsCPK13* and *OsCPK5* were detected in the control samples, suggesting that these genes might by regulated by circadian clock. Public expression data from the Mocker's Lab at Donald Danforth Plant Science Center (www.diurnal.mocklerlab.org) showed a diurnal oscillation of both *OsCPK13* and *OsCPK5* genes (correlations of 0.883 and 0.948 respectively) (Figure CI.3).

Natural variation in the expression of selected *OsCPK* genes.

The expression levels of the four selected *OsCPK* genes were analyzed by qRT-PCR in different rice cultivars and wild rice species, obtained from different geographical

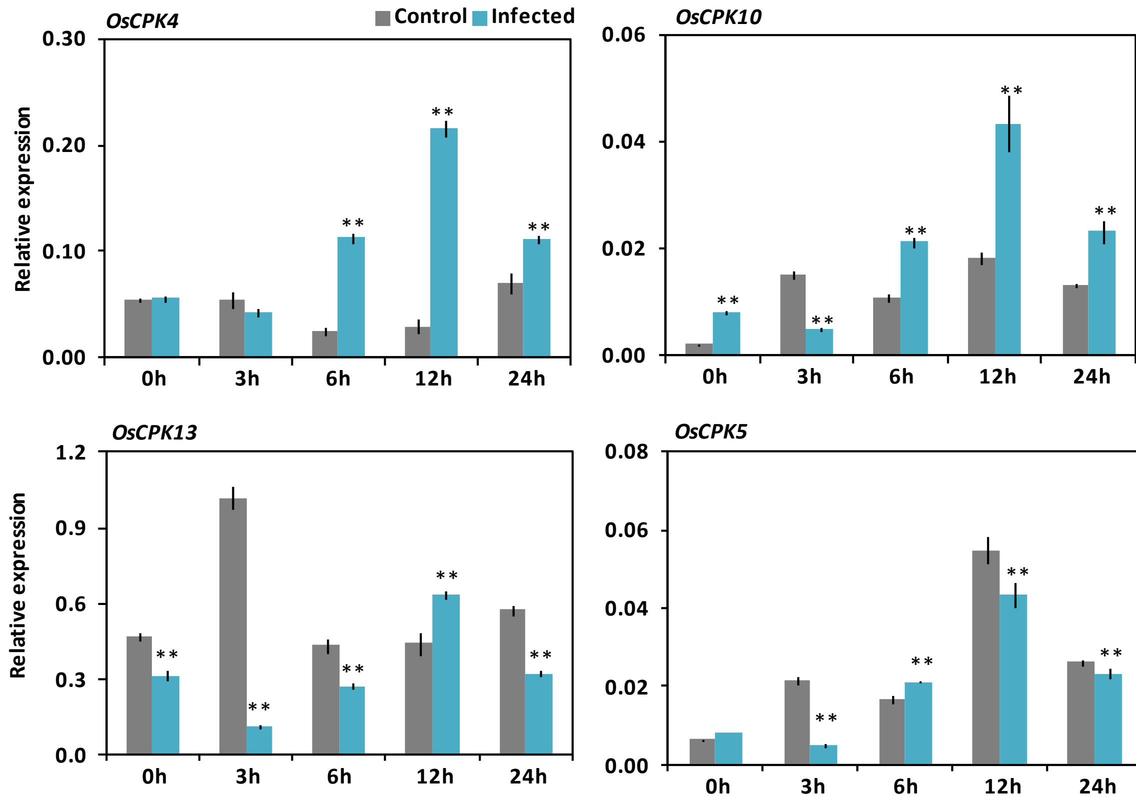

Figure CI.2: Expression levels of selected *OsCPKs* genes in response to *M.oryzae* infection. A pool of three leaves of 3 week-old rice plants (cv. Nipponbare) were locally inoculated with a *M. oryzae* spore suspension (10^5 spores/ml) and collected at the indicated period of time for total RNA extraction. Expression levels of the selected *OsCPK* genes were determined by qRT-PCR normalized to *OsUbi5*. Results are representative of three independent experiments. Asterisks represent significant differences (one-way ANOVA analysis, *P≤0.05, **P≤0.01).

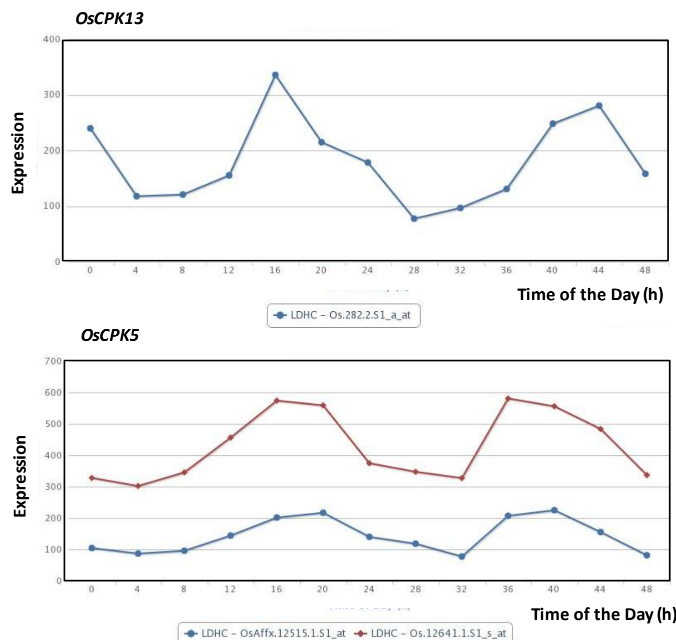

Figure CI.3: Circadian clock regulation of *OsCPK13* and *OsCPK5* gene expression. Dynamic gene expression during day time of the different probes matching with the *OsCPK13* and *OsCPK5* genes, with a 0.85 of correlation cut-off. Graphics obtained from the www.diurnal.mocklerlab.org webpage (Mocker's Lab, Donald Danforth Plant Science Center).

Natural variation in the expression of selected *OsCPK* genes.

The expression levels of the four selected *OsCPK* genes were analyzed by qRT-PCR in different rice cultivars and wild rice species, obtained from different geographical location, and showing different resistance or susceptibility phenotypes to pathogens. The collection of rice cultivars and wild species was already available in the group (Campo et al., 2013) (Table CI.2). The expression study was performed in plants grown for three weeks in control conditions. Results are shown in Figure CI.4. We observed differential expression levels for the four *OsCPK* genes among the cultivars and wild species, these differences being less pronounced among the different cultivated varieties than among the wild species. No important differences were observed associated to sub-speciation, showing similar levels the japonica, javanica and indica cultivars. Differences in the expression levels were also observed among each *OsCPK* gene, being always *OsCPK13* the one reaching the highest levels and *OsCPK10* the lowest. Despite the homogeneity, some cultivars and wild species showed notable differences for individual *OsCPKs*. For instance, most of the analyzed cultivars and wild-type species showed homogenous and low expression levels of the *OsCPK4* gene except for the Padi santan, some *javanica* cultivars and *O. nivara* wild species. In the case of *OsCPK10*, all the samples showed similar expression levels with a little increase in the IR64 and Co25 cultivars, and *O. nivara* and *O. barthii* species. Notice that the biggest differences were observed with the highly expressed *OsCPK13* gene, for which the two African rice species did not accumulated the corresponding transcripts at all. Being the *O. barthii* the parental species of *O. glaberrima*, it could be possible that an important change in the *OsCPK13* gene ocurred during the evolution. Finally, the *japonica* ARC13309 cultivar and *O. barthii* reached the highest *OsCPK5* expression levels. All together these results indicated that these *OsCPK* genes were conserved during the domestication process of rice among the analyzed varieties. The differences in the expression levels of the selected *OsCPK*s among the rice wild species are very interesting because they might be associated to the important phenotypic differences among them.

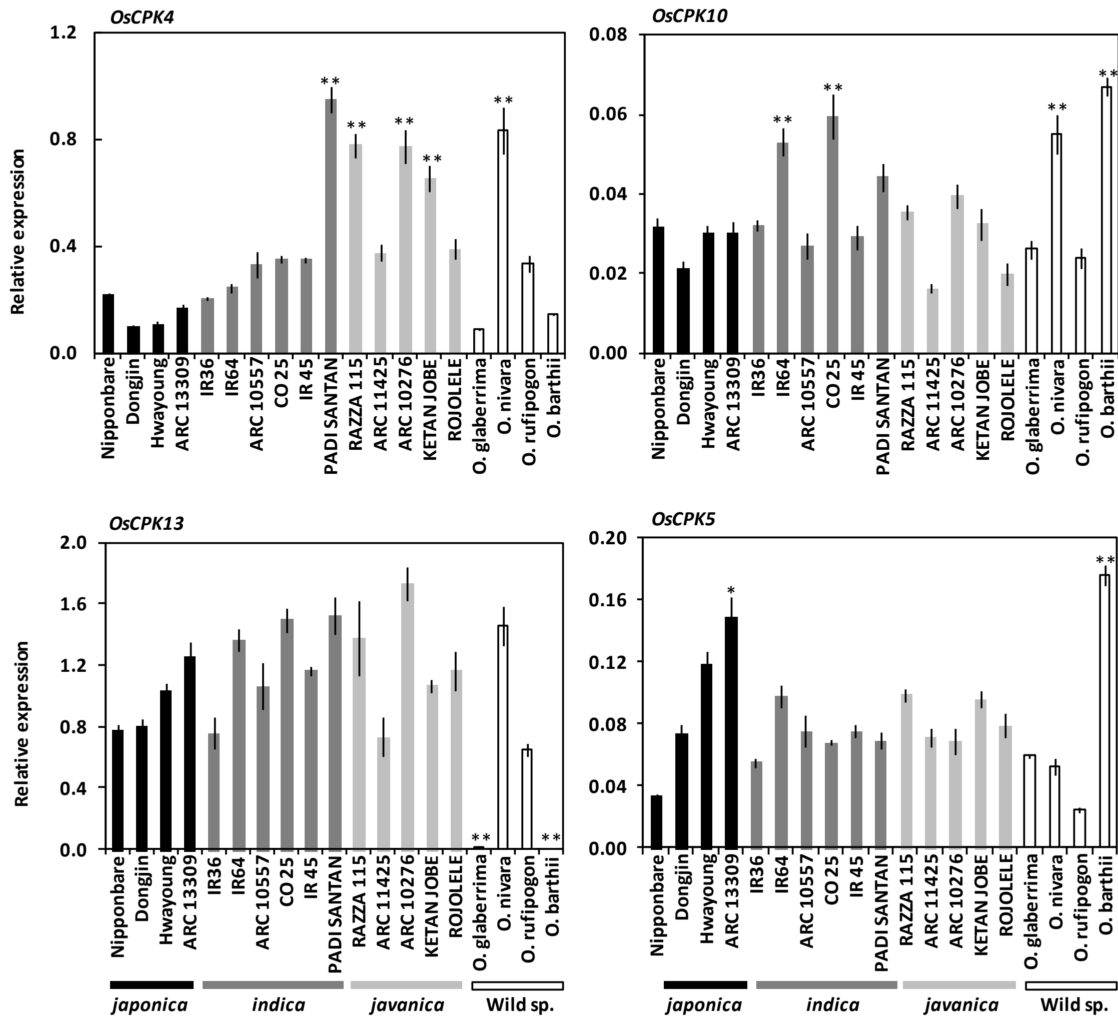

Figure CI.4: Expression levels of *OsCPKs* selected genes in different cultivated rice varieties and wild rice species. A pool of three leaves of 15 different cultivated rice varieties and 4 wild rice species were collected for total RNA extraction. Expression levels of the selected *OsCPK* genes were determined by qRT-PCR normalized to *OsUbi5*. Asterisks represent significant differences (one-way ANOVA analysis, Tukey's test, *P≤0.05, **P≤0.01). Information about cultivars and wild species is available in Table CI.2.

Discussion

The present work identifies four specific *OsCPK* genes as fungal elicitor responsive genes, namely the *OsCPK4*, *OsCPK5*, *OsCPK10* and *OsCPK13* genes. All of them showed similar accumulation dynamics in rice leaves associated to the *M. oryzae* elicitor treatment: a fast increase that reaches a maximum at 30 minutes before returning to

Table CI.2: *Oryza* cultivars and species used in this work, accession numbers and geographical region of its cultivation.

Species	Group cultivar	Name	ID/Accession*	Source/Country
O. sativa	*Japonica*	Nipponbare	IRGC12731	Japan
O. sativa	*Japonica*	Dongjin		
O. sativa	*Japonica*	Hwayoung		
O. sativa	*Japonica*	ARC 13309	IRGC22650	India
O. sativa	*Indica*	IR36	IRGC30416	Philippines
O. sativa	*Indica*	IR64	IRGC66970	Philippines
O. sativa	*Indica*	ARC 10557	IRGC42582	India
O. sativa	*Indica*	CO 25	IRGC3697	India
O. sativa	*Indica*	IR 45	IRGC47675	Philippines
O. sativa	*Indica*	PADI SANTAN	IRGC18402	Indonesia
O. sativa	*Javanica*	RAZZA 115	IRGC10641	Italy
O. sativa	*Javanica*	ARC 11425	IRGC21381	India
O. sativa	*Javanica*	ARC 10276	IRGC20821	India
O. sativa	*Javanica*	KETAN JOBE	IRGC25428	Indonesia
O. sativa	*Javanica*	ROJOLELE	IRGC9909	Indonesia
O. glaberrima	Wild		IRGC104042	Chad
O. nivara	Wild		IRGC100898	India
O. rufipogon	Wild		IRGC105616	China
O. barthii	Wild		IRGC104119	Chad

* Accessions from the International Rice Research Institute (IRRI).

basal levels after 6 hours of treatment. Therefore, the induction of these genes seems to be an early response to the perception of fungal elicitors on the leaf surface. It has long been known that elicitor treatment triggers different early and fast defense signaling responses (Denoux *et al*., 2008; Mandal *et al*., 2013). Thus, the induction of *CPK* genes is one of these early defense reactions activated upon the recognition of elicitors, also known as Pathogen Associated Molecular Patterns (PAMPs). This is a conserved response, because other *CPK*s genes have been reported to be rapidly induced within 5 min to 1 hour upon elicitor treatment, not only in rice but also in other plant species (Murillo *et al*., 2001; Romeis *et al*., 2001; Chico *et al*., 2002;

Akimoto-tomiyama *et al*., 2003; Wan *et al*., 2007; Coca and San Segundo, 2010). Interestingly, only a subset of genes of the complex *OsCPK* multigenic family was induced in response to *M. oryzae* elicitors, pointing to the specificity of this response. This specificity might be determined by the inductor, and accordingly, different *OsCPK* genes are induced by other fungal elicitors such as chitin (Wan *et al*., 2007) or N-acetylchitooligosaccharides (Akimoto-tomiyama *et al*., 2003). Only, the *OsCPK13* gene was found to be a general elicitor responder.

Our study also shows that the *OsCPK4* and *OsCPK10* genes are induced by pathogen infection. Both of them showed the same expression profile, being activated as soon as the fungal penetration structures are formed at 6 hpi, reached a maximum at 12 hpi, and returned to basal levels at 24 hpi, once the invasive phase of the *M. oryzae* life cycle is completed (Campos-Soriano and San Segundo, 2009). This timing coincides with the biotrophic growth of *M. oryzae*, in which the fungus grows within the host plant cells, surrounded by the invaginated plant plasma membrane and deriving nutrition from living plant cells. This expression profile suggests that *OsCPK4* and *OsCPK10* genes are not only responding to the fungal detection in the leaf surface, as elicitor treatment revealed, but also responding to the fungal leaf penetration since the induction is maintained until 24 hpi. Our expression data suggests that *OsCPK4* and *OsCPK10* are presumably defense related genes involved in the response to *M. oryzae* attack, particularly during its biothrophic phase. A similar expression profile was described for the *OsCPK9* gene induced by *M. oryzae* infection from 6 to 24 hpi with maximum accumulation levels at 12 hpi (Asano *et al*., 2005). Other rice *OsCPK* genes were reported as inducible by fungal infection, including the *OsCPK2*, *OsCPK15* and *OsCPK17* genes (Wan *et al*., 2007). Functional studies are required to determine the role of these genes in rice immunity.

Regarding the other *M. oryzae* elicitor-inducible *OsCPK5* and *OsCPK13* genes, they did not show a clear induction in response to the alive fungus as compared to control conditions. These observations suggest that they are not involved in the defense response to the fungal infection, at least during the biotrophic phase of *M. oryzae* infection. However, the expression of these genes under control conditions was highly variable and circadian clock dependent, which might mask a short response not

maintained for a long period of time. Surprisingly, we did not detect the induction upon pathogen recognition as expected from the elicitor results, probably due to the timing in which we monitored their expression in response to fungal infection. Further experiments are required to discard that these genes are not involved in the rice immune response.

The present study also shows a natural variation in the expression levels of these elicitor inducible *OsCPK* genes, which we could not associate to known pathogen resistant-susceptible phenotypes. The most important expression differences were observed amongst wild species, whereas the cultivated varieties showed more homogeneous expression levels. This might be due to the domestication process in which certain expression levels could be associated to a desirable character. Despite such homogeneity, some cultivars also showed notable differences for individual *OsCPKs.* Based on the information about all the registered rice varieties and species available at the International Rice Genebank collection (IRRI, www.irgcis.irri.org), we tried to establish some phenotypical associations with these differential expression levels. For the *OsCPK4* gene, the Razza 115, ARC 10276 and Ketan Jobe, *Javanica* cultivars, the Padi Santan Japonica cultivar, and the *O. nivara* species were the ones showing the highest expression levels. However, Ketan Jobe and Padi Santan are resistant to *M. oryzae* whereas ARC 10276 and Razza 155 are susceptible. Therefore, high expression levels are not directly correlated to blast disease resistance or susceptibility. Similarly, the more resistant varieties to blast disease are not the ones showing the highest expression levels of *OsCPK10* gene. Although, an interesting correlation to abiotic stress tolerance was found. The *O. barthii* species and the Co25 and IR64 *indica* cultivars accumulated the highest *OsCPK10* transcript levels, and are highly tolerant to drought stress and extreme temperature (Atwell et al., 2014; Lee et al., 2003). Finally, no correlations were also established for the *OsCPK13* and *OsCPK5* genes. This information is relevant for future rice breeding programs. All together, our studies identify the *OsCPK4* and *OsCPK10* genes as defense-related genes as candidates to modulate the resistance of rice plants to blast disease.

Experimental Procedures

Plant and fungal growth conditions

Rice plants were grown at 28ºC with a 14h/10h light/dark photoperiod during three weeks. Fungal strain *Magnaporthe oryzae* FR13 isolate (provided by D. Tharreau, CIRAD Montpelier, France) was grown in oatmeal agar (72.5g/L, 30mg/L cloramfenicol) for two weeks at 28ºC using a 16h/8h light/dark photoperiod. Spores were collected in sterile water, filtered with Miracloth (Calbiochem) and adjusted to the appropriate concentration using a Bürker counting chamber. *M. oryzae* elicitors were obtained as previously described (Casacuberta *et al*., 1992).

RNA isolation and qRT-PCR

Gene expression levels were determined from a pool of three leaves at the same developmental stage of 3-week-old soil-grown plants. Total RNA was extracted using TRIzol reagent (Invitrogen, Basel, Switzerland). DNAse treated RNA (1 µg) was retrotranscribed using the transcriptor first cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real-time PCR analyses were carried out in 96-well optical plates in a LightCycler® 480 System (Roche, Mannheim, Germany) according to the following program: 10 min at 95 °C, 45 cycles of 95 °C for 10s and 60 °C for 30s, and an additional cycle of dissociation curves to ensure a unique amplification. The reaction mixture contained 5µl of SYBR Green Master mix reagent (Roche), 2µl of 1:4 (Figures CI.1 and CI.2) or 1:5 (Figure CI.4) diluted cDNA sample and 300mM of each gene-specific primer (table CI.3) in a final volume of 10µl. The results for the gene expression were normalized to *OsUbi1* (LOC_Os06g46770) or *OsUbi5* (LOC_Os01g22490) genes. Three technical replicates were done for each sample.

Table CI.3: Primer sequences of genes used for gene expression analysis.

Gene name	Gene Locus	Primer sequences
OsCPK4	LOC_Os02g03410	For 5'-CGTGTGCAGCATGCAGATAA-3' Rev 5'-TGATTGCACGTATTCATCGCA-3'
OsCPK10	LOC_Os03g57450	For 5'-CAGAACAGTTTCAGCATCGGC-3' Rev 5'-CATTTTTTTCCCCGTTTCGAA-3'
OsCPK13	LOC_Os04g49510	For 5'-TGTCTTCCTGCCCAACGAAC-3' Rev 5'-TCAGAGTTGAGCAATGGCGT-3'
OsCPK5	LOC_Os02g46090	For 5'- GAGACGCACCTGGTGCACTA-3' Rev 5'- TCAAAGCTGCACTGTGGACG-3'
OsUbi1	LOC_Os06g46770	For 5'-TTCCCCAATGGAGCTATGGTT-3' Rev 5'-AAACGGGACACGACCAAGG-3'
OsUbi5	LOC_Os01g22490	For 5'-TAAGTGCGGCCTCACCTACG-3' Rev 5'-GGAGCCTACGCCTAAGCCTG-3'

References

Abbasi, F., Onodera, H., Toki, S., Tanaka, H., & Komatsu, S. (2004). OsCDPKI3, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. *Plant Molecular Biology*, **55**(4), 541–552.

Akimoto-tomiyama, C., Sakata, K., Yazaki, J., Nakamura, K., Shimbo, K., Yamamoto, K., Sasaki, T., Kishimoto, N., Shibuya, N., Minami, E. (2003). Rice gene expression in response to N-acetylchitooligosaccharide elicitor: comprehensive analysis by DNAmicroarray with randomly selected ESTs. *Plant Molecular Biology*, **52**(3), 537–551.

Asano, T., Hakata, M., Nakamura, H., Aoki, N., Komatsu, S., Ichikawa, H., Hirochika, H., Ohsugi, R. (2011). Functional characterisation of OsCPK21, a calcium-dependent protein kinase that confers salt tolerance in rice. *Plant Molecular Biology*, **75**(1), 179–191.

Asano, T., Hayashi, N., Kobayashi, M., Aoki, N., Miyao, A., Mitsuhara, I., Ichikawa, H., Komatsu, S., Hirochica, H., Kikuchi, S., Ohsugi, R. (2012). A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. *Plant Journal*, **69**(1), 26–36.

Asano, T., Tanaka, N., Yang, G., Hayashi, N., & Komatsu, S. (2005). Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: Comprehensive analysis of the CDPKs gene family in rice. *Plant and Cell Physiology*, **46**(2), 356–366.

Atwell, B. J., Wang, H., & Scafaro, A. P. (2014). Could abiotic stress tolerance in wild relatives of rice be used to improve Oryza sativa? *Plant Science : An International Journal of Experimental Plant Biology*, **215-216**, 48–58.

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., & San Segundo, B. (2014). Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. *Plant Physiology*, **165**(2), 688–704.

Campo, S., Peris-Peris, C., Siré, C., Moreno, A. B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C., San Segundo, B. (2013). Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. *New Phytologist*, **199**(1), 212–227.

Campos-Soriano, L., Gómez-Ariza, J., Bonfante, P., & San Segundo, B. (2011). A rice calcium-dependent protein kinase is expressed in cortical root cells during the presymbiotic phase of the arbuscular mycorrhizal symbiosis. *BMC Plant Biology*, **11**(1), 90.

Campos-Soriano, L., & San Segundo, B. (2009). Assessment of blast disease resistance in transgenic PRms rice using a gfp-expressing Magnaporthe oryzae strain. *Plant Pathology*, **58**(4), 677–689.

Casacuberta, J. M., Raventós, D., Puigdoménech, P., & San Segundo, B. (1992). Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. *MGG Molecular & General Genetics*, **234**(1), 97–104.

Cheng, S., Willmann, M. R., Chen, H., & Sheen, J. (2002). Update on Calcium Signaling through Protein Kinases . The Arabidopsis Calcium-Dependent Protein Kinase Gene Family 1. *Plant Physiology*, **129**, 469–485.

Chico, J. M., Raíces, M., Téllez-Iñón, M. T., & Ulloa, R. M. (2002). A calcium-dependent protein kinase is systemically induced upon wounding in tomato plants. *Plant Physiology*, **128**(1), 256–270.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., Dewdney, J. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. *Molecular Plant*, **1**(3), 423–45.

Fu, L., Yu, X., & An, C. (2013). Overexpression of constitutively active OsCPK10 increases Arabidopsis resistance against Pseudomonas syringae pv. tomato and rice resistance against Magnaporthe grisea. *Plant Physiology and Biochemistry*, **73**, 202–210.

Fu, L., Yu, X., & An, C. (2014). OsCPK20 positively regulates Arabidopsis resistance against Pseudomonas syringae pv. tomato and rice resistance against Magnaporthe grisea. *Acta Physiologiae Plantarum*, **36**(2), 273–282.

Harmon, A. C., Gribskov, M., Gubrium, E., & Harper, J. F. (2001). The CDPK superfamily of protein kinases. *New Phytologist*, **151**(1), 175–183.

Ho, S. L., Huang, L. F., Lu, C. A., He, S. L., Wang, C. C., Yu, S. P., Chen, J., Yu, S. M. (2013). Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. *Plant Molecular Biology*, **81**(4-5), 347–361.

Komatsu, S., Yang, G., Khan, M., Onodera, H., Toki, S., & Yamaguchi, M. (2007). Over-expression of calcium-dependent protein kinase 13 and calreticulin interacting protein 1 confers cold tolerance on rice plants. *Molecular Genetics and Genomics*, **277**(6), 713–723.

Kovach, M. J., Sweeney, M. T., & McCouch, S. R. (2007). New insights into the history of rice domestication. *Trends in Genetics*, **23**(11), 578–587.

Mandal, S., Kar, I., Mukherjee, A. K., & Acharya, P. (2013). Elicitor-Induced Defense Responses in Solanum lycopersicum against Ralstonia solanacearum. *The Scientific World Journal*, **2013**, 561056.

Manimaran, P., Mangrauthia, S. K., Sundaram, R. M., & Balachandran, S. M. (2015). Constitutive expression and silencing of a novel seed specific calcium dependent protein kinase gene in rice reveals its role in grain filling. *Journal of Plant Physiology*, **174**, 41–48.

Mehlmer, N., Wurzinger, B., Stael, S., Hofmann-Rodrigues, D., Csaszar, E., Pfister, B., Bayer, R., Teige, M. (2010). The Ca2+-dependent protein kinase CPK3 is required for MAPK-independent salt-stress acclimation in Arabidopsis. *Plant Journal*, **63**(3), 484–498.

Murillo, I., Jaeck, E., Cordero, Mj., & San Segundo, B. (2001). Transcriptional activation of a maize calcium-dependent protein kinase gene in response to fungal elicitors and infection. *Plant Molecular Biology*, **45**(2), 145–158.

Ray, S., Agarwal, P., Arora, R., Kapoor, S., & Tyagi, A. K. (2007). Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (Oryza sativa L. ssp. indica). *Molecular Genetics and Genomics*, **278**(5), 493–505.

Romeis, T., Ludwig, A. A., Martin, R., & Jones, J. D. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. *The EMBO Journal*, **20**(20), 5556–5567.

Saijo, Y., Kinoshita, N., Ishiyama, K., Hata, S., Kyozuka, J., Hayakawa, T., Nakamura, T., Shimamoto, K., Yamaya, T., Izui, K. (2001). A Ca(2+)-dependent protein kinase that endows rice plants with cold- and salt-stress tolerance functions in vascular bundles. *Plant & Cell Physiology*, **42**(11), 1228–1233.

Sang, T., & Ge, S. (2007). Genetics and phylogenetics of rice domestication. *Current Opinion in Genetics and Development*, ***17***, 533–538.

Wan, B., Lin, Y., & Mou, T. (2007). Expression of rice Ca2+-dependent protein kinases (CDPKs) genes under different environmental stresses. *FEBS Letters*, **581**(6), 1179–1189.

Wilson, R. a, & Talbot, N. J. (2009). Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. *Nature Reviews. Microbiology*, **7**(3), 185–195.

CHAPTER II

Functional characterization of *OsCPK4* in the rice defense response

Enhancing blast disease resistance by overexpression of the calcium-dependent protein kinase OsCPK4 in rice

Mireia Bundó and María Coca*

Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB. Edifici CRAG, Campus de la UAB, 08193 Bellaterra, Barcelona, Spain

*Corresponding author: email: maria.coca@cragenomica.es

Article submitted to Plant Biotechnology Journal (second round review).

Abstract

Rice is the most important staple food for more than half of the human population, and blast disease is the most serious disease affecting global rice production. In this work, the isoform OsCPK4 of the rice calcium dependent protein kinase family is reported as a regulator of rice immunity to blast fungal infection. It shows that overexpression of *OsCPK4* gene in rice plants enhances resistance to blast disease by preventing fungal penetration. The constitutive accumulation of OsCPK4 protein prepares rice plants for a rapid and potentiated defense response, including the production of reactive oxygen species, callose deposition and defense gene expression. *OsCPK4* overexpression leads also to constitutive increased content of the glucosilated salicylic acid hormone in leaves without compromising rice yield. Given that *OsCPK4* overexpression was known to confer also salt and drought tolerance in rice, the results reported in this paper demonstrate that OsCPK4 acts as a convergence component that positively modulates both biotic and abiotic signaling pathways. All together, our findings indicate that OsCPK4 is a potential molecular target to improve not only abiotic stress tolerance, but also blast disease resistance of rice crops.

Introduction

Rice blast disease, caused by the filamentous ascomycete fungus *Magnaporthe oryzae,* is the most important rice disease due to its severity and wide distribution (approximately 85 countries around the world) (Ou *et al.* 1987). *M. oryzae* attacks rice plants at all developmental stages, more often during the seedling stage, and it can infect leaves, stems, nodes, collars and panicles (Dean *et al.*, 2012). Rice blast causes severe crop loses varying from 10 to 85% depending on the area and climatology (Skamnioti and Gurr, 2009) (http://www.irri.org/research/better-rice-varieties/disease-and-pest-resistant-rice). Resistant cultivars and pesticides have traditionally been used to control this disease. However, the fungus *M. oryzae* overcomes host resistance quickly and resistant cultivars become ineffective after a few years (Lee *et al.*, 2009). Pesticide use, on the other hand, is costly and environmentally unfriendly. Being rice a paramount source of human food, new strategies providing long-term blast protection should therefore be developed. The study of the plant defense responses offers a vast field of possibilities to improve disease resistance in rice.

In addition to structural barriers and preformed antimicrobial compounds, plants have evolved inducible immune responses to defend themselves against pathogen attack. The defense response starts with the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) that activate the PAMP-triggered immunity (PTI) (Boller and He, 2009; Chisholm *et al.*, 2006; Jones and Dangl, 2006). Successful pathogens have evolved to suppress the PTI response by the action of effectors. But, plants in turn have evolved a second defense layer, known as effector-triggered immunity (ETI), consisting of resistance proteins that recognize these effectors (Jones and Dangl, 2006). Both PTI and ETI counteract the pathogen attack by inducing immune responses (Tsuda and Katagiri, 2010). The earliest defense reactions include changes in ion fluxes across membranes, an increase in the intracellular calcium concentration, the activation of protein kinases, or the synthesis of reactive oxygen species (ROS) (Baxter *et al.*, 2013; Lecourieux *et al.*, 2006; Meng and Zhang, 2013; Tena *et al.*, 2011; Torres, 2010; Seybold *et al.*, 2014). Forward reactions consist of transcriptional reprogramming, alterations in hormone status and cell-wall reinforcement through callose depositions and lignifications and in some cases even by

cell death at the site of infection (Liu *et al.*, 2014; Luna *et al.*, 2010; Navarro *et al.*, 2004; Tsuda and Katagiri, 2010). Defense responses locally activated in primary pathogen-infected plant tissues are often extended to distal non-infected tissues, conferring systemic acquired resistance (SAR) (Durrant and Dong, 2004; Ryals *et al.*, 1996). This resistance is long-lasting and effective against secondary attack by unrelated pathogens. SAR is associated to the signal molecule salicylic acid (SA) and the accumulation of pathogenesis-related (PR) proteins which are thought to contribute to resistance (Durrant and Dong, 2004).

Calcium influx is one of the earliest events upon pathogen recognition in plant defense response (Ranf *et al.*, 2011). Alterations in calcium concentration are sensed by calcium-binding proteins, including calmodulin, calcium-dependent protein kinases (CDPK or CPKs) and calcineurin B-like proteins, which relay the calcium signal into specific cellular and physiological responses (Harper *et al.*, 2004; Dodd *et al.*, 2010). CPKs represent unique calcium sensors able to translate calcium signals directly into phosphorylation events, because they combine in a single molecule a calcium binding domain and a serine/threonine kinase domain (Harper *et al.*, 2004). In this sense, genetic and biochemical studies have demonstrated that these plant proteins are important players in numerous signaling pathways and biological processes, including stress signaling cascades and immune signaling responses (Boudsocq and Sheen, 2013; Romeis and Herde, 2014; Schulz *et al.*, 2013)

CPKs are encoded by large gene families, the rice genome containing 31 *CPK* genes (Asano *et al.*, 2005; Ray *et al.*, 2007). In contrast to Arabidopsis CPKs, little is known about the functions of specific rice CPKs. Among the ones functionally characterized are the OsCPK13 (Saijo *et al.*, 2000), OsCPK12 (Asano *et al.*, 2012) and OsCPK9 (Wei *et al.*, 2014) proteins that have been reported as signaling components of abiotic stress responses; and the OsCPK10 (Fu *et al.,* 2013) and the OsCPK18 (Xie *et al.*, 2014) described as positive and negative regulators of *M. oryzae* resistance, respectively. Only OsCPK12 has been shown to be involved in both abiotic and biotic stress signaling (Asano et al., 2013). Recently our group reported that OsCPK4 positively regulates salt and drought stress adaptation (Campo *et al.*, 2014). Contrary to OsCPK12 that oppositely modulates the different signaling pathways; the present study reports that

OsCPK4 is also a positive regulator of immunity in rice. OsCPK4 overexpression confers enhanced resistance to blast disease in rice plants by preventing *M. oryzae* fungal penetration. The enhanced resistance phenotype is associated to the constitutive accumulation of conjugated SA and callose, and a fast and stronger activation of defense responses, including ROS production and defense gene expression, without compromising rice productivity.

Results

OsCPK4 expression is induced by *M. oryzae* infection in rice plants

A search for altered expression genes in a microarray-based global transcriptomic analysis of rice plants in response to *M. oryzae* elicitors (Campo *et al.*, 2013) identified the *OsCPK4* gene as an upregulated gene in leaves after 2 hours treatment (fold change= 1.94; p-value=0.0002). The *OsCPK4* gene (LOC_Os02g03410) encodes a CPK involved in the adaptation of rice plants to salinity and drought conditions (Campo *et al.*, 2014). To confirm that *OsCPK4* gene expression is altered during the defense response of rice plants, it was examined in leaves at different times after inoculation with *M. oryzae* spores (Figure CII.1a). *OsCPK4* expression was rapid and strongly induced in rice leaves at earlier stages of infection at 6 hours post-inoculation (hpi), coinciding with the formation of the fungal infective structure, named appresorium (Wilson and Talbot, 2009). *OsCPK4* activation increased until 12 hpi (approximately an 8 fold-increase) and started to decrease at 24 hpi, once fungal penetration had already occurred. These observations show that *OsCPK4* is an early response gene against *M. oryzae* infection in rice leaves.

OsCPK4 protein accumulation was also examined in blast infected leaves. In agreement with *OsCPK4* transcript levels, Western-blot analyses showed an increase in the accumulation of the encoded protein after pathogen inoculation (Figure CII.1b). These results indicate that *OsCPK4* transcriptional activation is translated in the protein accumulation, and suggest that the OsCPK4 protein is involved in the defense response of rice plants to *M. oryzae* infection.

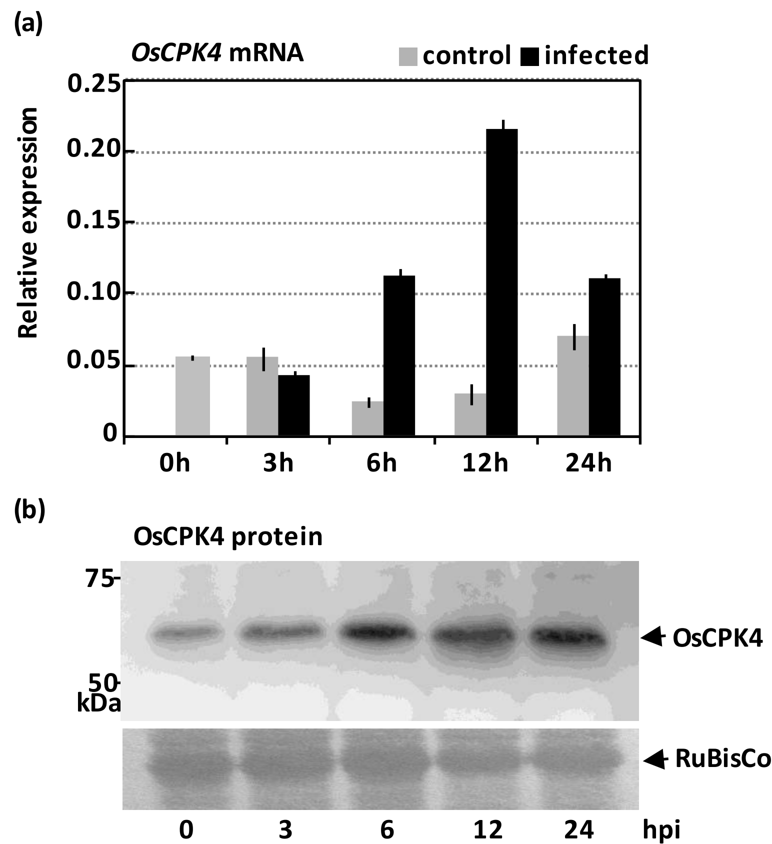

Figure CII.1: *OsCPK4* expression and protein accumulation in response to fungal infection. (a) Transcript levels were determined by qRT-PCR analysis in rice leaves (*Oryza sativa* cultivar Nipponbare) after inoculation with a *M. oryzae* spore suspension (10^5 spores/ml) at the indicated period of time. Specific primers were used to detect the *OsCPK4* mRNA levels that were normalized to the *OsUbi5* mRNAs. Error bars indicate SEM of three replicates. (b) OsCPK4 accumulation was determined by Western-blot analysis using specific anti-OsCPK4 antibodies at indicated period of time after inoculation. Lower panel corresponds to Ponceau staining of protein samples (40 µg per lane). Leaves from four different plants grown in soil for three-weeks were collected in a pool at each different time for total RNA (a) or total protein extraction (b). Results are representative of two independent experiments.

OsCPK4 overexpressor rice plants are more resistant to *M. oryzae* infection

To further investigate the function of *OsCPK4* in rice immunity we used the transgenic *OsCPK4* overexpressing rice plants previously described (Campo *et al.*, 2014). These plants were produced in the japonica cultivar Nipponbare, and expressed the *OsCPK4* full-length cDNA under the control of the strong and constitutive *ZmUbi1* promoter. Quantitative RT-PCR analyses confirmed that the expression of *OsCPK4* was indeed significantly enhanced in leaves of *OsCPK4-Ox* plants in comparison with wild-type or control empty vector plants (Figure CII.2a), resulting also in an increased accumulation of the corresponding protein (Figure CII.2b). The activity of the accumulated protein is

dependent on the presence of calcium (Figure CII.2c, d), suggesting that it remains as a latent protein in the rice leaves prone to be stimulated by calcium changes.

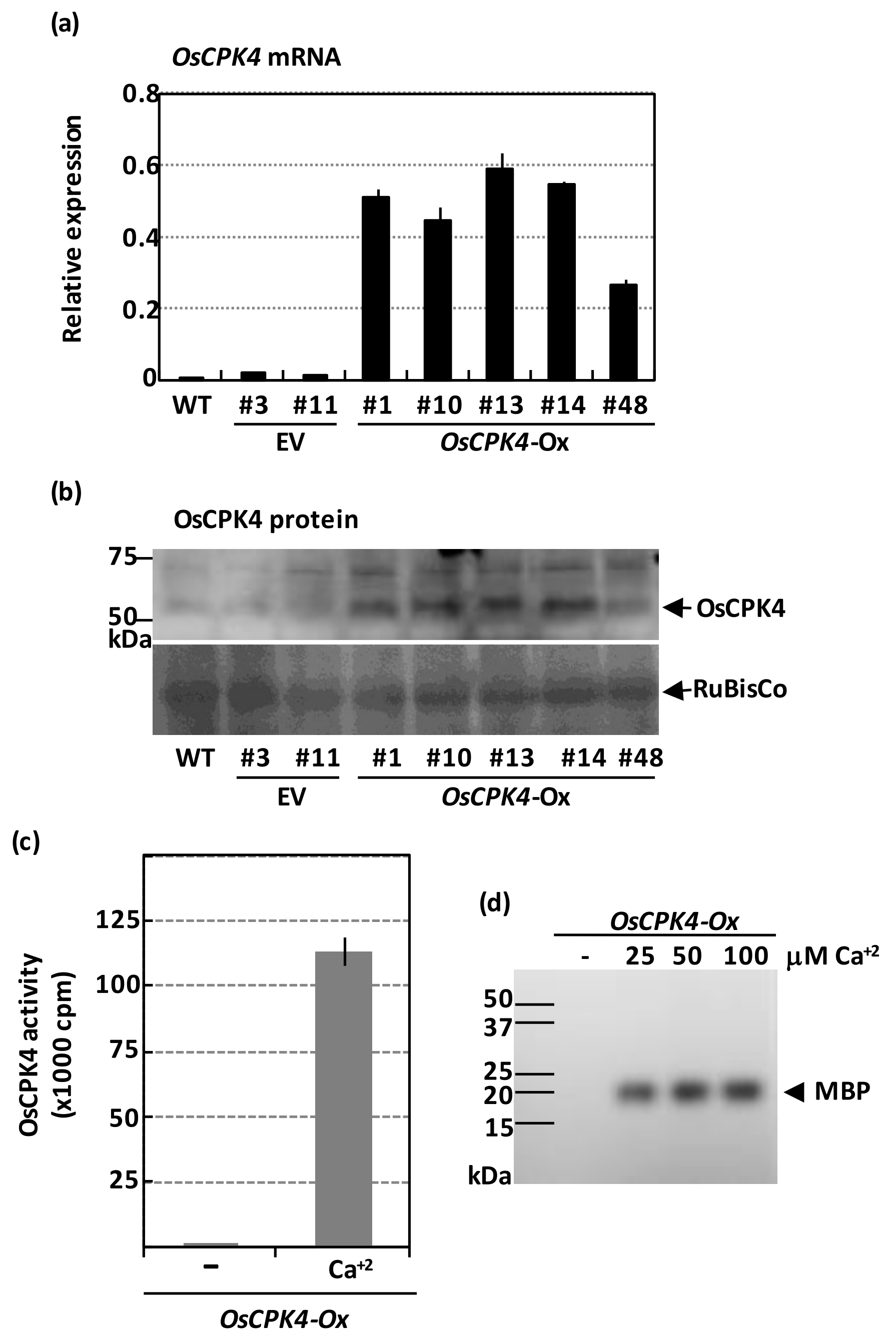

Figure CII.2: OsCPK4 accumulation and activity in transgenic rice leaves. (a) *OsCPK4* transcript levels as determined by qRT-PCR analysis normalized to *OsUbi1* transcripts. Values represent the mean and SEM of three replicates (b) OsCPK4 protein accumulation as determined by Western-blot analysis using anti-OsCPK4 antibodies. Lower panel shows the Ponceau staining of protein samples. Analyses were performed with leaves of wild-type (WT), empty vector (EV) and *OsCPK4*-overexpressor (*OsCPK4*-Ox) 3-week old plants. (c-d) Calcium dependent activity of immunoprecipitates from leaves of *OsCPK4*-Ox line #1 on casein (c) or myelin basic protein (MBP) (d). *In vitro* phosphorylation activity was determined by liquid scintillation counting and expressed in c.p.m (c) or monitored in SDS-PAGE (d). Calcium was added to the phosphorylation reactions at the free calcium concentration of 100 mM (c) or as indicated (d) or absence (-). Values are the mean of two independent measures of two independent assays and bars correspond to standard deviations.

The phenotype of *OsCPK4-Ox* lines, compared to wild-type or empty vector plants, was then characterized when challenged with the blast fungus using a detached leaf assay (Coca *et al.*, 2004). Following inoculation with the *M. oryzae* virulent strain FR13, the *OsCPK4*-Ox leaves developed less severe disease symptoms than control leaves (Figure CII.3a). At 7 dpi (days post-infection), extensive necrotic lesions with fungal sporulation were macroscopically observed on wild-type and empty-vector leaves, whereas only few lesions were developed on the *OsCPK4*-Ox leaves. The percentage of leaf area affected by blast lesions was determined by image analyses. The results revealed a statistically significant reduction on the lesion area of three independent transgenic lines as compared to control leaves (Figure CII.3b). In agreement with visual inspection, *OsCPK4*-Ox leaves contained significant less fungal biomass than control leaves, as determined by qPCR analysis of *M. oryzae* DNA (Figure CII.3c). The enhanced resistance phenotype to the blast fungus exhibited by *OsCPK4-Ox* leaves was then confirmed by whole plant infection assays. In this case, rice plants were spray-inoculated with a *M. oryzae* spore suspension, under experimental conditions similar to field conditions. The wild-type and empty vector control plants developed the typical blast disease lesions, whereas the *OsCPK4-Ox* plants showed clearly less and smaller infection lesions (Figure CII.3d). Further measure of disease severity showed that a higher percentage of *OsCPK4*-Ox plants exhibited resistant phenotype (around 22%) than wild-type or empty vector plants (around 5-10%), and a lower percentage exhibited highly susceptible phenotype (around 27%) than control plants (65%) (Figure CII.3e). Collectively, these results suggest that *OsCPK4* positively mediates enhanced resistance to blast fungal infection.

To gain more insight into the nature of the enhanced blast resistance observed in the *OsCPK4*-Ox plants, the infection process and fungal development in rice leaves was investigated by fluorescence microscopy analysis using a *GFP*-expressing *M. oryzae* virulent strain (GFP-Guy11). *GFP* expression is reported not to affect the pathogenicity of *M. oryzae* fungal strains (Campos-Soriano and San Segundo, 2009; Sesma and Osbourn, 2004). At early infection stages (12 hpi), *M. oryzae* spores were easily visualized on the leaf surface of the rice plants by fluorescence confocal microscopy (Figure CII.4a-d). Most of the spores on wild-type and empty vector leaves were germinated and produced short germ tubes that developed appresoria and invasive

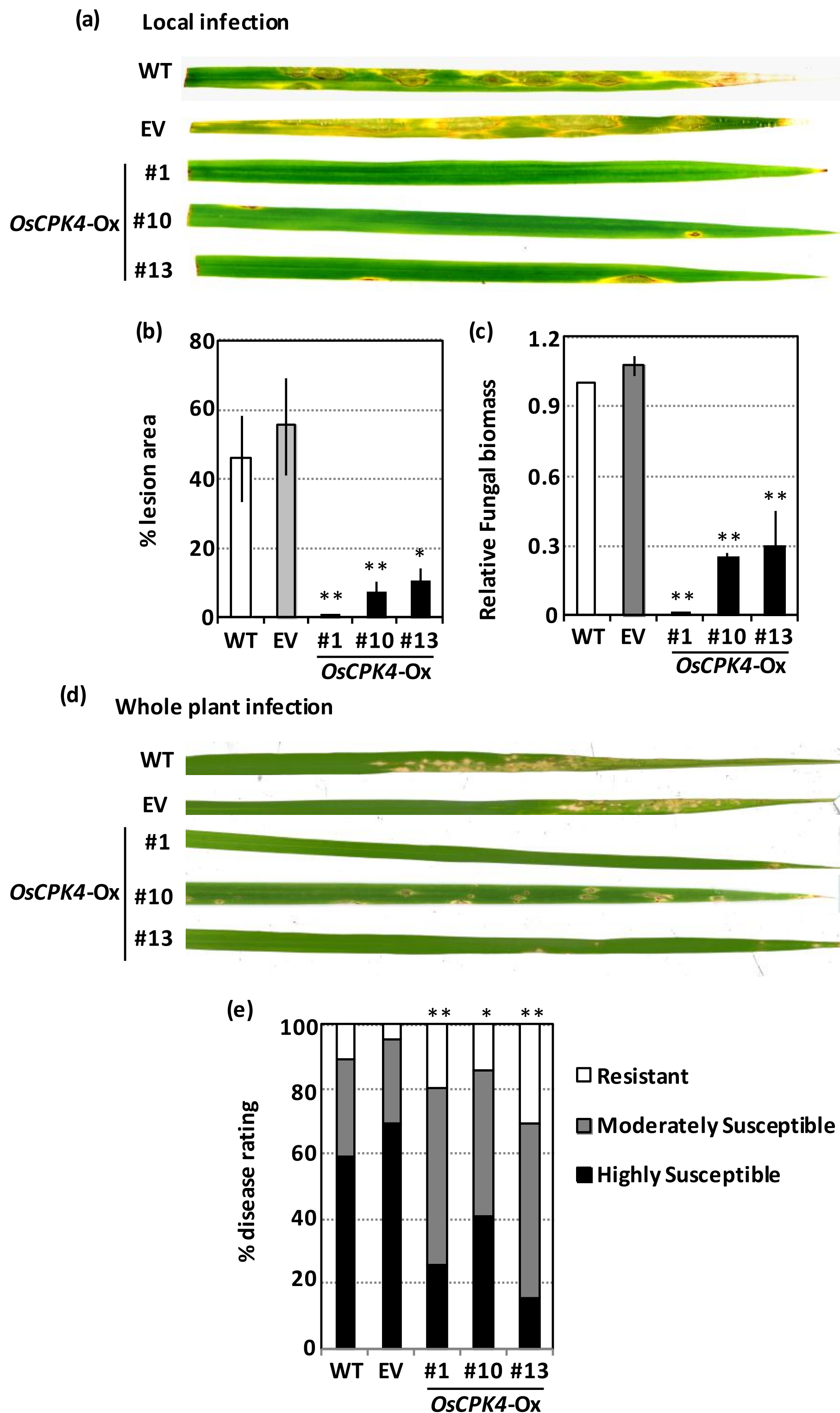

Figure CII.3: *OsCPK4* overexpressing plants are more resistant to *Magnaporthe oryzae* infection. (a) Rice disease lesions caused by *M. oryzae* locally inoculated (10^5 spores/ml) on leaves of wild-type (WT), empty vector (EV) and *OsCPK4*-Ox plants (lines #1, #10 and #13) at 7dpi. (b) Percentage average of lesion area per leaf of three independent assays with three replicates per line at 7 dpi. (c) Relative fungal amount as determined by qPCR of *M. oryzae* 26S rDNA gene compared to *OsUbi1* gene and referred to WT. Values correspond to the average of three independent assays in which three leaves were used for quantifications. (d) Disease lesions on leaves from spray-inoculated whole rice plants with *M. oryzae* spore suspension (10^5 spores/ml) at 7dpi. (e) Disease rating for ten plants per line at 7 dpi following the Standard Evaluation System for blast disease (IRRI, 2002) based on leaf lesion area percentage. Mean values of 2 independent assays. Asterisks represent significant differences (one-way ANOVA analysis and Tukey's test; *P≤0.05, **P≤0.01).

hyphae penetrating into epidermal cells (Figure CII.4a-b, e). However, *M. oryzae* spores on *OsCPK4*-Ox leaves germinated freely developing abnormal germ tubes, in some cases thick and highly vacuolated (Figure 4c) while, in others, thin and very long (Figure CII.4d), without visible evidences of penetration events (Figure CII.4f-g). These observations support that fungal penetration was impaired in *OsCPK4*-Ox leaves. After 2 dpi, infection lesions were visible under fluorescent microscopy in control leaves (Figure CII.4h), but not in *OsCPK*4-Ox leaves (Figure CII.4i). At later stages (7 dpi), *M. oryzae* completed its lifecycle in wild-type and empty vector leaves showing the typical blast lesions with a bright fluorescent mycelia growing and sporulating (Figure CII.4j-k). Only small necrotic spots were observed in the OsCPK4-Ox leaves (Figure CII.4l-m). Our observations indicate that OsCPK4-mediated resistance relies in the interference with fungal penetration rather than colonization.

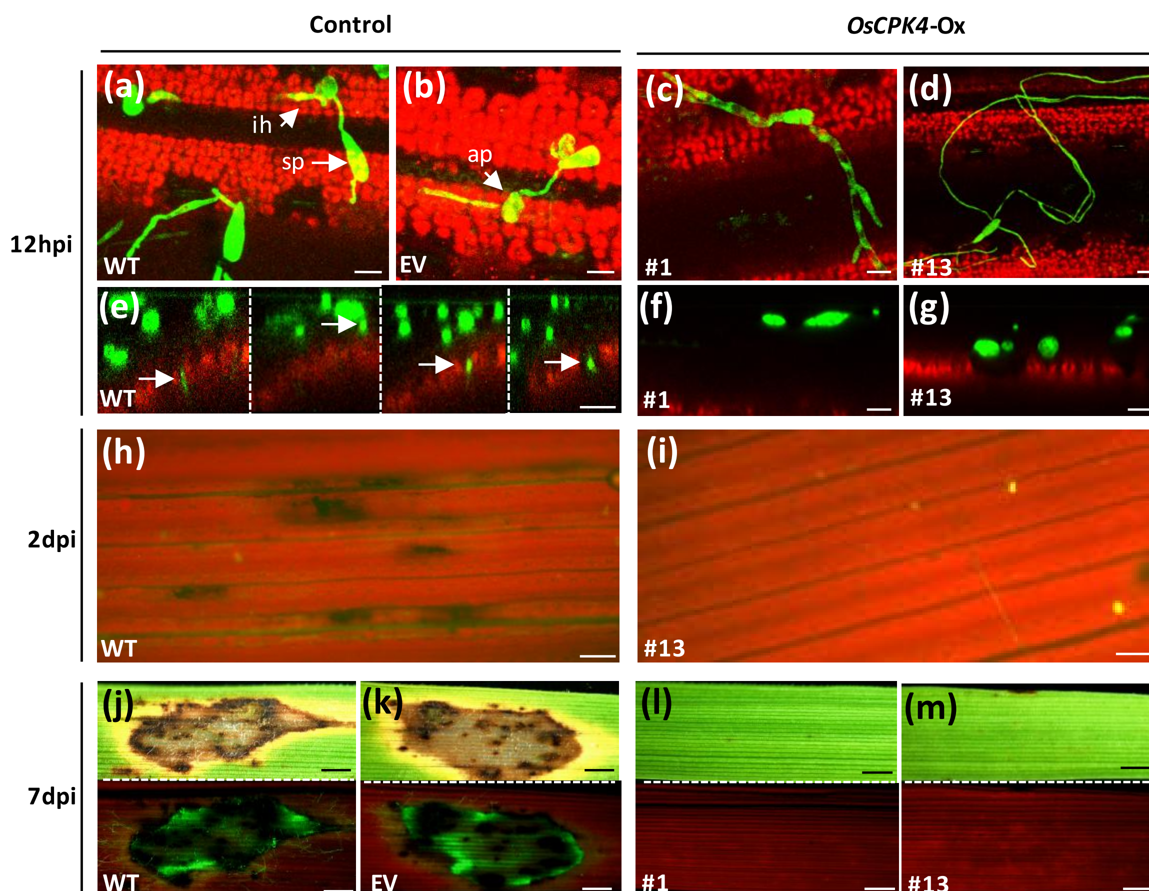

Figure CII.4: Microscopic analysis of *Magnaporthe oryzae* infection process on rice leaves. Representative images of *OsCPK4* overexpressor (lines 1 and 13), wild-type (WT) and control empty vector (EV) leaves inoculated with the GFP-*M. oryzae* spores (10^5 spores/ml). (a-g) Images of confocal laser microscopy of leaves at 12 hpi, corresponding to projections (a-d) and xz slides (e-g) Epifluorescence images at 2 dpi (h-i) or 7dpi (j-m, lower panels). (j-m, upper panels) Steroscopic bright field images. Bars = 10 µm (a-g), 100 µm (h-i), 1 mm (j-m). Key: sp, spore; ap, appresorium; ih, invasive hypha.

The resistance of *OsCPK4*-Ox plants to other rice pathogens was also evaluated. Seedlings were assayed against the seed-borne and soil-transmitted fungal pathogen *F. verticillioides*, which has been associated with the bakanae disease in rice (Wulff *et al.,* 2010). Our results indicate that *OsCPK4*-Ox seedlings are as susceptible to *F. vertillioides* infection as control wild-type and empty vector plants (Figure CII.5). Similarly, *OsCPK4*-Ox seedlings were equally susceptible as control seedlings when challenged with the bacterial pathogen *Dickeya dadantii*, previously known as *Erwinia chrysanthemi,* the causal agent of foot rot in rice (Goto, 1979; Mansfield *et al.*, 2012). These results suggest that the enhanced resistance to *M. oryzae* shown by *OsCPK4*-Ox plants is specific against this fungal pathogen, and that it does not affect their defense against other rice pathogens with different pathogenesis mechanisms.

Defense response is early activated in *OsCPK4* overexpressor rice plants

One of the earliest defense reactions is the production of ROS, a hallmark of successful pathogen recognition and activation of plant defense response (Torres, 2010). Since OsCPK4 interferes with the *M. oryzae* infection process at early stages, the ROS production during defense responses in *OsCPK4*-Ox rice leaves was investigated. ROS formation was monitored *in vivo* using the CM-H_2DCFDA probe, a non-invasive fluorescent ROS indicator (Kristiansen *et al.*, 2009). Microscopic analyses showed the induction of fluorescence in rice leaves in response to elicitor treatment, which was faster and stronger in the *OsCPK4* than in wild-type or control empty vector leaves (Figure CII.6a). Thirty minutes after elicitor treatment, fluorescence was barely visualized in the wild-type or empty vector leaves, but clearly visible in the leaves of two independent *OsCPK4*-Ox lines (Figure CII.6a, middle panels). At 1 hour treatment, the ROS formation was already detected in the wild-type and empty vector leaves, although a stronger fluorescent labeling was observed in the *OsCPK4* lines (Figure CII.6a, lower panels). Fluorescence quantification showed significant differences in intensity and timing of ROS formation between *OsCPK4*-Ox and control lines (Figure CII.6b). Similarly, ROS production was significantly stronger in the *OsCPK4*-Ox leaves compared to control leaves in response to *M. oryzae* spore inoculation (Figure CII.6c-d). These observations suggest that OsCPK4 accumulation mediates accelerated and potentiated ROS formation in response to *M. oryzae* infection in rice leaves. Another defense hallmark is the callose deposition to fortify cell walls that avoids pathogen

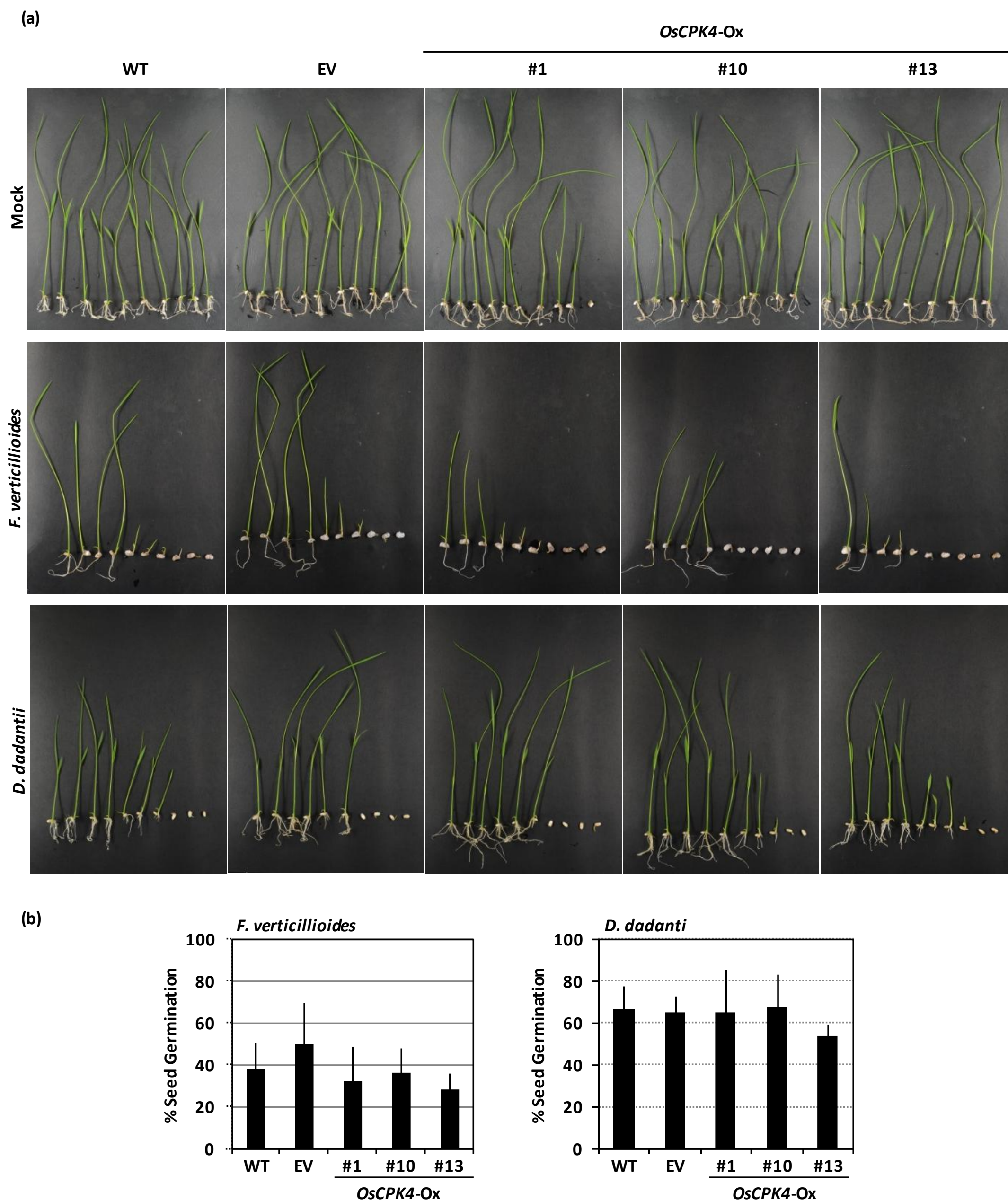

Figure CII.5: *OsCPK4* overexpressing plants are not more resistant to *Fusarium verticillioides* and *Erwinia chrysanthemi*. (a) Representative images of *F. verticillioides* and *E. chrysanthemi* infections, showing the seed germination in control conditions (first line), the phenotype with 10^3 spore/ml of *F. verticillioides* at 7dpi (secong line) and the phenotype with 10^7 cfu (colony forming unit)/ml of *E. chrysanthemi* at 7dpi (third line). (b) Percentage of seed germination in the *F. verticillioides* and *E. chrysanthemi* infeccion assays. Values represent means and SE of 5 independent infections assays with 10 seeds per line in each one.

penetration into the plant cell (Luna *et al.*, 2010; Voigt, 2014). Given that *OsCPK4* overexpression prevents fungal penetration, the callose accumulation was analyzed in *OsCPK4*-Ox leaves. Callose was clearly visualized after aniline blue staining as intense blue-green fluorescence under UV light in the epidermal cell walls of *OsCPK4*-Ox leaves

(Figure CII.7a). Quantification of fluorescent leaf area indicated that callose was more abundantly accumulated in the cell walls of *OsCPK4*-Ox leaves inoculated with *M. oryzae* spores (24 hpi) than in non-inoculated leaves (Figure CII.7b).

Under the same experimental conditions, callose fluorescence was not detected in control plant leaves. These observations indicate that *OsCPK4* overexpression mediates the constitutive accumulation of callose, and its stronger deposition in response to pathogen infection in rice leaves.

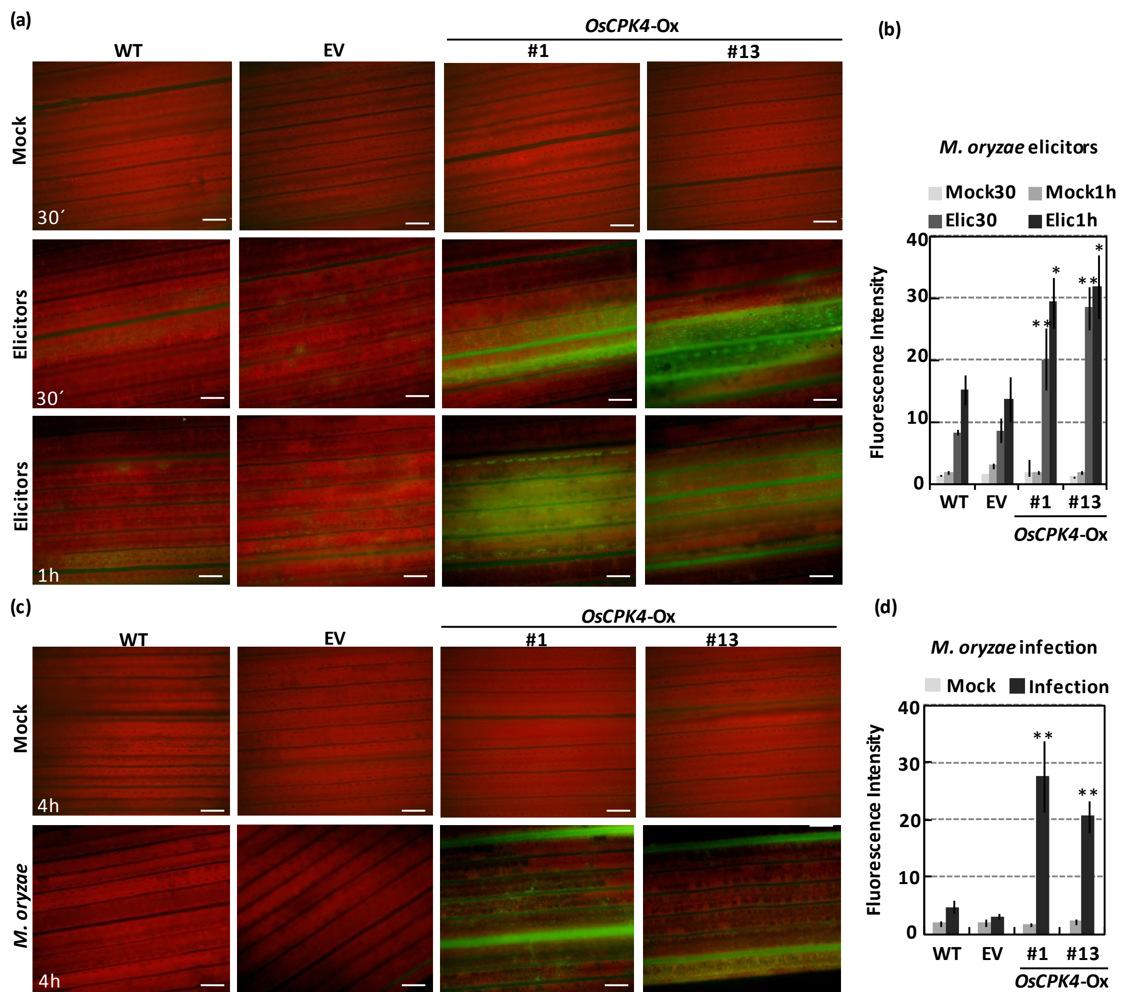

Figure CII.6: Rapid and strong ROS formation in *OsCPK4* overexpressing leaves during defense response. Representative epifluorescence microscopy images of wild-type (WT), control empty vector (EV) and *OsCPK4* overexpressor (*OsCPK4*-Ox, lines 1 and 13) leaves after 1 h vacuum infiltration with a 10 µM CM-H_2DCFDA solution and treated with (a) *M. oryzae* elicitors (1%) or mock solution; and (c) spore suspension (10^5 spores/ml) or mock solution for the indicated period of time. (b, d) Quantitative comparison of fluorescence intensities in elicitor treated leaves (b) and fungal-inoculated leaves (d). Values represent the average intensities, and error bars the SD of three independent leaves. Asterisks denote significant differences (One-way ANOVA analysis and Tukey's test, *P≤0.05, **P≤0.001). Results are representative of two independent experiments. Scale bar = 200 µm.

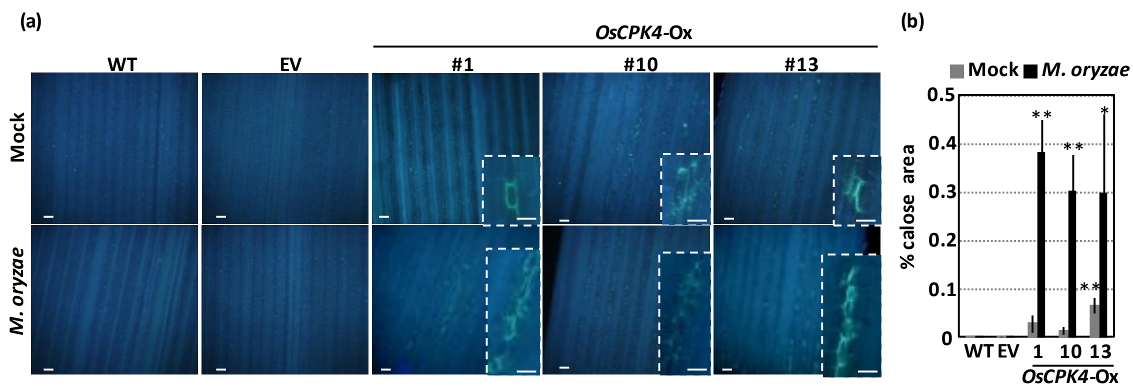

Figure CII.7: Callose deposition in *OsCPK4* overexpressing rice leaves. (a) Images of wild-type (WT), empty-vector (EV) or *OsCPK4*-overexpressing (*OsCPK4*-Ox) leaves (lines #1, #10 and #13) from three-week old plants locally inoculated with *M. oryzae* spore suspensions (10^5 spores/ml) or mock solution. Leaves were stained with aniline blue and visualized under UV epifluorescence microscopy at 24 hpi. Magnifications are shown in inset boxes. Bars correspond to 100 µm, and 50 µm in inset boxes (b) Mean values of the percentage of fluorescent area per leaf of three independent replicas per line in three independent assays (total 9 leaves per line). Asterisks denote significant differences (One-way ANOVA analysis and Tukey's test, *P≤0.05, **P≤0.001).

Defense gene expression is potentiated in *OsCPK4* overexpressor rice plants

To further investigate the mechanism underlying OsCPK4-mediated disease resistance, the expression profile of rice defense genes was analyzed in the transgenic plants in response to *M. oryzae* infection. First, the expression of the widely used defense marker *OsPBZ1* and *OsPR5* genes was monitored. These genes encode two SA-regulated pathogenesis related proteins from the PR10 and PR5 families (Datta *et al.*, 1999; Jwa *et al.*, 2006; Midoh and Iwata, 1996; Rakwal *et al.*, 2001). Stronger induction of these two defense genes was observed in *OsCPK4*-Ox plants when compared against wild-type or empty vector control plants upon pathogen challenge (Figure CII.8a-b). These observations suggest that the *OsCPK4*-Ox plants developed a potentiated defense compared to control plants.

Similarly, the analysis of defense signaling components *OsNPR1/OsNH1* and *OsWRKY45* genes showed stronger induction in the *OsCPK4*-Ox plants than in the control plants (Figure CII.8c-d). The two genes encode a transcriptional cofactor and transcriptional factor of the SA-mediated defense pathway (Chern *et al.*, 2001; Shimono *et al.*, 2012). Additionally, upstream components, such as the *OsEDS1* gene

encoding an activator of SA signaling (Wiermer *et al.*, 2005), or the *OsSID2* gene plants encoding the isochorismate synthase enzyme responsible for part of SA synthesis in (Wildermuth *et al.*, 2001), also showed stronger activation in *OsCPK4-Ox* plants pathway in *OsCPK4*-Ox plants that might mediate its enhanced resistance to *M. oryzae*.

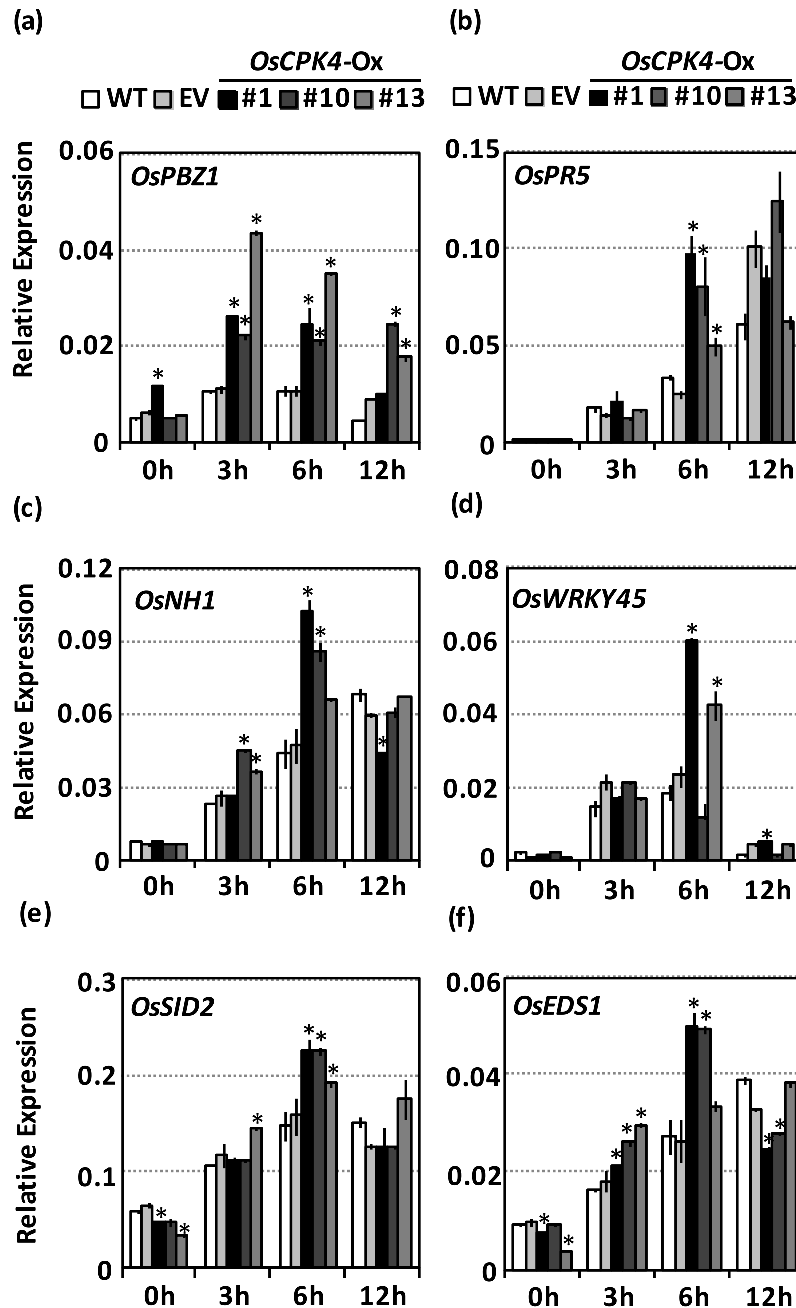

Figure CII.8: Defense gene expression in *OsCPK4* overexpressor plants in response to *Magnaporthe oryzae* infection. Leaves of wild-type (WT), empty vector (EV) and *OsCPK4*-Ox (lines #1, #10, #13) plants were locally inoculated with a *M. oryzae* spore suspension (10^5 spores/ml), and collected in a pool of 4 leaves at the indicated period of time. Expression levels of indicated defense-related genes were determined by qRT-PCR and normalized to *OsUbi1*. Asterisks denote significant differences (one-way ANOVA and Tukey's test, *P≤0.01). Results are representative of two independent experiments.

Overexpression of *OsCPK4* leads to increased SA content without compromising rice productivity

The observed strong induction of *OsSID2* gene expression, as well as of other genes related to SA defense signaling, prompted us to quantify the SA content in the *OsCPK4*-Ox lines. We determined the levels of free SA and its glucose conjugate (SAG) under control conditions. No significant differences in free-SA levels were detected, but *OsCPK4-Ox* leaves accumulated up to twice as much SAG as compared to the control empty vector or wild-type leaves (Figure CII.9). Our results indicate that the overexpression of *OsCPK4* leads to the accumulation of SAG in rice leaves under control conditions, which in turn results in the strong activation of downstream SA-mediated defense upon pathogen infection, as revealed by our gene expression studies.

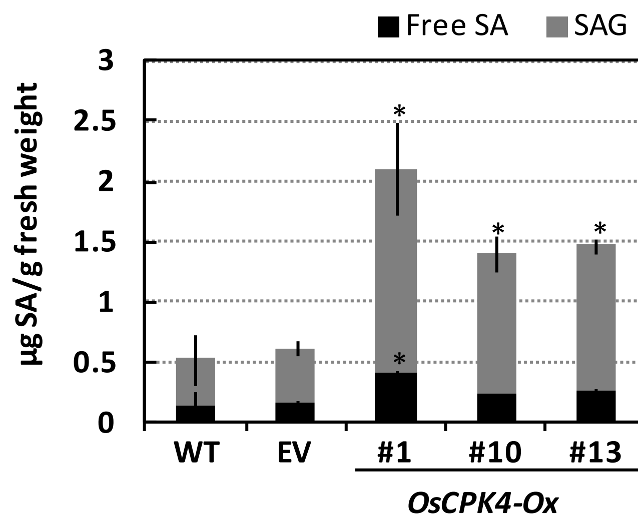

Figure CII.9: Increased content of total SA, free SA and glucoside conjugate (SAG) in *OsCPK4* overexpressor plants. Data are mean values of two independent quantifications in a pool of 3 leaves from three-week old wild-type (WT), empty vector (EV) or *OsCPK4*-overexpressor (*OsCPK4*-Ox) plants. Asterisk denotes significant differences (one-way ANOVA analysis, *P<0.05).

The constitutive accumulation of SA is often associated to disease resistance but is also accompanied by fitness costs; that is, a penalty in plant growth and productivity (Takatsuji, 2014). To determine the effects of detected high SAG levels in *OsCPK4* rice plants, several fitness parameters of plant growth under controlled conditions were analyzed. *OsCPK4*-Ox plants showed similar appearance than control wild-type and empty vector plants (Figure CII.10a). They reached the same height at heading time

(Figure CII.10b), flowered at the same period of time after sowing (Figure CII.10c) and, more importantly, produced similar grain yield in two different experiments in which plants were grown under random distribution (Figure CII.10d). Hence, despite that OsCPK4-mediated SAG accumulation, our observations indicate that *OsCPK4* overexpression does not have a negative impact in the growth and productivity of rice plants.

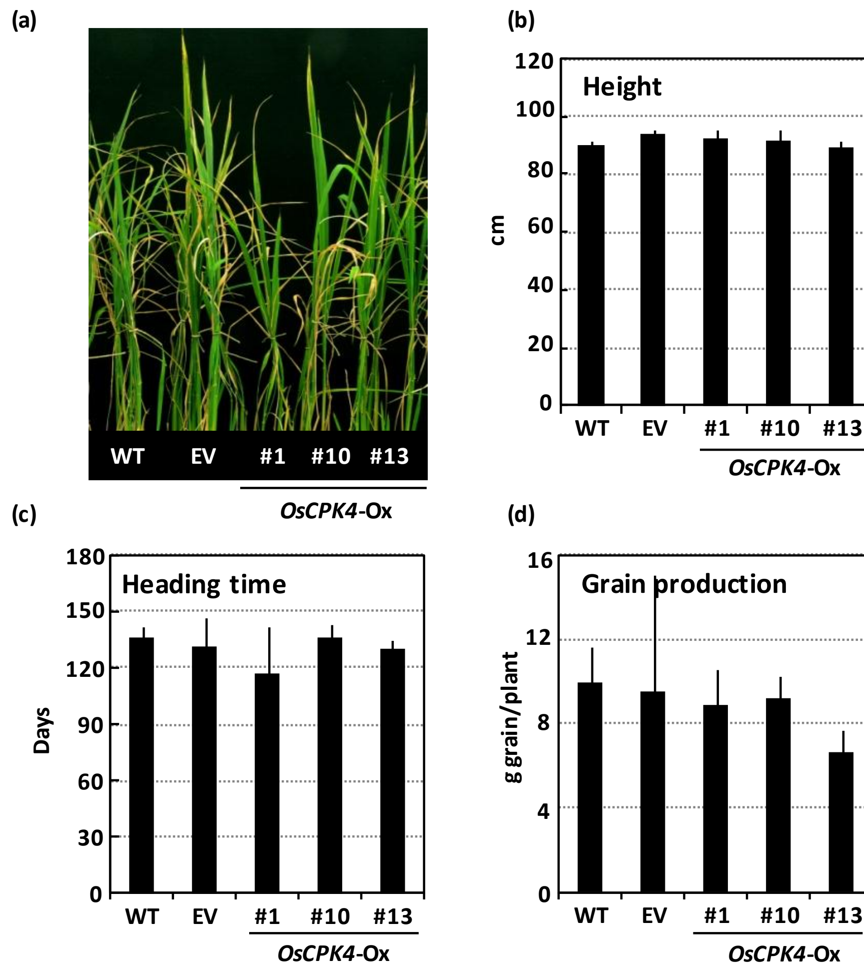

Figure CII.10: Plant performance of *OsCPK4*-overexpressing rice plants. (a) Phenotypic appearance of wild-type (WT), empty vector (EV) and *OsCPK4*-overexpressor (OsCPK4-Ox) rice plants 60 days after sowing. (b) Average height of plants at heading time. (c) Average time of heading (days after sowing). (d) Average grain yield production per plant grown under randomized distribution. Parameters were recorded for four different plants per each of the three independent analyzed lines. Results are representative of two independent experiments. No significant differences were measured for these parameters.

Discussion

The present study reveals that the isoform OsCPK4 from the multigenic family of rice CPKs has a function in the innate immunity of rice plants. Given that OsCPK4 was also known to participate in the salt and drought stress responses (Campo *et al.*, 2014), our results demonstrate that OsCPK4 is a signaling component that positively modulates both abiotic and biotic stress responses in rice plants. This work shows that the expression of the *OsCPK4* gene was rapidly induced in rice leaves when challenged with the *M. oryzae* pathogen, and that *OsCPK4* overexpression conferred enhanced resistance to rice blast disease, together supporting that OsCPK4 mediates the immune response to blast fungus in rice plants. OsCPK4 accumulation is induced at early stages of the infection process, coinciding with pathogen penetration, and suggesting that this protein acts at the earliest signaling events initiated upon pathogen recognition. Among the earliest immune reactions, calcium influxes are included (Ranf *et al.*, 2011; Blume *et al.*, 2000; Jeworutzki *et al.*, 2010), which occur through plasma membrane calcium channels activated by the recognition via pathogen recognition receptors (PRRs) of pathogen-associated molecular patterns (PAMPs) (Kurusu *et al.*, 2005). Since OsCPK4 is localized at the plant plasma membrane (Campo *et al.*, 2014), our hypothesized mechanistic model is that OsCPK4 acts as calcium sensor of changes stimulated by pathogen perception that triggers the downstream defense signaling events mediated by phosphorylation cascades (Figure CII.11). In agreement with the proposed mechanism of action, *OsCPK4*-Ox plants that accumulate constitutively increased levels of the protein exhibited a rapid and potentiated defense response upon pathogen infection. These plants accumulate the full OsCPK4 protein, including the calcium binding regulatory domain, ready to be stimulated by calcium upon pathogen sensing. Thus, *OsCPK4*-Ox plants showed fast and enhanced ROS production, increased callose deposition, and strong defense gene expression when challenged with the *M. oryzae* fungal pathogen. As a result, these plants showed an enhanced disease resistance phenotype against *M. oryzae* as determined by visual inspection, fungal growth quantification, and disease lesion measurement. Blast disease resistance was shown not only in detached leaf assays but also in whole plant infection assays. These results support that OsCPK4 participates in the signal transmission initiated by

pathogen perception, and the constitutive increased accumulation of OsCPK4 leads to an accelerated and amplified defense signal.

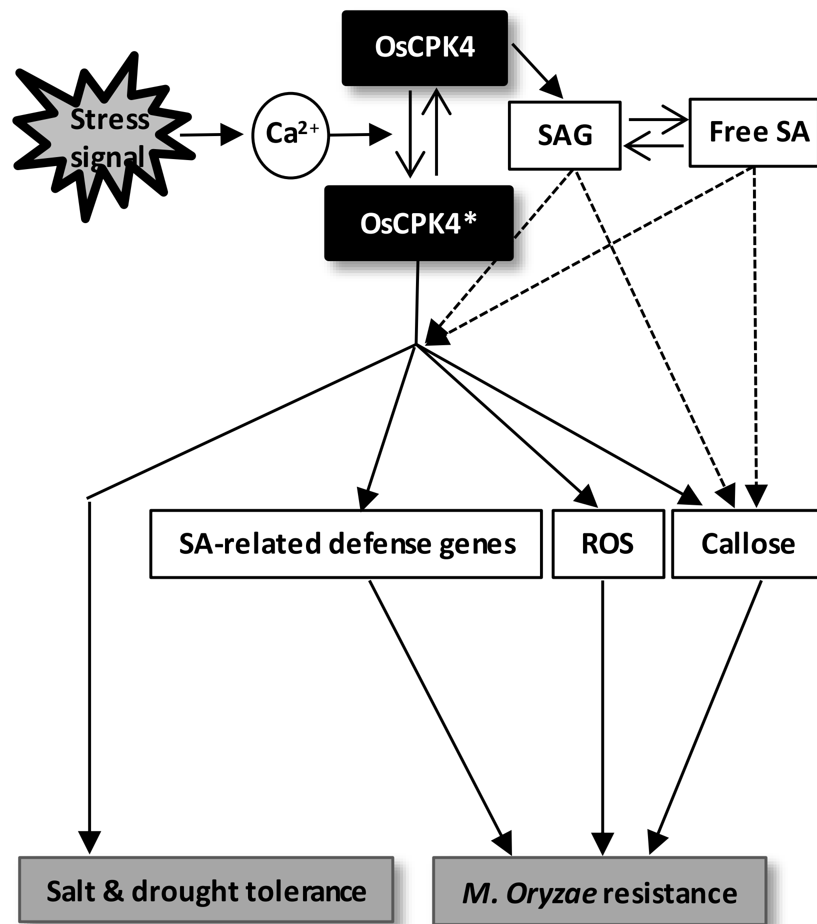

Figure CII.11: Model for OsCPK4-mediated defense responses. Stress induces Ca^{2+} changes that activate OsCPK4 protein. The activated OsCPK4 protein regulates ROS production, callose deposition and SA-regulated defense gene expression, resulting in resistance to *Magnaporthe oryzae* infection. OsCPK4 also mediated accumulation of SAG.

Our results showed that ROS production was stronger and faster in *OsCPK4*-Ox plants upon elicitor or pathogen perception. ROS levels might reach toxic thresholds for *M. oryzae*, leading to fungal penetration blockage as observed under confocal microscopy. However, the importance of ROS in defense reactions is not only due to their toxicity to pathogens, but also to their role as signaling molecules for local and systemic responses (Mittler et al., 2009). ROS mediate the defensive response through oxidative waves that activate signal transduction through phosphorylation cascades, accompanied of hormonal signalling and the expression of defense-related genes

(Shetty *et al.*, 2008; Baxter *et al.*, 2013). Therefore, the increased ROS production might contribute to the enhanced defense responsiveness observed in *OsCPK4*-Ox plants. Be as toxic compound or as signaling molecules, ROS production seems to contribute to the enhanced resistance of *OsCPK4*-Ox plants, and to be activated by OsCPK4 in response to PAMP stimulation. Connections between ROS production and CPKs have been already described in the literature, these studies reporting that ectopic expression of constitutively active CPK variants resulted in increased production of ROS (Dubiella *et al.*, 2013; Kobayashi *et al.*, 2007; Romeis *et al.*, 2001; Xing *et al.*, 2001). Moreover, NADPH oxidases playing a central role in the oxidative burst during immune responses have been reported as CPK targets in potato and Arabidopsis (Dubiella *et al.*, 2013; Kobayashi *et al.*, 2007). Similarly for rice, the plasma membrane NADPH oxidases might be potential targets of the plasma membrane associated OsCPK4 protein, triggering a fast and strong oxidative burst upon pathogen attack in the plants that constitutively accumulated increased levels of OsCPK4 protein. Other sources for ROS production also exist in plant cells, such as the peroxidases identified in Arabidopsis as major contributors to ROS production during responses to fungal elicitors (Daudi *et al.*, 2012), and they might be also potential OsCPK4 targets. Future studies will address OsCPK4 target identification.

OsCPK4 overexpressor plants accumulate increased SAG levels, the glucosylated form of SA. SAG is considered a likely storage form of physiologically active free SA, which is accumulated in the vacuole to serve as a source of free SA when required in dicotyledonous plants (Dean *et al.*, 2005; Seo *et al.*, 1995). Although in rice plants, SAG has been proposed to have *per se* a role in activating defenses for induced resistance (Umemura *et al.*, 2009). This increased accumulation of SAG prepared *OsCPK4*-overexpressing rice plants for a strong activation of SA-mediated defense signaling upon *M. oryzae* infection. As a result, intense activation of components of the SA pathway was detected, including the biosynthetic gene *OsSID2*, the Os*NH1* and *OsWRKY45* transcriptional activator genes, and the end-products *OsPBZ1* and *OsPR5*. Another immune response associated to SA is the callose deposition, being promoted by SA (Yi *et al.*, 2014). In agreement with the high SAG content, callose was also accumulated in the *OsCPK4*-Ox. Callose might represent a physical barrier that prevents fungal penetration leading to the observed resistant phenotype of *OsCPK4*-Ox

plants. Our results reveal that OsCPK4 contributes to the accumulation of SAG and callose in rice plants under non-inductive conditions.

Our data suggest that the rice plants overexpressing *OsCPK4* are sensitized or preconditioned for a robust and fast immune response by accumulating a signaling component that can be immediately activated upon exposure to stress. Defense responses usually have fitness costs associated to resource allocation for defensive compounds or the toxicity of the defensive products (van Hulten *et al.*, 2006), and the strategies to improve disease resistance in plants based in the constitutive activation of defenses are accompanied by negative effects on plant growth and yield (Gust *et al.*, 2010; Takatsuji, 2014). In this sense, we have shown that the overexpression of the *OsCPK4* gene in rice plants does not have a negative impact on plant performance, at least under containment conditions. The growth, flowering time, and yield fitness parameters of these plants are not significantly different than those of the wild-type plants. This is in agreement with the observation that OsCPK4 overexpressing rice plants did not show constitutive expression of defense related genes or ROS accumulation under non-inductive conditions, although they do accumulate SAG and callose. This is consistent with the already reported global transcriptomic analyses showing that overexpression of OsCPK4 in rice plants has a low impact in the rice transcriptome (Campo *et al.*, 2014). All together, our results support that OsCPK4 might be a good target for blast protection while maintaining rice yield.

OsCPK4-Ox rice plants are also more tolerant to salt and drought stress (Campo *et al.*, 2014). SA, in addition to modulate the immune response in plants, is also known to improve the tolerance to salt and drought stress by preventing membrane damage among other mechanisms (Farooq *et al.*, 2009; Jayakannan *et al.*, 2013). Moreover, SA inhibits lipid peroxidation, thus protecting cell membranes (Dinis *et al.*, 1994; Lapenna *et al.*, 2009). Therefore, the improved tolerance to drought and salinity of *OsCPK4*-Ox rice plants associated to a reduction of lipid peroxidation could be mediated by the increased content of SAG. This is an interesting result since tradeoffs between defense and abiotic stress tolerance have been frequently reported (Sharma *et al.*, 2013). For instance, OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance (Asano *et al.*, 2012). However crosstalk between biotic and abiotic signaling

pathways can result not only in negative but also in positive functional outcomes (Sharma *et al.*, 2013). Our studies demonstrate that OsCPK4 acts as a convergence component that positively modulates both biotic and abiotic signaling pathways, presumably modulating SA levels, and suggesting that it is a good molecular target to improve tolerance to different stresses in rice plants.

Experimental procedures

Plant and fungal growth conditions

OsCPK4 overexpressor rice plants were previously generated and described (Campo *et al.*, 2014). They were grown at 28ºC with a 14h/10h light/dark photoperiod. Fungal strains of *M. oryzae* FR13 isolate (provided by D. Tharreau, CIRAD Montpelier, France) and Guy11-GFP (provided by A. Sesma, CBGP Madrid, Spain) were grown in oatmeal agar (72.5g/L, 30mg/L cloramfenicol) for two weeks at 28ºC using a 16h/8h light/dark photoperiod. Their spores were collected in sterile water, filtrated with Miracloth (Calbiochem), and adjusted to the appropriate concentration using a Bürker counting chamber. *M. oryzae* elicitors were obtained as previously described (Casacuberta *et al.*, 1992). *F. verticillioides* and *D. dadantii* strains were grown as previously described (Gómez-Ariza *et al.*, 2007).

RNA isolation and qRT-PCR

Gene expression levels were determined from a pool of four leaves at the same developmental stage of 3-week-old soil-grown plants. Total RNA was extracted using TRIzol reagent (Invitrogen, Basel, Switzerland). DNAse treated RNA (1 µg) was retrotranscribed using the transcriptor first cDNA synthesis kit (Roche, Mannheim, Germany). qRT-PCR analyses were carried out in 96-well optical plates in a LightCycler® 480 System (Roche, Mannheim, Germany) according to the following program: 10 min at 95 °C, 45 cycles of 95 °C for 10s and 60 °C for 30s, and an additional cycle of dissociation curves to ensure a unique amplification. The reaction mixture contained 5µl of SYBR Green Master mix reagent (Roche), 2µl of 1:4 diluted cDNA sample and 300 nM of each gene-specific primer (table CII.1) in a final volume of 10µl. The results for the gene expression were normalized to *OsUbi1* (LOC_Os06g46770) and *OsUbi5*

(LOC_Os01g22490) genes as indicated. Three technical replicates were done for each sample.

Table CII.1: Primer sequences of genes used for gene expression analysis.

Gene name	Identifier	Primer sequences
OsCPK4	LOC_Os02g03410	For 5'-CGTGTGCAGCATGCAGATAA-3' Rev 5'-TGATTGCACGTATTCATCGCA-3'
OsCPK4 (transgene)	LOC_Os02g03410	For 5'- TCCAAGAGGACCTCCAAATCC-3' Rev 5'- AAATGTTTGAACGATCCCCG-3'
OsUbi1	LOC_Os06g46770	For 5'-TTCCCCAATGGAGCTATGGTT-3' Rev 5'-AAACGGGACACGACCAAGG-3'
OsUbi5	LOC_Os01g22490	For 5'-TAAGTGCGGCCTCACCTACG-3' Rev 5'-GGAGCCTACGCCTAAGCCTG-3'
oscpk4(mutant)	TRIM-M0060083	For 5'-CAGCAAGAGAAGAGAGGAGA-3' Rev 5'-GCGAGTGTGGGTGAGGGTAT-3' RB 5'-ACTCATGGCGATCTCTTACC-3'
26S-M.oryzae	AB026819	For 5'-TACGAGAGGAACCGCTCATTCAGATAATTA-3' Rev 5'-TCAGCAGATCGTAACGATAAAGCTACTC-3'
OsPBZ1	LOC_Os12g36880	For 5'-GCGATGGCTCCTGTGTGG-3' Rev 5'-CTCCGGCGACAGTGAGCT-3'
OsPR5	LOC_Os03g46070	For 5'-GACGACCAGACGAGCACCTT-3' Rev 5'-GTCCCTCATGGGCAGAAGAC-3'
OsNH1	LOC_Os01g09800	For 5'-TGAAAGAAGGGACCCACAAC-3' Rev 5'-AGGTGGATTTGCACCAGAAC-3'
OsWRKY45	LOC_Os05g25770	For 5'-CAATCGTCCGGGAATTCG-3' Rev 5'-GCCTTTGGGTGCTTGGAGT-3'
OsSID2	LOC_Os09g19734	For 5'-GAACCAAGGCTCTTGCTGTTG-3' Rev 5'-CCGTGGCGGTATCAAGTGA-3'
OsEDS1	LOC_Os09g22450	For 5'-AGACATCATCCCCCGCATAC-3' Rev 5'-CCTTCTGTGGCAGATGCAAG-3'

Protein extracts, CPK activity and immunoblot analysis

Protein extracts were obtained from membrane-enriched fractions prepared from leaves in a pool of at least four plants. Samples were ground in liquid nitrogen, thawed in two volumes of extraction buffer (10% sucrose, 50 mM TrisHCl pH7.5, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 1mM PMSF) and centrifugated at 15,000g for 20 min at 4 °C. The pellet was resuspended in 2 volumes of elution buffer (1% Triton X-100, 25

mM TrisHCl pH7.5, 1mM $MgCl_2$, 1 mM PMSF) using a cooled sonication bath. Protein extracts were recovered from the supernatant after centrifugation as before, quantified, separated in SDS-PAGE, and transferred to nitrocellulose membranes. Western blot analyses were performed using anti-OsCPK4 antibodies as described (Campo *et al.*, 2014). Antibodies were raised against the N-terminal variable domain of OsCPK4 (Met1 to Arg58) to specifically recognize this isoform of the conserved OsCPK family protein.

The calcium dependent kinase activity was analyzed as described with minor modifications (Boudsocq *et al.*, 2012). These include that total protein was extracted from rice leaves and immunoprecipitated for 2 hours with specific anti-OsCPK4 antibodies bound to Dynabeads® with the antibody coupling kit (Life Technologies); that the phosphorylation substrates were β-casein peptide (Sigma) and myelin basic protein (Invitrogen); and that the unincorporated radioactive nucleotides were discarded using MicroSpin G-25 columns (GE Healthcare). The concentration of free calcium in each buffer was calculated using MaxChelator (http://maxchelator.stanford.edu/).

Disease resistance assays with rice pathogens

M. oryzae infections were performed using a detached leaf infection assay as described (Coca *et al.*, 2004), or a whole plant infection assay by spraying the fungal spores with an aerograph at 2 atmospheres of pressure. Infection assays were carried out with three week-old plants grown in soil, using three pots with 10 plants each per line, and 2 ml of spore suspension (10^5 spores/ ml) per pot. The plants were maintained for 16h in a closed plastic bag for high humidity conditions after inoculation. Lesion areas were measured by image analysis software Assess v.2.0 at 7 dpi. Fungal biomass in rice infected leaves was determined at 7dpi by qPCR using specific primers for the *26S* ribosomal RNA gene of *M. oryzae*, and normalized to *OsUbi1* gene as described (Qi and Yang, 2002). DNA (15ng per qPCR reaction) was obtained from the rice infected leaves as described (Murray and Thompson 1980), but using MATAB as extraction buffer (0.1 M Tris HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000, and 0.5% sodium sulphite). Disease symptoms on whole plant infection assays were scored at 7dpi following the Standard evaluation system for blast

rice disease (IRRI, 2002). Three biological replicates were done for each line and three technical replicates per sample.

Infection assays with *F. verticillioides* were performed as previously described with minor modifications (Bundó *et al*., 2014), including a seed germination period of 8 hours previous inoculation with 10^3 spores/ml suspensions.

Assays with *D. dadantii* were done as described with minor modifications (Gómez-Ariza et al., 2007), reducing the seed germination period to 8 hours and increasing the inoculation doses to 10^7 CFU.

Fluorescence Microscopy

Confocal laser scanning microscopy was performed using an Olympus FV1000 microscope (Tokyo, Japan). GFP was excited with an argon ion laser emitting at 488 nm and fluorescence detected at 500-550 nm. Chlorophyll autofluorescence was visualized at 600-700 nm. Lesions were also observed under a Zoom Stereo Microscope Olympus SZX16 fitted with an Olympus DP72 Digital Camera.

For ROS detection, leaf segments from at least three different plants were infiltrated with a 10 μM solution of the fluorescent probe CM-H_2DCFDA (Molecular Probes) in 100 mM phosphate buffer pH7.2 for 2 hours. The leaves were then treated with a 1% *M. oryzae* elicitor solution in sterile water or inoculated with a 10^5 spores/ml suspension. ROS was monitored over the time using an Axiophot Zeiss epifluorescent microscope, and fluorescent signals were quantified by image analysis using the ImageJ software.

Callose accumulation was visualized by fluorescence under epifluorescence microscopy after aniline-blue staining of leaf segments from at least three different plants as previously described (Luna *et al.*, 2010). The fluorescent area per leaf segment was quantified also using the ImageJ software.

Salicylic acid quantification

Free SA and SAG content in rice leaves was determined as previously described with some minor modifications (Coca and San Segundo, 2010). Total SA was obtained from 1g of fresh-grinded leaves by two consecutive methanol and ethanol extractions (3 ml

each). After alcohol evaporation, the extracts were resuspended in water and separated into two parts, one to determine free-SA and the other for SAG. SAG samples were digested with 10U/ml of β-glucosidase from almonds (Sigma) at 37ºC during 16h. After digestion, the samples were filled up to 1 ml with milli-Q water, and HCl 37% (50 µl) was added. They were subjected to two consecutives extractions with ethyl acetate: cyclopentane: isopropanol (2ml, 50:50:1). Organic phases were evaporated and resuspended in methanol (25 µl) for the HPLC analysis using a Zorbax Eclipse XDB-C18 column (Agilent Technologies). Two biological replicates were done for each independent line.

Acknowledgments

We thank B. San Segundo for scientific advice; M. Alborno, S. Campo and L. Campos-Soriano for critical reading of the manuscript; and M. Amenós for technical assistance with confocal microscopy. This work was supported by grants BIO2009-08719 and BIO2012-32838 from "Ministerio de Economía y Competitividad" (MINECO, Spain) with European Regional Development Funds (FEDER).

References

Asano, T., Hayashi, N., Kobayashi, M., Aoki, N., Miyao, A., Mitsuhara, I., Ichikawa, H., Komatsu, S., Hirochika, H., Kikuchi, S. and Ohsugi, R. (2012) A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. *Plant J.* **69**, 26-36.

Asano, T., Tanaka, N., Yang, G., Hayashi, N. and Komatsu, S. (2005) Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the CDPKs gene family in rice. *Plant Cell Phys.* **46**, 356-366.

Baxter, A., Mittler, R. and Suzuki, N. (2013) ROS as key players in plant stress signalling. *J. Exp. Bot*. **65**, 129-1240.

Blume, B., Nürnberger, T., Nass, N. and Scheel, D. (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell*, **12**, 1425-1440.

Boller, T. and He, S.Y. (2009) Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science*, **324**, 742-744.

Boudsocq, M., Droillard, M.J., Regad, L., and Laurière, C. (2012). Characterization of Arabidopsis calcium-dependent protein kinases: activated or not by calcium? Biochem. J. **447:** 291-299.

Boudsocq, M. and Sheen, J. (2013) CDPKs in immune and stress signaling. *Trends Plant Sci*, **18**, 30-40.

Bundó, M., Montesinos, L., Izquierdo, E., Campo, S., Mieulet, D., Guiderdoni, E., Rossignol, M., Badosa, E., Montesinos, E., San Segundo, B., and Coca, M. (2014). Production of cecropin A antimicrobial peptide in rice seed endosperm. BMC Plant Biol. **14**:102.

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., and San Segundo, B. (2014) Overexpression of a calcium-dependent protein kinase confers salt and drought tolerance in rice by preventing membrane lipid peroxidation. *Plant Phys*. **165**, 688-704.

Campo, S., Peris-Peris, C., Siré, C., Moreno, A.B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C. and San Segundo, B. (2013) Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. *New Phyt.*, **199**, 212-227.

Campos-Soriano, L. and San Segundo, B. (2009) Assessment of blast disease resistance in transgenic PRms rice using a gfp-expressing Magnaporthe oryzae strain. *Plant Path.*, **58**, 677-689.

Casacuberta, J.M., Raventos, D., Puigdomenech, P. and San Segundo, B. (1992) Expression of the gene encoding the PR-like protein PRms in germinating maize embryos. *Mol. Gen. Genet.* **234**, 97-104.

Chern, M.S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X. and Ronald, P.C. (2001) Evidence for a disease-resistance pathway in rice similar to the NPR1-mediated signaling pathway in Arabidopsis. *Plant J.* **27**, 101-113.

Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, **124**, 803-814.

Coca, M., Bortolotti, C., Rufat, M., Penas, G., Eritja, R., Tharreau, D., del Pozo, A.M., Messeguer, J. and San Segundo, B. (2004) Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus Magnaporthe grisea. *Plant Mol. Biol.* **54**, 245-259.

Coca, M. and San Segundo, B. (2010) AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. *Plant J.* **63**, 526-540.

Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G.S., Muthukrishnan, S. and Datta, S.K. (1999) Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to Rhizoctonia solani causing sheath blight disease. *Theor Appl Genet*, **98**, 1138-1145.

Daudi, A., Cheng, Z., O`Brien, J.A., Mammarella, N., Khan, S., Ausubel, F.M. and Bolwell, G.P. (2012) The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. *Plant Cell*, **24**, 275-287.

Dean, J., Mohammed, L. and Fitzpatrick, T. (2005) The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. *Planta*, **221**, 287-296.

Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G.D. (2012) The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.*, **13**, 414-430.

Dinis, T.C.P., Madeira, V.M.C. and Almeida, L.M. (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Of Bioch. Biophys*. **315**, 161-169.

Dodd, A.N., Kudla, J., and Sanders, D. (2010) The language of calcium signaling. *Ann. Rev. Plant Biol*. **61**, 593-620.

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.P., Schulze, W.X. and Romeis, T. (2013) Calcium-dependent protein kinase/NADPH oxidase

activation circuit is required for rapid defense signal propagation. *Proc Nat Ac Sci USA* **110**, 8744-8749.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Ann. Rev. Phytopat.* **42**, 185-209.

Farooq, M., Basra, S.M.A., Wahid, A., Ahmad, N. and Saleem, B.A. (2009) Improving the drought tolerance in rice (*Oryza sativa* L.) by exogenous application of salicylic acid. *J. Agr. Crop Sci.* **195**, 237-246.

Fu, L., Yu, X., and An C. (2013) Overexpression of constitutively active *OsCPK10* increases *Arabidopsis* resistance against *Pseudomonas syringae pv. tomato* and rice resistance against *Magnaporthe grisea*. *Plant Phys. Biochem.* **73**: 201-210.

Gómez-Ariza, J., Campo, S., Rufat, M., Estopa, M., Messeguer, J., San Segundo, B., and Coca, M. (2007) Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. *Mol. Plant Microbe Interact.* **20**: 832-842.

Goto, M. (1979) Bacterial foot rot of rice caused by a strain of *Erwinia chrysanthemi*. Phytopathol. **69**: 213-216.

Gust, A.A., Brunner, F. and Nürnberger, T. (2010) Biotechnological concepts for improving plant innate immunity. *Curr. Op. Biotech*. **21**, 204-210.

Harper, J.F., Breton, G. and Harmon, A. (2004) Decoding Ca2+ signals through plant protein kinases. *Ann. Rev. Plant Biol.* **55**, 263-288.

IRRI, International Rice Research Institute. (2002) Standard Evaluation System for Rice.

Jayakannan, M., Bose, J., Babourina, O., Rengel, Z. and Shabala, S. (2013) Salicylic acid improves salinity tolerance in Arabidopsis by restoring membrane potential and preventing salt-induced K(+) loss via a GORK channel. *J Exp Bot*, **64**, 2255-2268.

Jeworutzki, E., Roelfsema, M.R., Ansch++tz, U., Krol, E., Elzenga, J.T., Felix, G., Boller, T., Hedrich, R. and Becker, D. (2010) Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca2+-associated opening of plasma membrane anion channels. *Plant J.* **62**, 367-378.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323-329.

Jwa, N.S., Agrawal, G.K., Tamogami, S., Yonekura, M., Han, O., Iwahashi, H. and Rakwal, R. (2006) Role of a defense/stress-related marker genes, proteins and a secondary metabolites in a defining rice self-defense mechanisms. *Plant Phys. Bioch.*, **44**, 261-273.

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N. and Yoshioka, H. (2007) Calcium-dependent protein kinases regulate the

production of reactive oxygen species by potato NADPH oxidase. *Plant Cell*, **19**, 1065-1080.

Kristiansen, K.A., Jensen, P.E., MØller, I.M. and Schulz, A. (2009) Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H_2DCFDA and confocal laser microscopy. *Physiologia Plantarum*, **136**, 369-383.

Kurusu, T., Yagala, T., Miyao, A., Hirochika, H. and Kuchitsu, K. (2005) Identification of a putative voltage-gated Ca2+ channel as a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. *Plant J.* **42**, 798-809.

Lapenna, D., Ciofani, G., Pierdomenico, S.D., Neri, M., Cuccurullo, C., Giamberardino, M.A. and Cuccurullo, F. (2009) Inhibitory activity of salicylic acid on lipoxygenase-dependent lipid peroxidation. *Bioch. Biophys. Acta*, **1790**, 25-30.

Lecourieux, D., Ranjeva, R. and Pugin, A. (2006) Calcium in plant defence-signalling pathways. *New Phytol.*, **171**, 249-269.

Lee, F., Cartwright, R.D., Jia, Y. and Correll, J.C. (2009) Field resistance expressed when the Pi-ta gene is compromised by *Magnaporthe oryzae*. In *Advances in Genetics, Genomics and Control of Rice Blast Disease* (Wang, G.L. and Valent, B., eds): Springer Netherlands, pp. 281-289.

Liu, W., Liu, J., Triplett, L., Leach, J.E., and Wang, G.L. (2014) Novel insights into rice innate immunity against bacterial and fungal pathogens. *Annu. Rev. Phytopathol.* **52**: 213-214.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B. and Ton, J. (2010) Callose deposition: A multifaceted plant defense response. *Mol. Plant-Micr. Interact*. **24**, 183-193.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S.V., Machado, M.A., Toth, I., Salmond, G., and Foster, G.D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* **13**: 614-629.

Meng, X. and Zhang, S. (2013) MAPK cascades in plant disease resistance signaling. *Ann. Rev. Phytopathol*. **51**, 245-266.

Midoh, N. and Iwata, M. (1996) Cloning and characterization of a probenazole-inducible gene for an intracellular pathogenesis-related protein in rice. *Plant Cell Phys.* **37**, 9-18.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T. and Jones, J.D.G. (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant Phys*. **135**, 1113-1128.

Ou SH. (1987) Rice Diseases. Commonwealth Mycological Institute, Surrey, UK.

Qi, M. and Yang, Y. (2002) Quantification of *Magnaporthe grisea* during infection of rice plants using real-time polymerase chain reaction and Northern blot/phosphoimaging analyses. *Phytopathology*, **92**, 870-876.

Rakwal, R., Agrawal, G.K. and Agrawal, V.P. (2001) Jasmonate, salicylate, protein phosphatase 2A inhibitors and kinetin up-regulate OsPR5 expression in cut-responsive rice (*Oryza sativa*). *J. Plant Phys.*, **158**, 1357-1362.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J. and Scheel, D. (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* **68**, 100-113.

Ray, S., Agarwal, P., Arora, R., Kapoor, S. and Tyagi, A. (2007) Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. *indica*). *Mol Genet Genomics*, **278**, 493-505.

Romeis, T. and Herde, M. (2014) From local to global: CDPKs in systemic defense signaling upon microbial and herbivore attack. *Curr. Op. Plant Biol.* **20**, 1-10.

Romeis, T., Ludwig, A.A., Martin, R. and Jones, J.D. (2001) Calcium-dependent protein kinases play an essential role in a plant defence response. *EMBO J.* **20**, 5556-5567.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell*, **8**, 1809-1819.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K. and Izui, K. (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.* **23**, 319-327.

Schulz, P., Herde, M. and Romeis, T. (2013) Calcium-dependent protein kinases: hubs in plant stress signaling and development. *Plant Phys.* **163**, 523-530.

Seo, S., Ishizuka, K. and Ohashi, Y. (1995) Induction of salicylic acid β-Glucosidase in tobacco leaves by exogenous salicylic acid. *Plant Cell Phys*. **36**, 447-453.

Sesma, A. and Osbourn, A.E. (2004) The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature*, **431**, 582-586.

Seybold, H., Trempel, F., Ranf, S., Scheel, D., Romeis, T. and Lee, J. (2014) Ca^{2+} signalling in plant immune response: from pattern recognition receptors to Ca^{2+} decoding mechanisms. *New Phytologist*, **204**, 782-790.

Sharma, R., De Vleesschauwer, D., Sharma, M.K. and Ronald, P.C. (2013) Recent advances in dissecting stress-regulatory crosstalk in rice. *Mol. Plant* **6**, 250-260.

Shetty, N., JØrgensen, H., Jensen, J., Collinge, D. and Shetty, H.S. (2008) Roles of reactive oxygen species in interactions between plants and pathogens. *Eur J Plant Pathol*, **121**, 267-280.

Shimono, M., Koga, H., Akagi, A., Hayasi, N., Goto, S., Sawada, M., Kurihara, T., Matsushita, A., Sugano, S., Jiang, C., Kaku, H., Inoue, H. and Takatsuji, H. (2012) Rice WRKY45 plays important roles in fungal and bacterial disease resistance. *Mol. Plant Pathol.* **13**, 83-94.

Skamnioti, P. and Gurr, S.J. (2009) Against the grain: safeguarding rice from rice blast disease. *Trends Biotech.* **27**, 141-150.

Takatsuji, H. (2014) Development of disease-resistant rice using regulatory components of induced disease resistance. *Front Plant Sci.* **5**, 630.

Tena, G., Boudsocq, M. and Sheen, J. (2011) Protein kinase signaling networks in plant innate immunity. *Curr. Op. Plant Biol.* **14**, 519-529.

Torres, M.A. (2010) ROS in biotic interactions. *Physiologia Plantarum*, **138**, 414-429.

Tsuda, K. and Katagiri, F. (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. *Curr. Op. Plant Biol.* **13**, 459-465.

Umemura, K., Satou, J., Iwata, M., Uozumi, N., Koga, J., Kawano, T., Koshiba, T., Anzai, H. and Mitomi, M. (2009) Contribution of salicylic acid glucosyltransferase, OsSGT1, to chemically induced disease resistance in rice plants. *Plant J.* **57**, 463-472.

van Hulten, M., Pelser, M., van Loon, L.C., Pieterse, C.M.J. and Ton, J. (2006) Costs and benefits of priming for defense in Arabidopsis. *Proc. Nat. Ac. Sci. USA* **103**, 5602-5607.

Voigt, C.A. (2014) Callose-mediated resistance to pathogenic intruders in plant defense-related papillae. *Front. Plant Sci.* **5**, 168.

Wei, S., Hu, W., Deng, X., Zhang, Y., Liu, X., Zhao, X., Luo, Q., Jin, Z., Li, Y., Zhou, S., Sun, T., Wang, L., Yang, G. and He, G. (2014) A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. *BMC Plant Biol.*, **14**, 133.

Wiermer, M., Feys, B.J. and Parker, J.E. (2005) Plant immunity: the EDS1 regulatory node. *Curr. Op. Plant Biol.* **8**, 383-389.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562-565.

Wilson, R.A. and Talbot, N.J. (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nat Rev Micro*, **7**, 185-195.

Wulff, E.G., Sorensen, J.L., Lubeck, M., Nielsen, K.F., Thrane, U., Torp, J. (2010) *Fusarium* spp. Associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environ. Microbiol.* **12:** 649-657.

Xie, K., Chen, J., Wang, Q. and Yang, Y. (2014) Direct phosphorylation and activation of a mitogen-activated protein kinase by a calcium-dependent protein kinase in rice. *Plant Cell*, **26**, 3077-3089.

Xing, T., Wang, X.J., Malik, K. and Miki, B.L. (2001) Ectopic expression of an Arabidopsis calmodulin-like domain protein kinase-enhanced NADPH oxidase activity and oxidative burst in tomato protoplasts. *Mol. Plant-Microbe Interac.* **14**, 1261-1264.

Yi, S.Y., Shirasu, K., Moon, J.S., Lee, S.G. and Kwon, S.Y. (2014) The Activated SA and JA signaling pathways have an influence on flg22-triggered oxidative burst and callose deposition. *Plos One*, **9**.

CHAPTER III

Functional characterization of *OsCPK10* in the rice defense response and drought stress

Abstract

Plants are constantly exposed to different stresses that may affect their growth and development. The stress perception triggers the activation of signaling pathways integrated into a complex network which communicate different pathways between them. ROS, plant hormones and kinases have been proposed to be the key components of the crosstalk between different stress responses. Calcium-dependent protein kinases are important signaling components that have been described to participate in multiple plant stress responses in collaboration with ROS and plant hormones, among other signaling components. In this work, the rice isoform *OsCPK10* is reported as a positive modulator of the rice resistance to blast disease and drought stress tolerance. Constitutive accumulation of a HA-tagged OsCPK10 protein entails rice plants with a higher antioxidant capacity leading to an enhanced tolerance to oxidative stress. It is shown that OsCPK10 improved the ROS scavenging activity in rice plants during desiccation by modulating the accumulation of Catalase A, which reduced the lipid peroxidation degree, and prevented the integrity of cell membranes, resulting in drought tolerance. These results suggest that OsCPK10 improved the tolerance to blast disease and drought stress by prevention of the ROS-caused damages. Here, OsCPK10 appears as a convergent component that positively modulates both biotic and abiotic stress signaling pathways, opening new possibilities to improve rice tolerance to stress.

Introduction

Rice is the staple food for half of the world population. Unfortunately rice, as other crops, is exposed to different environmental stresses that constrain their growth and development, and culminates in harvest losses. Nowadays, in the context of the global climate change, these negative environmental factors may be more pronounced and damaging. For this reason, the study of plant responses against stresses is very necessary at this time, in order to develop new strategies to mitigate the effects of the coming environmental problems and guarantee food security. Blast disease, caused by the ascomycete fungus *Magnaporthe oryzae*, is one of the most devastating diseases in rice culture worldwide (Dean *et al*., 2012). The fungus can infect all the parts of the plant provoking its death in juvenile stages, or causing the total loss of grain in panicle infections. Annual losses of rice grain due to blast disease vary between 10-35% (Skamnioti and Gurr, 2009). Many efforts have been done in the generation of blast disease resistance rice varieties, based in the presence of resistance genes (Ballini *et al*., 2008; Wang *et al*., 2014). But some years later, these resistance varieties are overcome by new *M. oryzae* strains that are not recognized by the resistance genes (Huang *et al*., 2014). Thus, a better understanding of plant defense might provide new strategies to combat this disease.

Another important negative factor for rice cultivation is drought. Around 75% of rice is cultivated in irrigated ecosystems (Bouman *et al*., 2007). The lack of water affects the growth and development of rice plants during their entire life cycle, but during the reproductive and grain filling phases is more damaging (Farooq *et al*., 2012). Drought stress is a growing problem worldwide, affecting 50% of world production of rice every year (Mackill, 2010).

Even more, multiple stresses can occur simultaneously under field conditions both biotic and abiotic, and rice plants have to give an integrate response to this adverse condition. The perception of the stresses by the plant activates different signaling pathways that lead to physiological, cellular and molecular adaptive responses. Interactions between the different stress-induced signaling pathways could be synergistic and/or antagonist, and result in a desirable cross-tolerance or a detrimental

susceptibility. The crosstalk between signaling pathways involves phytohormones, transcription factors, kinase cascades and reactive oxygen species (ROS) (Ben Rejeb *et al*., 2014; Kissoudis *et al*., 2014).

Calcium-dependent protein kinases (CDPKs or CPKs) are plant proteins involved in different stress responses although some of them are related to developmental processes (Ludwig *et al*., 2004; Boudsocq and Sheen, 2013; Shulz *et al*., 2013; Ray *et al*., 2013). These proteins are characterized by combining in a single polypeptide chain a calmodulin domain with four EF-hand Ca^{2+} binding motifs and a kinase domain, which confers them the ability to perceive Ca^{2+} fluctuations and rapidly translate them into a phosphorylation signal (Harmon *et al*., 2001; Harper *et al*., 2004). Considering that Ca^{2+} acts as a second messenger in most of the plant stress responses, CPKs are well suited to be involved in the interaction between signaling pathways. They are members of multigenic families with 31 different isoforms in rice, for which a functional diversification has been proposed (Asano *et al*., 2005; Ray *et al*., 2007). Few rice OsCPK isoforms have been functionally characterized but mainly in relation to a single stress response (Saijo *et al*., 2000; Asano *et al*., 2011; Fu *et al*., 2013; Wei *et al*., 2014). Only the OsCPK12 was shown to oppositely modulate both biotic and abiotic stress responses (Asano *et al*., 2012), and more recently, the OsCPK4 to positively mediate both blast disease resistance and drought and salt tolerance (Campo *et al*., 2014; Bundó and Coca, 2015). In the present study, OsCPK10 is reported as a positive regulator of both biotic and biotic stress responses. We show that *OsCPK10* overexpression in rice plants confers blast disease resistance and drought tolerance through an enhanced ROS scavenging capacity. The connection of OsCPK10 to the ROS scavenging mechanisms confirms the involvement of CPKs and ROS in the crosstalk between biotic and abiotic stresses, opening new possibilities to improve rice tolerance to stress.

Results

OsCPK10 expression is induced by both biotic and abiotic stresses in rice plants

A search for altered gene expression in rice leaves in response to *M. oryzae* elicitors using a previously described microarray global transcriptomic analysis (Campo *et al*. 2013), identified the *OsCPK10* gene as an upregulated gene at 30 minutes after treatment (Fold Change = 1.32, p-value = 0.031). This data suggests that this gene might be involved in the defense response of rice plants. Further gene expression analyses showed that *OsCPK10* was also upregulated in rice leaves inoculated with *M. oryzae* fungal spores. As shown in Figure CIII.1A, *OsCPK10* transcripts started to be accumulated in rice leaves at 6 hours post inoculation (hpi), reached a maximum at 12 hpi, and then decreased progressively until 24 hpi. This accumulation kinetic timely coincides with the initial invasive growth of *M. oryzae* in the foliar epidermal cells, which starts with the appressoria formation at 6 hpi and continues with invasive hyphae growth and ramification into the live host cell until 12-24 hpi (Kankanala *et al*., 2007; Wilson and Talbot 2009; Campos-Soriano *et al*., 2013). Thus, the *OsCPK10* expression profile can be associated to the pathogen recognition signal during initial infection phases. A detailed analysis of *OsCPK10* promoter sequences (1375 bp upstream of the coding sequence, just at the end of previous locus LOC_Os3g57430) identified several stress-responsive regulatory elements (Figure CIII.1B, Table CIII.1), which are known to contribute to the expression of stress-related genes at transcriptional level. Among them, the most frequently found in *OsCPK10* promoter were the ABRE (ABA-responsive elements) and DRE (dehydratation-responsive elements) elements. These motifs are considered as major interdependent regulatory elements of gene expression in response to dehydratation stress (Narusaka *et al*., 2003). These observations suggest that *OsCPK10* gene expression might be regulated by abiotic stress. Therefore, *OsCPK10* transcript accumulation was monitored in rice plants in response to drought stress. A time maintained induction of *OsCPK10* gene in rice leaves under air drying stress was observed, which differed from the transient induction detected in the control leaves, probably due to the drought stress imposed by opening the plant containers (Figure CIII.1C). In roots, a weak and transient

induction was detected at early time points of treatments (Figure CIII.1D). *OsCPK10* gene expression was also induced by ABA treatment, this hormone playing an important role in the adaption of plants to drought conditions (Figure CIII.1E). Together, these results show that *OsCPK10* is induced by both biotic and abiotic stress, suggesting that this gene might be involved in the responses to different stress in rice plants.

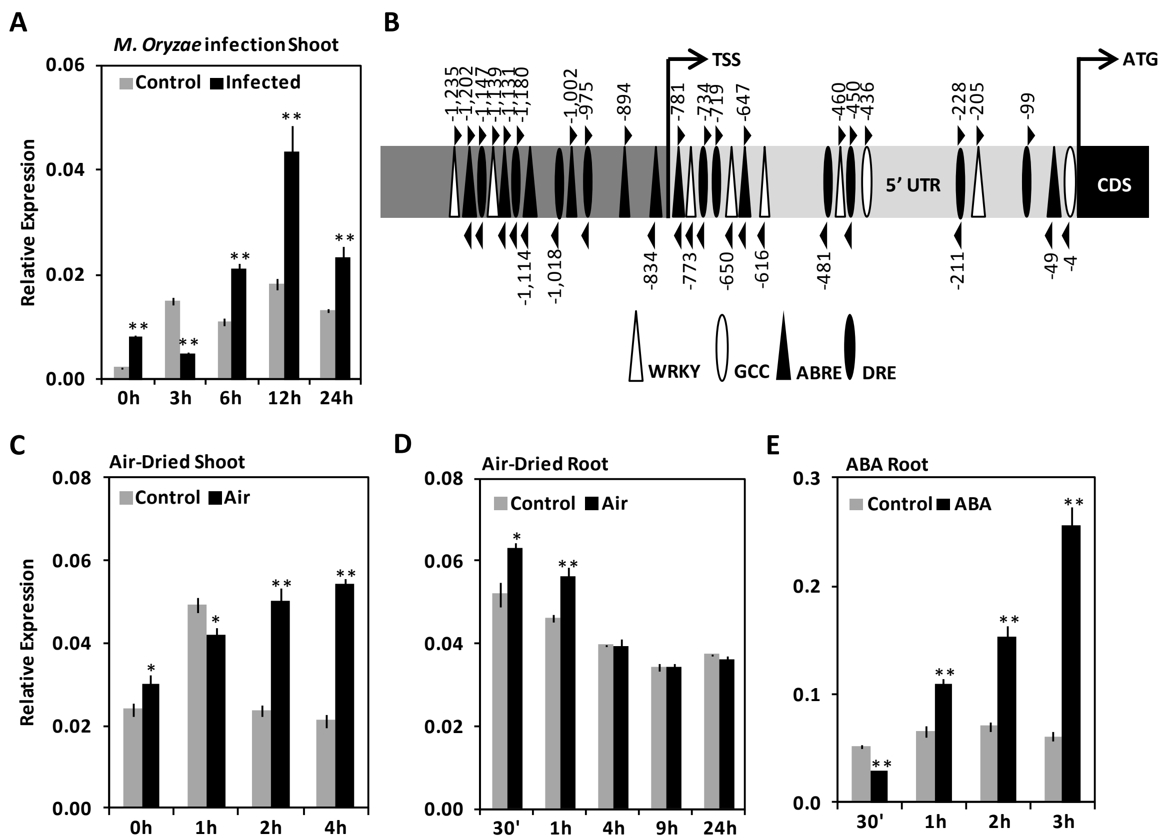

Figure CIII.1: Expression of *OsCPK10* gene in response to biotic and abiotic stress rice plants. A, *OsCPK10* expression in response to *M. oryzae* infection (10^5 spores/ml) in rice leaves at the indicated period of time post inoculation. B, Diagram of the *OsCPK10* promoter region showing the position of the biotic and abiotic stress-related *cis*-elements. C-D, *OsCPK10* expression in response to air drying stress of rice shoots (C) and roots (D). E, *OsCPK10* expression levels in rice roots in response to ABA (100 µM) treatment. *OsCPK10* transcript levels were determined by qRT-PCR and normalized to *OsUbi5* mRNA levels. Values represent means and SD of three replicates. Asterisks indicate significant differences (one-way ANOVA analysis, *P<0.05, ** P<0.01).

Table CIII.1: *cis*-related motifs identified in the 1375 bp upstream region of *OsCPK10* gene. The PLACE database (Prestridge, 1991; Higo *et al.*, 1999) was used to perform the analysis. Stress related abiotic and biotic stress responsive motifs are listed by alphabetical order.

Motif name	Number	Sequence	Description
ABRELATERD1	2	ACGTG	ABRE-like sequence required for etiolation-induced expression of *erd1* (early responsive to dehydration) in Arabidopsis.
ABRERATCAL	3	MACGYGB	ABRE-related sequence identified in the upstream regions of 162 Ca^{2+}-responsive upregulated genes. M=C/A; Y=T/C; B=T/C/G
ACGTABREMOTIFA2OSEM	2	ACGTGKC	Experimentally determined sequence requirement of ACGT-core of motif in ABRE of rice gene. K=G/T
ACGTATERD1	8	ACGT	ACGT sequence required for etiolation-induced expression of *erd1* in Arabidopsis.
BOXLCOREDCPAL	2	ACCWWCC	Consensus of the putative "core" sequences of box-L-like sequences in carrot (D.c.) *PAL1* promoter region. W=A/T
CBFHV	1	RYCGAC	Binding site of barley (H.v.) CBF1 and CBF2; CBFs are also known as dehydration-responsive element binding proteins (DREBs). R=A/G; Y=C/T
DPBFCOREDCDC3	2	ACACNNG	bZIP transcription factors, DPBF-1 and 2 (Dc3promoter-binding factor-1 and 2) binding core sequence; *Dc3* expression is normally embryo-specific, and also can be induced by ABA.
DRE2COREZMRAB17	1	ACCGAC	DRE2 core found in maize (Z.m.) *rab17* gene promoter; *rab17* is expressed during late embryogenesis, and is induced by ABA.
DRECRTCOREAT	1	RCCGAC	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) cis-acting element found in many genes in Arabidopsis and in rice. R=G/A
GCCCORE	2	GCCGCC	Core of GCC-box found in many pathogen-responsive genes such as *PDF1.2*, *Thi2.1*, and *PR4*; Has been shown to function as ethylene-responsive element.
LTRECOREATCOR15	1	CCGAC	Core of low temperature responsive element (LTRE) of *cor15a* gene in Arabidopsis (A.t.); ABA responsiveness.
MYB1AT	3	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in Arabidopsis. W=A/T

MYB2CONSENSUSAT	1	YAACKG	MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in Arabidopsis. Y=C/T; K=G/T.
MYBCORE	3	CNGTTR	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2. ATMYB2 is involved in regulation of genes that are responsive to water stress in Arabidopsis.
MYCCONSENSUSAT	9	CANNTG	MYC recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes in Arabidopsis. N=A/T/G/C
PROXBBNNAPA	1	CAAACACC	"prox B (proximal portion of B-box) found in *napA* gene of *Brassica napus* (B.n.); Required for seed specific expression and ABA responsiveness; ABRE mediated transactivation by ABI3 and ABI3-dependent response to ABA.
SEBFCONSSTPR10A	2	YTGTCWC	Binding site of the potato silencing element binding factor (SEBF) gene found in promoter of pathogenesis-related gene (*PR-10a*). W=A/T, Y=C/T
WBBOXPCWRKY1	1	TTTGACY	"W box" found in amylase gene in sweet potato, *alpha-Amy2* genes in wheat, barley, and wild oat, *PR1* gene in parsley, and a transcription factor gene in Arabidopsis; Y=C/T
WBOXATNPR1	1	TTGAC	"W-box" found in promoter of *Arabidopsis thaliana* (A.t.) *NPR1* gene; They were recognized specifically by salicylic acid-induced WRKY DNA binding proteins.
WBOXNTCHN48	1	CTGACY	W box identified in the region between -125 and -69 of a tobacco class I basic chitinase gene *CHN48*; NtWRKY1, NtWRKY2 and NtWRKY4 bound to W box; NtWRKYs possibly involved in elicitor-respsonsive transcription of defense genes in tobacco. Y=C/T
WBOXNTERF3	5	TGACY	W box found in the promoter region of a transcriptional repressor *ERF3* gene in tobacco; May be involved in activation of *ERF3* gene by wounding. Y=C/T.
WRKY71OS	7	TGAC	A core of TGAC-containing W-box; Binding site of rice *WRKY71*, a transcriptional repressor of the gibberellin signaling pathway; Parsley WRKY proteins bind specifically to TGAC-containing W box elements within the Pathogenesis-Related Class10 (*PR-10*) genes.

OsCPK10 localizes in the plasma membrane

The OsCPK10 protein shows the typical CPK structure of four functional domains: a calmodulin domain with four EF-hand calcium-binding sites, a junction autoinhibitory domain, a Ser-Thr kinase domain, and a variable N-terminal domain (Chen *et al*., 2002; Harper *et al*., 2004; Asano *et al*., 2005). The N-terminal domain of OsCPK10 is the largest of the rice CPK family (131 aminoacid residues), and contains a predicted myristoilation site and a palmitoilation site (NMT-The Myr Predictor http://mendel.imp.ac.at/myristate/SUPLpredictor.htm; CSS-Palm 2.0, Ren *et al*., 2008) (Figure CIII.2A). Myristoilation and palmitoylation motifs at the beginning of CPKs have been reported as responsible for their membrane association (Martin and Busconi, 2000; Lu and Hrabak, 2002; Coca and San Segundo, 2010; Witte *et al*., 2010; Campos-Soriano *et al*., 2011). To localize OsCPK10 in the plant cell, the *OsCPK10-GFP* fusion gene was transiently expressed in *Nicotiana benthamina* leaves via Agroinfiltration (Figure CIII.2B-J). OsCPK10-GFP protein was visualized at the cell periphery, likely the plasma membrane (Figure CIII.2D-J), whereas GFP alone was ubiquitously distributed inside the epidermal cells (Figure CIII.2B-C). To confirm the plasma membrane localization, leaves expressing the *OsCPK10-GFP* gene were treated with a hypertonic solution of mannitol to induce plasmolysis. The OsCPK10-GFP protein conserved the plasma membrane localization in the shrunken protoplasm, clearly visualized in the typical Hetchian strands that anchor the membrane to the cell wall (Figure CIII.2F, G). Moreover, *OsCPK10-GFP* transformed cells were stained with the lipophilic probe FM4-64 that fluoresces intensely upon binding to plasma membrane. As shown in Figure CIII.2H-J, the red fluorescence of the FM4-64 staining perfectly overlapped with the OsCPK10-GFP green fluorescence, resulting in a yellow staining of the plasma membrane when images were merged. These results showed plasma membrane localization for the OsCPK10 protein.

OsCPK10HA protein is accumulated in rice plants

To characterize the biological function of *OsCPK10* gene in plants, transgenic rice plants for overexpression of a HA-tagged version of *OsCPK10* gene were generated. The plants were produced in the japonica cultivar Nipponbare via *Agrobacterium thumefaciens*, using a pCAMBIA 1300-derived vector containing the *OsCPK10* full-

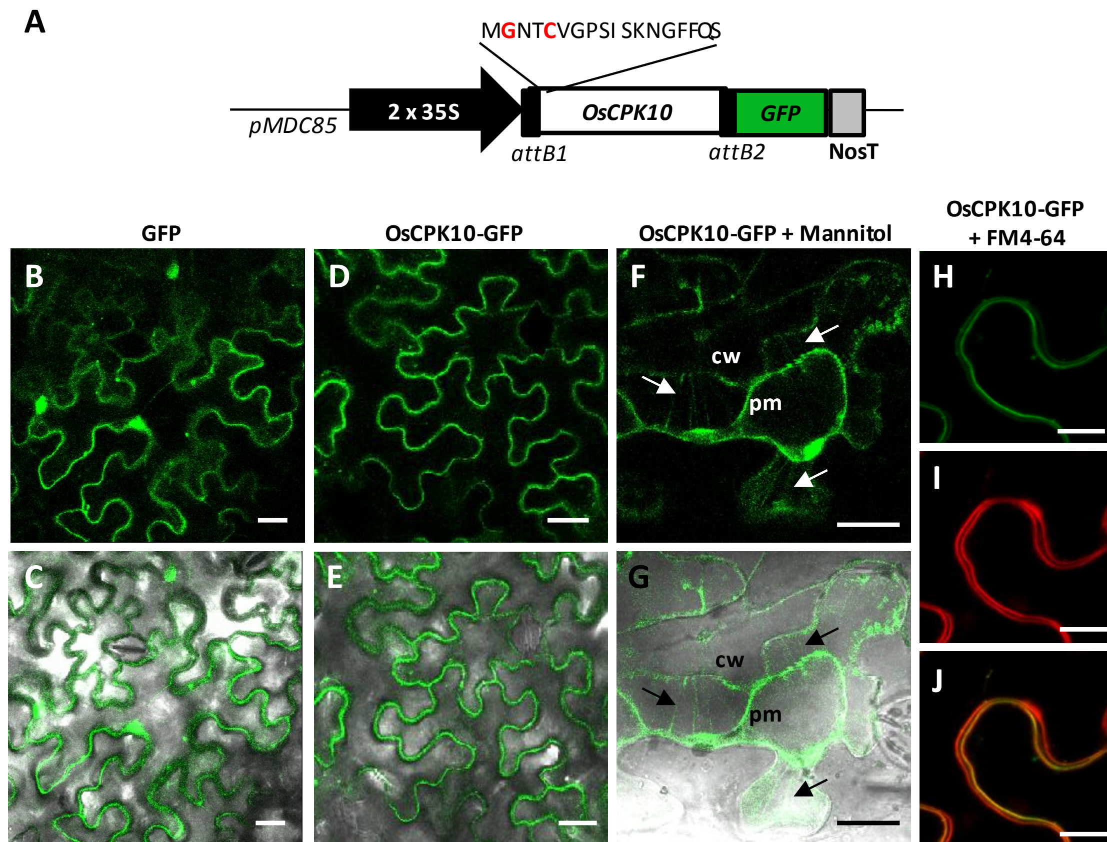

Figure CIII.2: Plasma membrane localization of OsCPK10. Confocal fluorescence microscopy of *Nicotiana benthamiana* epidermal cells transformed with the *OsCPK10-GFP* gene via *Agrobacterium*. Images were taken 48h after agroinfiltration. A, Schematic representation of the *OsCPK10-GFP* gene construct used for agroinfiltration. The predicted N-terminal myristoilation and palmitoylation sites are indicated in red. B and C, GFP protein control localization. D and E, OsCPK10-GFP fusion protein localization. F and G, Plasmolysed cell transformed with *OsCPK10-GFP* gene after 15 min of treatment with mannitol. Arrows indicate the Hetchian strands attaching the plasma membrane (pm) to the cell wall (cw). H, I and J, *N. benthamiana* transformed cell with *OsCPK10-GFP* gene stained with the lipophilic dye FM4-64. B, D, F, H, I and J are fluorescence images; C, E, and G are the merged images with bright field; and J, the merged images of the green (H) and red (I) fluorescent images. Scale bar corresponds to 20 µm (A-G) or 10 µm (H-J).

length cDNA (1,800 bp) extended in C-terminal with the sequences encoding the HA epitope under the control of the strong and constitutive maize *ubiquitin1* promoter and the nopaline synthase terminator (Figure CIII.3A). Thus, the construct was designed for the production of a full length OsCPK10 protein preserving its regulatory domains, namely junction and calmodulin domains, and HA-tagged in C-terminal to avoid interference with the N-terminal localization signals. Five independent transgenic lines were obtained that accumulated the recombinant protein as determined by immunoblot analysis (Figure CIII.4), and three of them were selected to

obtain homozygous lines in the progeny plants. However, no homozygous lines could be identified, either in T_2 or in T_3 generation plants. All the selected lines contained a single transgene insertion as estimated by qPCR in comparison with the *Sucrose Phosphate Synthase* (*SPS*) gene (data not shown). Segregation ratios in hygromycin selection media were about 50% of resistant plants, suggesting a negative effect of the transgene in homozygosis.

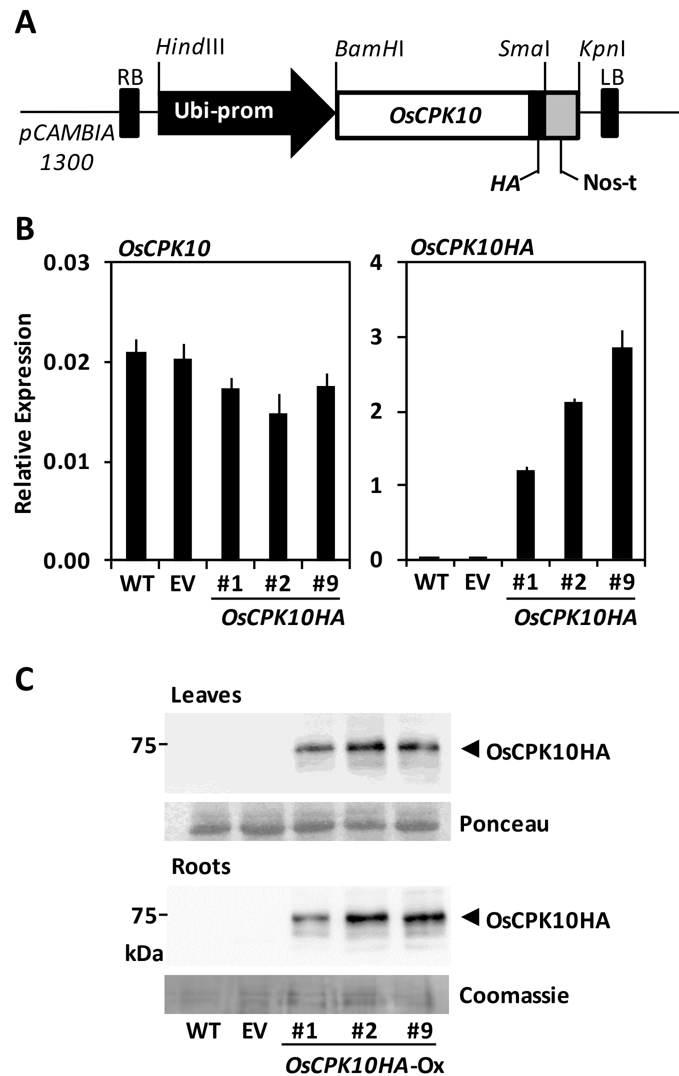

Figure CIII.3: OsCPK10HA accumulation in transgenic rice plants. A, Schematic representation of the pUbi::*OsCPK10HA::nos* transgene used for rice transformation. B, Transcript levels of *OsCPK10* and *OsCPK10HA* in leaves of wild-type (WT), empty vector (EV) and the indicated lines of *OsCPK10HA* rice plants as determined by qRT-PCR analysis using *OsUbi5* mRNAs for normalization. Values are the means and SD of three replicates. C, OsCPK10HA protein accumulation in leaves (upper panel) and roots (lower panel) from indicated plants as determined by Western-blot analysis using specific anti-HA antibodies.

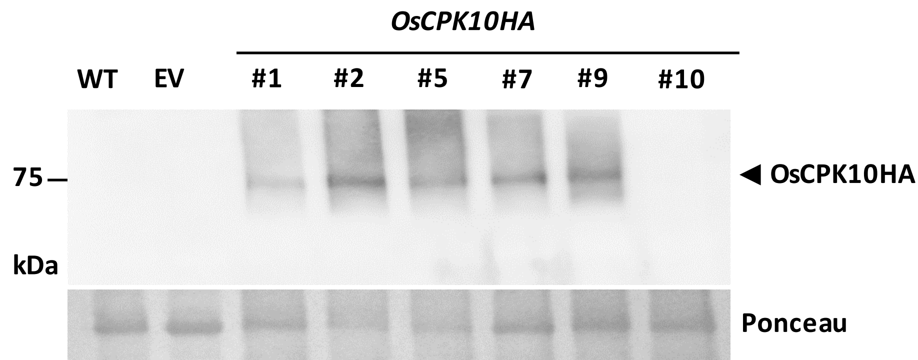

Figure CIII.4: OsCPK10HA accumulation in T_0 transgenic plants. Immunoblot analysis of protein extracts from leaves of wild-type (WT), empty vector and the indicated lines of *OsCPK10HA* transgenic plants (T_0 generation) using specific anti-HA antibodies.

Quantitative RT-PCR analysis confirmed that *OsCPK10HA* transcripts were accumulated in the hemyzygous plants in T3 generation (lines #1, #2 and #9), without affecting *OsCPK10* endogenous expression (Figure CIII.3A, B). The tagged protein was detected by immunoblot analysis using anti-HA antibodies in the roots and shoots of the selected plants (Figure CIII.3C). These results demonstrated that the generated transgenic lines expressed the *OsCPK10HA* gene, and the tagged-protein is accumulated in the rice plant tissues. These *OsCPK10HA* hemyzygotic plants showed a normal phenotypic appearance, quite similar to wild-type or empty vector control plants, when grown under greenhouse conditions (Figure. CIII.5A). Several growth parameters were measured in two independent growing seasons with plants randomly distributed, and no statistically significant differences were observed among the lines and compared with control plants. They flowered at the same time (Figure CIII.5B), reaching the same height at heading time (Figure CIII.5C), produced similar grain yield (Figure CII.5D), and the seed weight is similar (Figure CIII.5E). Therefore, the expression of *OsCPK10HA* in hemizygosis appears not to have deleterious effects on rice plant performance.

No insertional mutants in *OsCPK10* gene were found in the publically available rice mutant collections; hence all our studies were carried out with *OsCPK10HA* rice plants.

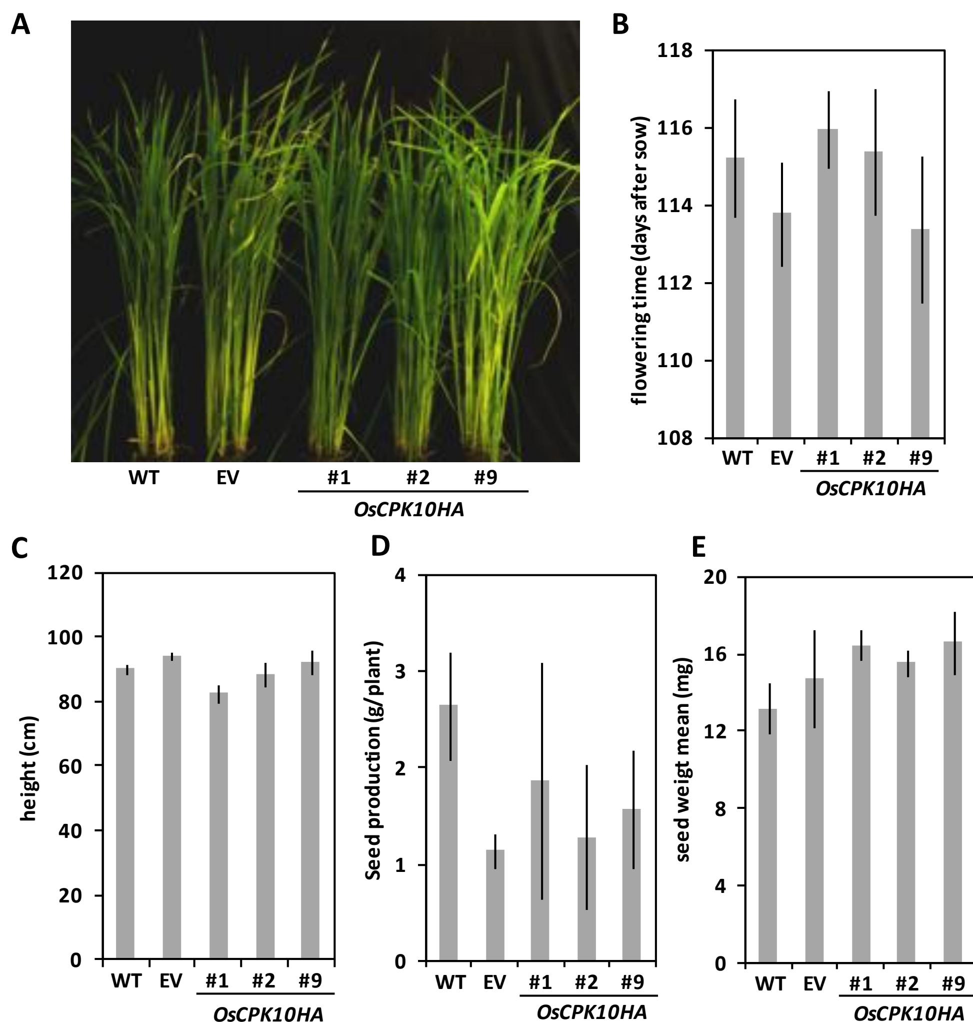

Figure CIII.5: Performance of *OsCPK10HA* rice plants. A, Phenotypic appearance of wild-type (WT), empty vector (EV) and *OsCPK10HA* (lines #1, #2 and #9) rice plants at 127 days after sowing. B, Height of plants at heading time. C, Flowering time (days after sowing). D, Average grain yield per plant grown under randomized distribution. E, Seed weight. Values are the mean of five different plants per line ± SD, and are representative of two independent assays. Parameters were recorded for five different plants per line. Results are representative of two independent experiments. No significant differences were measured for these parameters.

OsCPK10HA expression enhances blast disease resistance in rice plants.

The effects of *OsCPK10HA* expression on the rice defense response were assessed by inoculating the transgenic plants with the blast fungal pathogen. As shown in Figure CIII.6A, *OsCPK10HA* plants exhibited healthier appearance at 7 dpi as compared with wild-type or empty vector control plants, these ones showing a wilting phenotype. A close inspection showed extensive necrotic lesions with fungal sporulation on wild-

type and empty-vector control leaves, whereas only few restricted lesions were developed on the *OsCPK10HA* leaves (Figure CIII.6B). The percentage of leaf area affected by blast lesions was determined by image analysis, the results revealing a statistically significant reduction on the lesion area of the tree independent transgenic lines as compared with the control leaves (Figure CIII.6C). Further measures of disease severity showed that a higher percentage of *OsCPK10HA* plants exhibited resistant phenotype (around 20%) than wild-type or empty vector plants (0%), and a lower percentage exhibited highly susceptible phenotype (around 20%) than control plants (70%) (Figure CIII.6D). Consistently with the visual inspection, *OsCPK10* leaves bore significant less fungal biomass than control leaves, as determined by qPCR of *M. oryzae* DNA (Figure CIII.6E). Collectively, these results show that *OsCPK10HA* positively mediates enhanced resistance to blast disease.

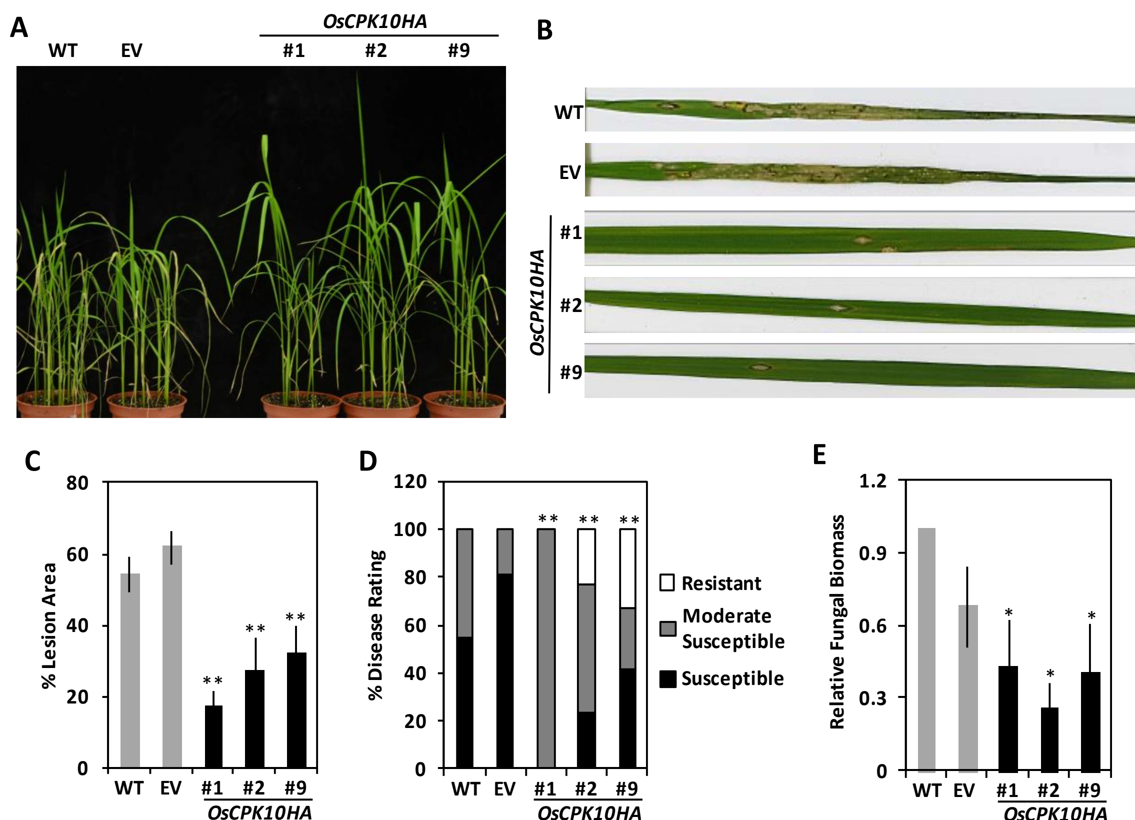

Figure CIII.6: Resistance of *OsCPK10HA* plants to *Magnaporthe oryzae* infection. A, Phenotype of wild-type (WT), empty vector (EV) and three independent lines of *OsCPK10HA* rice plants (#1, #2, #9) at 7 days post inoculation (dpi) with a *M. oryzae* spore suspension (10^5 spore/ml). B, Blast disease leaf symptoms in detail. C, Average of leaf lesion area percentage of leaves. D, Average of disease rating percentage according to the Standard Evaluation System for Blast Disease (IRRI, 1996). E, Relative fungal amount as determined by qPCR of *M.oryzae* 26S rDNA gene compared to rice *Ubiquitin1* gene and referred to WT. Values are means ± SE of two independent assays with 10 plants per line at 7dpi. Asterisks indicate significant differences (one-way ANOVA analysis, *P<0.05, ** P<0.01).

OsCPK10HA expression improves drought tolerance in rice plants

To investigate whether OsCPK10 also has a role in the adaptation of rice plants to water stress, the *OsCPK10HA* plants were assessed for drought tolerance. For that, transgenic, empty vector and wild-type plants were grown under fully watered regime for 22 days (Figure CIII.7A, 7B D22), and then deprived of irrigation for 12 days (Figure CIII.7A, 7B D34). At this time, all the plants were severely affected by the water deficit showing pale color, dried leaves and wilting phenotype (Figure CIII.7B, D34). Plants were then returned to regular watering conditions for recovering (Figure CIII.7A). Fifteen days later, only *OsCPK10HA* showed green leaves and survived to the drought treatment (Figure CIII.7B, D49). These results were consistently reproduced in three independent experiments, *OsCPK10HA* plants showing a survival score about of 25% to 44% significantly higher than the 0% of the control plants (Figure CIII.7C). This improved performance of *OsCPK10HA* plants was also shown by measuring their fresh weight after recovery. As compared with control plants, a clear increased on the fresh weight was observed for the same dry weight in all the lines (Fig. CIII.7D). These results indicate that *OsCPK10HA* expression increases drought tolerance in rice plants.

To evaluate whether the enhanced drought tolerance was caused by a better water retention ability, the water loss rates were calculated at early times of rice plant desiccation. Results in Figure CIII.7E showed no significant differences among the control and transgenic lines, suggesting that the exhibited drought tolerance of *OsCPK10HA* plants is not mediated by a reduction of the water loss.

OsCPK10HA expression improves oxidative stress tolerance in rice plants by increasing their antioxidant activity

Diverse biotic and abiotic stresses trigger the rapid production of ROS that act as stress signaling molecules due to their capacity to diffuse membranes; although their uncontrolled production can reach phytotoxic levels and cause oxidative damage of membranes and other components (Torres, 2010; Barna *et al*., 2012; Mittler *et al*., 2011; Choudhury *et al*., 2013; Mittler and Blumwald, 2015). To investigate the ROS production in the *OsCPK10HA* plants, transgenic seedlings were air-dried during 4 hours and the H_2O_2 levels were determined in leaves (Figure CIII.8A). *OsCPK10HA* plants accumulated less H_2O_2 during the air dry treatment compared to the wild-type

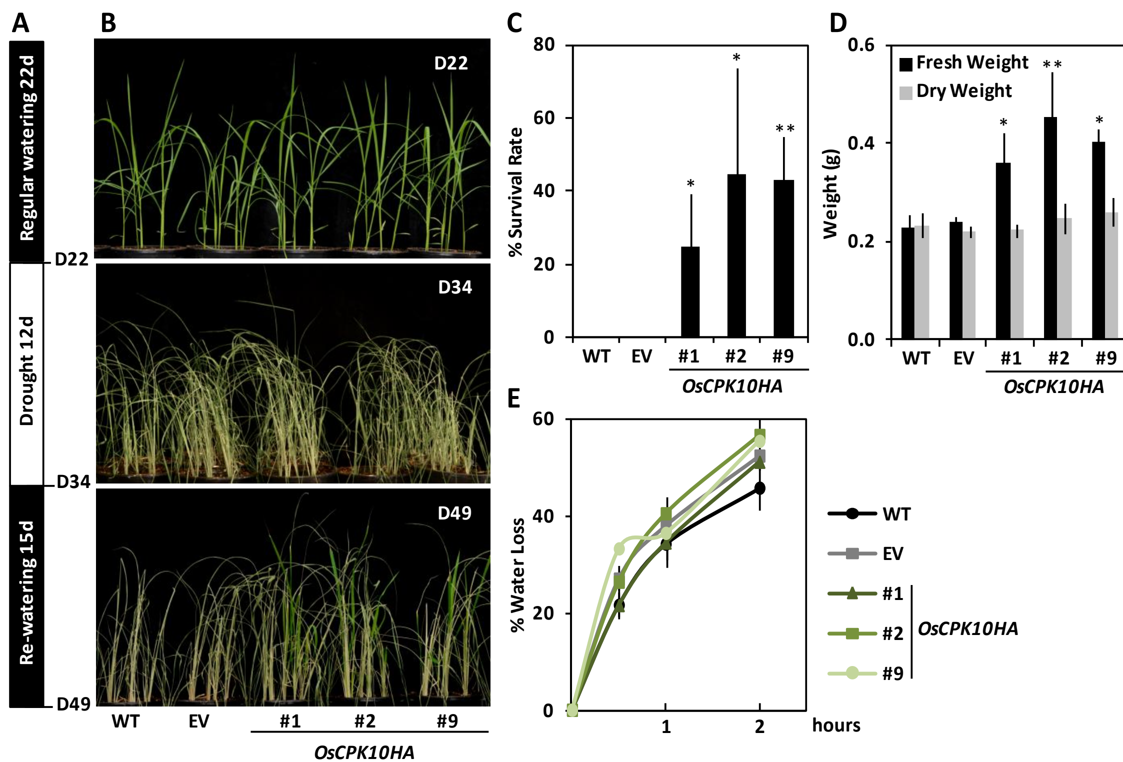

Figure CIII.7: Drought tolerance of *OsCPK10HA* rice plants. A, Diagram of the experimental design for drought tolerance assays. B, Phenotypical appearance of wild-type (WT), empty vector (EV) and three lines (#1, #2, #9) of *OsCPK10HA* transgenic plants at the indicated phases of the drought tolerance assay. C, D, Survival rates (C) and fresh and dry weights (D) of plants after rewatering (D49). Values are the means ± SE of three independent assays with five plants per line. E, Water loss rate of air-dried 10 day-old seedlings (9 seedlings per line). Asterisks show significant differences (one-way ANOVA analysis, *$P \leq 0.05$, **$P \leq 0.01$).

and EV plants. This observed reduction in hydrogen peroxide accumulation might be due to a higher capacity to be metabolized by the *OsCPK10HA* plants.

To further investigate their ROS scavenging capacity, leaf pieces of the transgenic and control plants were exposed to the oxidative agent methyl viologen (MV). After 4 days of treatment, the *OsCPK10HA* leaves showed significantly higher chlorophyll content than the empty vector or wild-type leaves (Figure CIII.8B). Visual inspection confirmed that the leaf pieces of the *OsCPK10HA* plants remained green at the end of the treatment whereas control leaves were totally whitish (Figure CIII.8C). These data indicated that the photosynthetic apparatus was less damaged by MV-induced oxidative stress in the lines accumulating OsCPK10HA. Therefore, OsCPK10HA plants showed an improved tolerance to oxidative stress, suggesting they have a better antioxidant capacity.

Catalase proteins are among the main H_2O_2 scavengers in plant cells (Jiang and Zhang, 2002; Du *et al*., 2008; Ye *et al*., 2011). Thus, the catalase levels were monitored in the *OsCPK10HA* transgenic lines under control conditions or in response to drought stress by western-blot analysis. Immunodetection using anti-catalase antibodies showed an immunoreactive polypeptide around 55 kDa, with a higher intensity in the air-dried samples of *OsCPK10HA* leaves than in wild-type or empty vector leaves (Figure CIII.8D). These results suggest that catalase proteins are accumulated to higher levels in the *OsCPK10HA* leaves in response to drought stress than in control plants, thus leading to a higher catalase activity. As a result, the *OsCPK10HA* leaves showed a higher hydrogen peroxide detoxifying capacity and, as observed in Figure CIII.8A., accumulated lower H_20_2 levels than control plants.

Three catalase genes have been identified in the rice genome, namely *OsCAT-A* (LOC_Os02g02400), *OsCAT-B* (LOC_Os06g51150) and *OsCAT-C* (LOC_Os03g03910). The expression levels of the three genes were analyzed by qRT-PCR in the shoots of dessicated seedlings. As shown in Figure CIII.8E, the *OsCAT-A* transcripts accumulated to a higher extend in the *OsCPK10HA* leaves than in the control plants, especially in the lines #1 and #2 those that showed higher catalase protein accumulation in the western-blot analysis (Figure CIII.8D). The *OsCAT-B* and *OsCAT-C* transcripts accumulated to lower levels than the *OsCAT-A* transcripts, without significant differences among the analyzed lines (Figure CIII.8E). These results point to the rice Catalase A as the responsible for the higher detoxifying capacity depicted by *OsCPK10HA* plants.

OsCPK10HA prevents membrane damage during drought stress in rice plants

The higher ability to detoxify H_2O_2 observed in the *OsCPK10HA* plants might provide protection against the oxidative damage that accompanies drought stress, which might cause lipid peroxidation and perturbation of the cell membrane functioning. To further investigate the mechanism underlying drought tolerance in *OsCPK10HA* plants, the lipid peroxidation levels were examined in the transgenic plants in comparison to wild-type plants. Lipid peroxidation was measured as malondialdehyde (MDA) content, MDA being a typical breakdown product of peroxidized polysanturated fatty acids in

plant membranes (Campo *et al*., 2014). As shown in Figure CIII.8F, the MDA content was increased in response to the desiccation treatment in wild-type plants, whereas the *OsCPK10HA* plants maintained a low MDA content upon desiccation. The MDA content of desiccated *OsCPK10HA* plants was significantly reduced to more than a half in comparison to desiccated wild-type plants. These results indicate a lower degree of lipid peroxidation caused by desiccation in the *OsCPK10HA* plants, possibly due to their higher ROS detoxifying capacity.

As a measurement of membrane damage caused by desiccation, the electrolyte leakage was evaluated in the transgenic and wild-type plants. Interestingly, the *OsCPK10HA* leaves showed significant lower values of electrolyte leakage than wild-type plants not only upon desiccation but also in control conditions (Figure CIII.8G). These results indicated that OsCPK10HA preserves membrane integrity, especially during drought stress.

Discussion

This study shows that *OsCPK10* is induced by *M. oryzae* infection, drought stress, as well as by ABA treatment, in rice plants. This transcriptional activation is consistent with the presence of various biotic and abiotic stress-related elements in the promoter, including GCC, WRKY, ABRE and DRE motifs. The transcriptional regulation of *CPK* genes in response to stress has been extensively documented in the literature, which in most cases correlates with the functional involvement in the stress induced response (Coca and San Segundo, 2010; Asano *et al*., 2011; Fu *et al*., 2013; Campo *et al*., 2014; Fu *et al*., 2014). These results suggest that OsCPK10 is involved in both the biotic and abiotic stress responses of rice plants.

Transgenic rice plants constitutively expressing a recombinant *OsCPK10* gene that encodes an HA-tagged protein were produced. The tag was added at the C-terminal of the protein to avoid any interference with myristoylation and palmitoylation sites found at the N-terminal of the protein, these sequences mediating the association to membranes in other reported CPK proteins (Martin and Busconi, 2000; Lu and Hrabak, 2002; Coca and San Segundo, 2010; Witte *et al*., 2010; Campos-Soriano *et al*., 2011).

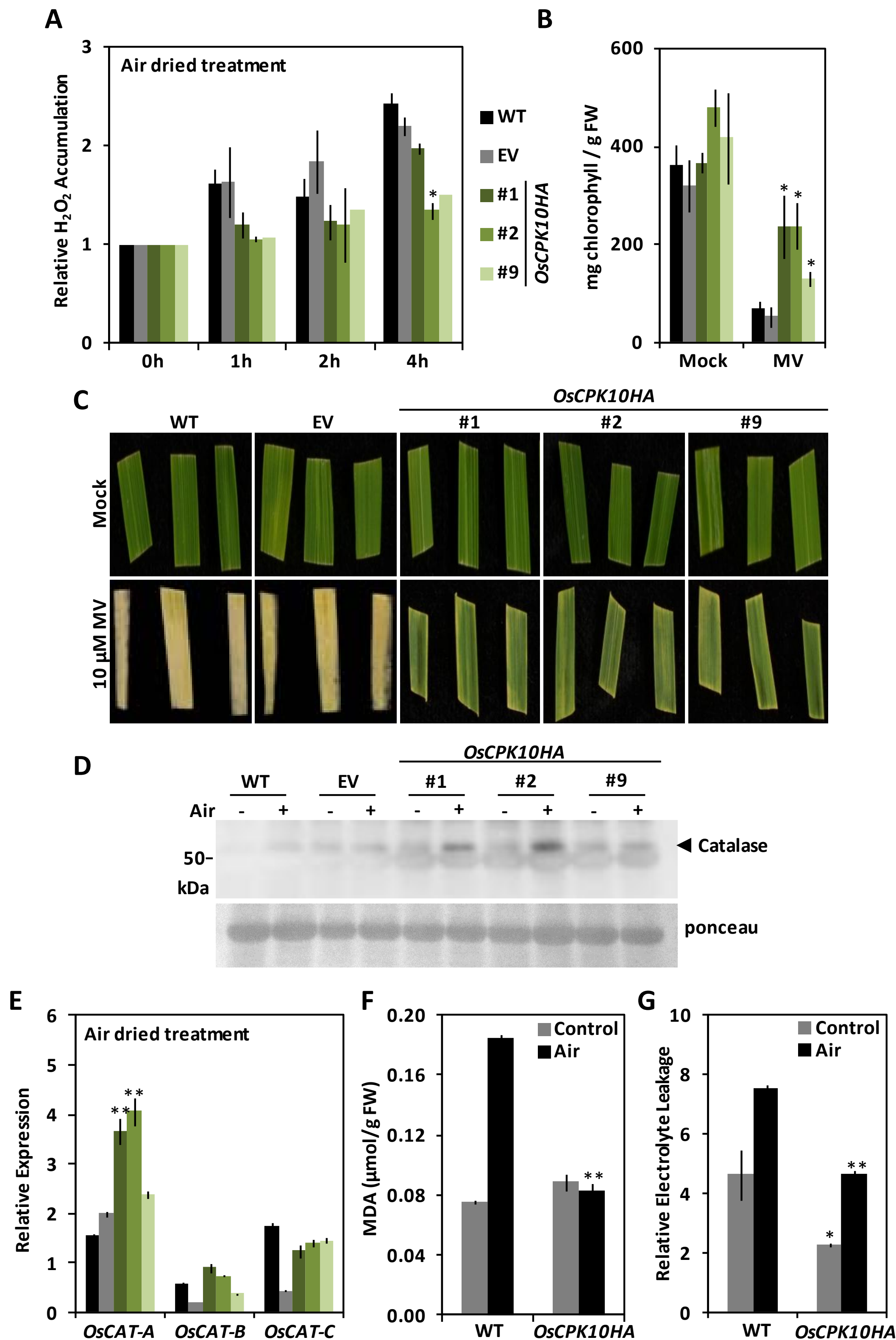

Figure CIII.8: Higher H_2O_2 detoxifying capacity of *OsCPK10HA* rice plants in response to drought stress. A, H_2O_2 content in air-dried wild-type (WT), empty vector (EV) and three lines of *OsCPK10HA* rice shoots at the indicated times. Values are the means ± SEM of the increments in H_2O_2 concentration in pools of three 10 days-old seedlings for each line and two independent assays. B, Chlorophyll content of mock or 10 µM methylviologen (MV) treated leaf fragments for 4 days. Values are the means ± SEM of three independent assays with four biological replicates. C, Representative images of leaf fragments treated with mock or MV for 4 days. D, Western-blot analysis of catalase accumulation in rice shoots untreated (-)

or air dried treated (+) for 2h. E, Transcript levels of the three rice *catalase* genes as determined by qRT-PCR analysis, and normalized to the *OsUbi5* in 2h-air dried shoots. Same color code as in A and B panels. Values are the means ± SD of three technical replicates for RNA samples from pools of three seedlings per line. F, MDA content, and G, Relative electrolyte leakage of WT and *OsCPK10HA* line #2 seedlings in control conditions or after 4h-air dried treatment. Values are the means ± SEM of three biological replicates from a pool of three seedlings. Asterisks show significant differences (one-way ANOVA analysis, *P≤0.05, **P≤0.01).

Indeed, OsCPK10 was localized associated to the plasma membrane when transiently produced in *N. benthamiana* epidermal cells. By means of an HA-tag, the accumulation of the OsCPK10HA protein was detected in the produced transgenic lines. The produced OsCPK10HA protein contained the four typical domains of CPK proteins, including the two regulatory ones, the junction autoinhibitory, and the calmodulin domain. Thus, the accumulated protein should preserve their previously reported calcium regulation (Fu *et al*., 2013). Presumably, the *OsCPK10HA* expressing plants accumulated constitutively the protein in an inactive state but prone to be activated by calcium signals. Homozygous lines accumulating the OsCPK10HA could not be obtained, although hemyzygous showed a normal appearance and reproduced as wild-type plants. These results suggest that high accumulation levels of OsCPK10HA protein might impair plant viability. A potential function of OsCPK10 in the plant development might be inferred from these observations.

The constitutive accumulation of the OsCPK10HA protein conferred rice plants with an enhanced resistance to the blast disease, as determined by visual inspection, fungal growth quantification, and disease lesion measurement. These results confirmed a previous report that showed that rice plants accumulating a constitutive active OsCPK10 form were more resistant to *M. oryzae* infection (Fu *et al*., 2013). Thus, the fully HA-tagged protein appears to be a functional protein, the HA-tag not interfering with its activity. Hence, OsCPK10 is a positive modulator of the rice defense response to the blast fungus.

OsCPK10HA plants also showed an improved tolerance to drought stress, since the transgenic plants were able to recover from a desiccation episode upon returning to sufficient water conditions. These results demonstrate that OsCPK10 plays also positive role in the adaptation of rice plants to drought stress. Drought tolerance can be achieved by different mechanisms contributing to dehydratation avoidance or

dehydratation tolerance (Verslues *et al*., 2006; Osakabe *et al*., 2014). Drought tolerant plants avoiding dehydratation are able to retain the tissue water content by stomatal closure and osmotic adjustment. *OsCPK10HA* plants suffered similar dehydratation than control plants, as indicated by the water retention curves, these data suggesting that dehydratation avoidance is not the drought adaptive strategy followed by these transgenic plants. Thus, OsCPK10 seems to assist a dehydration tolerance strategy to improve the performance of rice plants upon drought stress. The mechanisms underlying dehydratation tolerance prevent cellular damages caused by water loss, including the synthesis of protective proteins, ROS detoxification and other metabolic changes. In the case of *OsCPK10HA* plants, drought tolerance appeared to be mediated by an efficient ROS detoxification capacity. This drought tolerance mechanism has been widely documented (Kumar *et al*., 2014; Nakabayashi *et al*., 2014; Fang *et al*., 2015; Yin *et al*., 2015). We show here that OsCPK10 promotes an increased accumulation of Catalase A in response to desiccation stress, this enzyme catalyzing the decomposition of the highly reactive hydrogen peroxide, and protecting from its toxic effects. Drought tolerance improvement afforded by the accumulation of Catalase A in rice tissues has been already reported (Joo *et al*., 2014). The molecular mechanism through which OsCPK10 modulates the accumulation of the Catalase A remains to be solved. A transcriptional regulation through other signaling components might be a possibility, in agreement with the elevated *CatA* transcript levels detected in OsCPK10HA plants. Although other possibilities can also be considered, such as a direct OsCPK10-Catalase A interaction that stabilizes Catalase A. The interaction between a catalase protein and a CPK protein has been already shown in Arabidopsis plants, this interaction associated also to drought stress tolerance (Zou *et al*., 2015). Be that as it may, the increased accumulation of Catalase A promoted by OsCPK10 improved the antioxidant capacity of the *OsCPK10HA* rice plants, that reduced the levels of lipid peroxidation and preserved the membrane integrity upon desiccation. The oxidation of membrane polyunsaturated fatty acids by the excess of ROS associated to different abiotic stresses is known to provoke cell membrane damage and to increase membrane leakage (Wong-ekkabut *et al*., 2007; Bhattacharjee, 2014; Ayala *et al*., 2014). Preservation of the integrity and stability of cell membranes is a major determinant of drought tolerance in plants (Bajji, 2002; Farooq *et al*., 2009). In

fact, QTLs of membrane stability have been found in drought tolerant rice (Tripathy, 2000).

This increased ROS scavenging activity promoted by OsCPK10 might also benefit blast disease resistance. Lipid peroxidation also has an important effect during the infection processes, their levels being increased in rice leaves infected with *M. oryzae* (Ohta *et al*., 1991). Several lipid molecules are precursors of the defense signaling hormones ethylene and jasmonates, their peroxidation might be interfering with the defense response. When lipid peroxidation reached a threshold upon stress, cells commit suicide leading to necrosis (Spiteller, 2003), which might impede the propagation of biotrophic pathogens but benefit necrotophic pathogens (Glazebrook, 2005). Considering that *M. oryzae* is a hemibiotrophic fungus, a reduction on lipid peridoxidation as occurred in *OsCPK10HA* plants might impair the necroprophic phase of *M. oryzae* infection associated to lesions development in leaves.

Together, our results demonstrate that OsCPK10 positively modulates blast disease resistance and drought tolerance. Hence, OsCPK10 appears as a convergent component of biotic and abiotic stress responses. Recently, two other OsCPKs have been reported as common signaling components of both signaling pathways, namely OsCPK4 and OsCPK12. OsCPK4 contributes positively to blast resistance and to drought tolerance (Campo *et al*., 2014; Bundó and Coca, 2015), whereas OsCPK12 shows an antagonistic function mediating susceptibility to blast disease and salinity resistance (Asano *et al*., 2012). Therefore, these data point to CPKs as regulators of the interaction between biotic and abiotic signaling pathways. ROS, hormones and calcium signals appear as shared components between both abiotic and biotic stress signaling (Kissoudis *et al*., 2014; Barrios-Perez and Brown, 2014; Bostock 2014). OsCPK10 is transcriptionally regulated by ABA, functionally activated by calcium and a regulator of ROS levels, thus it is related to the major components of the regulatory stress networks. These observations suggest that OsCPK10 could be a master regulator of stress signaling pathways, thus providing new insights into the regulation of stress signaling network which offers new possibilities in the design of new and efficient strategies for rice crop improvement.

Experimental procedures

Plant materials, growth conditions, and stress treatments

Rice (*Oryza sativa* var. Nipponbare) was grown at 28ºC and 16h light/8h dark photoperiod. For drought stress treatments, plants were grown in sealed jars at 100% humidity for 10 days and left to air dried for the required period of time. ABA treatments were done also with 10 day-old seedlings by adding a 100 µM solution. Three technical and biological replicates were analyzed in each treatment.

Production of transgenic rice plants

For the expression of the *OsCPK10HA* gene, the full length OsCPK10 coding sequence extended in C-terminal with the sequences encoding the HA epitope was obtained. This DNA fragment was generated by PCR amplification from the Rice Genome Resource Center clone J013164K19, using the primers indicated in Table CIII.2, which introduced a *BamH*I restriction site at the 5´end (forward primer), and a *Sma*I restriction site and the HA-epitope sequences at the 3´end (reverse primer), just before the stop codon of the cDNA. The PCR fragment was cloned into the *BamH*I and *Sma*I sites of a pCAMBIA1300-derived vector containing the maize *Ubiquitin1* promoter (pUbi) and the *Nopaline synthase* terminator (Nos-t) previously described (Campo *et al*., 2014). The derived construct was verified by DNA sequencing. *Agrobacterium tumefaciens* strain EHA105 was transformed with the final vector for rice transformation, and transgenic rice plants (*O. sativa* cv. Nipponbare) were produced as previously described (Sallaud *et al*., 2003). The hygromycin resistance encoded in the T-DNA region was used as selection marker. The transgene insertion copies were estimated by qPCR using the *SPS* as reference gene as previously described (Bundó *et al*., 2014; Yang *et al*., 2005)

RNA isolation and qRT-PCR anaysis

Total RNA was extracted using TRIzol reagent (Invitrogen). DNAse treated RNA (1 µg) was retrotranscribed using the transcriptor first cDNA synthesis kit (Roche). qRT-PCR analyses were carried out in 96-well optical plates in a LightCycler® 480 System (Roche, Mannheim, Germany) according to the following program: 10 min at 95 °C, 45 cycles of 95 °C for 10s and 60 °C for 30s, and an additional cycle of dissociation curves to ensure

a unique amplification. The reaction mixture contained 5µl of SYBR Green Master mix reagent (Roche), 2µl of 1:4 diluted cDNA sample and 300 nM of each gene-specific primer (Table CIII.2) in a final volume of 10 µl. The results for the gene expression were normalized to *OsUbi5* (LOC_Os01g22490) gene. Three technical replicates were done for each sample.

Table CIII.2: Primer sequences of genes used for gene expression analysis. Restriction sites sequences are underlined and HA sequence is in bold.

Gene name	Gene Locus	Primer sequences
OsCPK10 (qRT-PCR)	LOC_Os03g57450	For 5'-CAGAACAGTTTCAGCATCGGC-3' Rev 5'-CATTTTTTTCCCCGTTTCGAA-3'
OsCPK10HA (qRT-PCR transgene)	LOC_Os03g57450	For 5'-ACCCATACGATGTTCCAGATTACG-3' Rev 5'-AAATGTTTGAACGATCCCCG-3'
OsCPK10-CDS + *HA* (cloning to pCAMBIA 1300)	LOC_Os03g57450	For 5'-GGATCCAATGGGGAACACGTGCGTC-3'(BamHI) Rev 5'-GCCCCGGGCTA**AGCGTAATCTGGAACATCG TATGGG**TATGGAAGACAACATATCGATCT-3'(*Sma*I)
OSCPK10-CDS (cloning to pMDC85)	LOC_Os03g57450	For 5'-GGATCCGGGAACACGTGCGT-3'(*Eco*RI) Rev 5'-GCGGCCGCTGGAAGACAACATATCGAT-3'(*Not*I)
OsUbi5 (qRT-PCR)	LOC_Os01g22490	For 5'-TAAGTGCGGCCTCACCTACG-3' Rev 5'-GGAGCCTACGCCTAAGCCTG-3'
26S-M.oryzae (qPCR)	AB026819	For 5'-TACGAGAGGAACCGCTCATTCAGATAATTA-3' Rev 5'-TCAGCAGATCGTAACGATAAAGCTACTC-3'
OsUbi1 (qPCR)	LOC_Os06g46770	For 5'-TTCCCCAATGGAGCTATGGTT-3' Rev 5'-AAACGGGACACGACCAAGG-3'
OsRab21 (qRT-PCR)	LOC_Os12g36880	For 5'-CGAGCGCAATAAAAGGAAAAA-3' Rev 5'-GAACGCCATCACACATTCACA-3'
OsCATA (qRT-PCR)	LOC_Os02g02400	For 5'-ACCTACACCTTCGTCACCCG-3' Rev 5'-GTGGAACTTGACGTACCTGGC-3'
OsCATB (qRT-PCR)	LOC_Os06g51150	For 5'-CCCCACATCCAGTTCGATTC-3' Rev 5'-CTTGTAGGGATCCATGGCGT-3'
OsCATC (qRT-PCR)	LOC_Os03g03910	For 5'-GATGGTGCTCAACCGCAAC-3' Rev 5'-AGCTGCTCGTTCTCCGAGAA-3'

Subcellular localization of OsCPK10

A construct for the production of a GFP C-terminal fusion protein was obtained by introducing the *OsCPK10* coding sequence into the pMDC85 plant expression vector (Curtis and Grossniklaus, 2003). For this, the *OsCPK10* coding sequence without the stop codon was previously cloned into pENTR3C plasmid (Invitrogen) after PCR amplification, using the primers indicated in Table CIII.2 which introduced a *Eco*RI

restriction site at the 5´end (forward primer) and a *Not*I restriction site at the 3´end (reverse primer), from the clone J013164K19 (Rice Genome Resource Center).

The *OsCPK10-GFP* fusion gene was transiently expressed in *Nicotiana benthamiana rdr6IR* mutant leaves (Schwach *et al*., 2005) by agroinfiltration using the *A. tumefaciens* strain EHA105 as previously described (Campo *et al*., 2013). Observations were performed at 48 hours after infiltration.

Confocal laser scanning microscopy was performed using an Olympus FV1000 microscope. The GFP was excited with an Argon ion laser emitting at 488 nm and fluorescence detected at 500-550 nm. To confirm plasma membrane localization, leaf cells were plasmolysed with 0.75 M mannitol during 15 min, or stained with 10 µM solution of the liophilic dye FM4-64 (Molecular Probes). Fluorescence was observed immediately after washing by exciting with a 543 nm argon ion laser.

Protein extracts and immunoblot analysis

Protein extracts for OsCPK10HA immunodetection were obtained from a pool of at least 4 different plants as previously described (Bundó and Coca, 2015). For catalase immunodetection, the protein extracts were obtained from the soluble fractions after centrifugation of shoot samples resuspended in two volumes of extraction buffer (50mM sodium-phosphate buffer pH7, 1mM EDTA, 1% w/v insoluble polyvinyl-polypyrrolidone). Western blot analyses were performed using anti-HA (Sigma, H6908) and anti-Catalase (Abcam, 1877) antibodies.

Rice blast disease resistance assays

M. oryzae infections with FR13 strain (provided by Dr. D. Tharreau, CIRAD, Montpellier France) were performed using the whole plant infection assay previously described (Bundo and Coca, 2015).

Drought tolerance assays

Rice plants were grown in soil at 28ºC, 14h light/10h darkness photoperiod under normal watering conditions for three weeks. At this moment, drought stress was applied by stopping the watering until desiccation symptoms were visible, such as wilting and whitening. Then, plants were rewatered for two weeks. Three independent

assays were performed, with five plants per line. Drought tolerance was evaluated by survival rate, fresh weight and dry weight, and it was measured at the end of the assay. To determine the water loss of the plants, 10-day old seedlings were air-dried and weighted at 0h, 30 minutes, 1h and 2h. Water loss percentage was calculated with the formula lost weight/initial weight ×100. Three biological and technical replicates were done.

Determination of hydrogen peroxide content

The content of H_2O_2 in control or air-dried shoots was determined as described in Velikova *et al*., 2000. Briefly, frozen and pulverized shoots (500 mg) were homogenized in 300 µl of 0.1% (w/v) TCA and centrifugated for 15 minutes at 13,000g. The recovered supernatants (500 µl) were mixed with 500 µl of 10 mM sodium-phosphate buffer pH 7.5 and 1 ml of 1M potassium iodide, and the absorbance at 390 nm was determined. The H_2O_2 concentration was calculated using de extinction coefficient $\varepsilon = 0.28\ \mu M^{-1}\ cm^{-1}$.

Oxidative stress tolerance assays

Tolerance assays to the oxidative agent MV (Sigma) were done with leaf fragments of ten-day old seedlings. Leaf fragments were incubated in sterile water (mock) or 10 µM MV solution (MV treatment), both solutions supplemented with 0.02% Tween-20, during 4 days at 28ºC and 16h light/8h darkness photoperiod, at 200 lux of light intensity. At the end of the assay, chlorophyll content was measured following the protocol described in Lichtenthaler and Buschmann, 2001.

MDA content and relative electrolyte leakage was determined as described in Campo *et al*., 2014, using pools of three biological replicates of control and air dried seedlings of ten-day old.

References

Asano, T., Hakata, M., Nakamura, H., Aoki, N., Komatsu, S., Ichikawa, H., Hirochika, H., Ohsugi, R. (2011). Functional characterisation of OsCPK21, a calcium-dependent protein kinase that confers salt tolerance in rice. *Plant Molecular Biology*, **75**(1), 179–191.

Asano, T., Hayashi, N., Kobayashi, M., Aoki, N., Miyao, A., Mitsuhara, I., Ichikawa, H., Komatsu, S., Hirochika, H., Kikuchi, S., Ohsugi, R. (2012). A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. *Plant Journal*, **69**(1), 26–36.

Asano, T., Tanaka, N., Yang, G., Hayashi, N., & Komatsu, S. (2005). Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: Comprehensive analysis of the CDPKs gene family in rice. *Plant and Cell Physiology*, **46**(2), 356–366.

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*. **2014**, 31.

Bajji, M., Kinet, J.-M., & Lutts, S. (2002). The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regulation*, **36**(1), 61–70.

Ballini, E., Morel, J.-B., Droc, G., Price, A., Courtois, B., Notteghem, J.-L., & Tharreau, D. (2008). A Genome-Wide Meta-Analysis of Rice Blast Resistance Genes and Quantitative Trait Loci Provides New Insights into Partial and Complete Resistance. *Molecular Plant-Microbe Interactions*, **21**(7), 859–868.

Barna, B., Fodor, J., Harrach, B. D., Pogány, M., & Király, Z. (2012). The Janus face of reactive oxygen species in resistance and susceptibility of plants to necrotrophic and biotrophic pathogens. *Plant Physiology and Biochemistry*, **59**, 37–43.

Barrios Perez, I., & Brown, P. J. (2014). The Role of ROS Signaling in Cross-Tolerance: From Model to Crop. *Frontiers in Plant Science*. **5**, 754

Bhattacharjee, S. (2014). Membrane lipid peroxidation and its conflict of interest: the two faces of oxidative stress. *Current Science*, **107**(11), 1811–1823.

Bostock, R. M., Pye, M. F., & Roubtsova, T. V. (2014). Predisposition in Plant Disease: Exploiting the Nexus in Abiotic and Biotic Stress Perception and Response. *Annual Review of Phytopathology*, **52**(1), 517–549.

Boudsocq, M., & Sheen, J. (2013). CDPKs in immune and stress signaling. *Trends in Plant Science*, **18**(1), 30–40.

Bouman, B. A. M., Lampayan, R. M., & Toung, T. P. (2007). *Water management in irrigated rice: coping with water scarcity*. Los Baños; Philippines: International Rice Research Institute.

Bulgakov, V. P., Gorpenchenko, T. Y., Shkryl, Y. N., Veremeichik, G. N., Mischenko, N. P., Avramenko, T. V, Fedoreyev, S.A., Zhuravlev, Y. N. (2011). CDPK-driven changes in the intracellular ROS level and plant secondary metabolism. *Bioengineered Bugs*, **2**(6), 327–330.

Bundó, M., & Coca, M. (2015). Enhancing blast disease resistance by overexpression of the calcium-dependent protein kinase OsCPK4 in rice. *Plant Biotechnology Journal*, (Under revision).

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., & San Segundo, B. (2014). Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. *Plant Physiology*, **165**(2), 688–704.

Campo, S., Peris-Peris, C., Siré, C., Moreno, A. B., Donaire, L., Zytnicki, M., Notredame, C., Llave, C., San Segundo, B. (2013). Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. *New Phytologist*, **199**(1), 212–227.

Campos-Soriano, L., Gómez-Ariza, J., Bonfante, P., & San Segundo, B. (2011). A rice calcium-dependent protein kinase is expressed in cortical root cells during the presymbiotic phase of the arbuscular mycorrhizal symbiosis. *BMC Plant Biology*, **11**(1), 90.

Campos-Soriano, L., Valè, G., Lupotto, E., & San Segundo, B. (2013). Investigation of rice blast development in susceptible and resistant rice cultivars using a gfp-expressing Magnaporthe oryzae isolate. *Plant Pathology*, **62**(5), 1030–1037.

Cao, W.-H., Liu, J., He, X.-J., Mu, R.-L., Zhou, H.-L., Chen, S.-Y., & Zhang, J.-S. (2007). Modulation of Ethylene Responses Affects Plant Salt-Stress Responses. *Plant Physiology*, **143** (2), 707–719.

Cheng, S., Willmann, M. R., Chen, H., & Sheen, J. (2002). Update on Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family 1. *Plant Physiology*, **129**, 469–485.

Choudhury, S., Panda, P., Sahoo, L., & Panda, S. K. (2013). Reactive oxygen species signaling in plants under abiotic stress. *Plant Signaling & Behavior*, **8**(4).

Coca, M., & San Segundo, B. (2010). AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. *The Plant Journal : For Cell and Molecular Biology*, **63**(3), 526–540.

Curtis, M. D., & Grossniklaus, U. (2003). A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. *Plant Physiology*, **133**(2), 462–469.

Dean, R., Van Kan, J. a L., Pretorius, Z. a., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, **13**(4), 414–430.

Ding, Y., Cao, J., Ni, L., Zhu, Y., Zhang, A., Tan, M., & Jiang, M. (2013). ZmCPK11 is involved in abscisic acid-induced antioxidant defence and functions upstream of ZmMPK5 in abscisic acid signalling in maize. *Journal of Experimental Botany*, **64**(4), 871–884.

Du, Y.-Y., Wang, P.-C., Chen, J., & Song, C.-P. (2008). Comprehensive Functional Analysis of the Catalase Gene Family in Arabidopsis thaliana. *Journal of Integrative Plant Biology*, **50**(10), 1318–1326.

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.-P., Schulze, W. X., Romeis, T. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proceedings of the National Academy of Sciences of the United States of America*, **110**(21), 8744–9.

Fang, Y., Liao, K., Du, H., Xu, Y., Song, H., Li, X., & Xiong, L. (2015). A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. *Journal of Experimental Botany*.

Farooq, M., Basra, S. M. A., Wahid, A., Ahmad, N., & Saleem, B. A. (2009). Improving the Drought Tolerance in Rice (Oryza sativa L.) by Exogenous Application of Salicylic Acid. *Journal of Agronomy and Crop Science*, **195**(4), 237–246.

Farooq, M., Hussain, M., Wahid, A., & Siddique, K. (2012). Drought Stress in Plants: An Overview. In R. Aroca (Ed.), *Plant Responses to Drought Stress: From Morphological to Molecular Features* (pp. 1–33). Springer-Verlag Berlin Heidelberg.

Fu, L., Yu, X., & An, C. (2013). Overexpression of constitutively active OsCPK10 increases Arabidopsis resistance against Pseudomonas syringae pv. tomato and rice resistance against Magnaporthe grisea. *Plant Physiology and Biochemistry*, **73**, 202–210.

Fu, L., Yu, X., & An, C. (2014). OsCPK20 positively regulates Arabidopsis resistance against Pseudomonas syringae pv. tomato and rice resistance against Magnaporthe grisea. *Acta Physiologiae Plantarum*, **36**(2), 273–282.

Glazebrook, J. (2005). Contrasting Mechanisms of Defense against Biotrophic and Necrotrophic Pathogens. *Annual Review of Phytopathology*, *43*(1), 205–227.

Harmon, A. C., Gribskov, M., Gubrium, E., & Harper, J. F. (2001). The CDPK superfamily of protein kinases. *New Phytologist*, **151**(1), 175–183.

Harper, J. F., Breton, G., & Harmon, A. (2004). Decoding Ca(2+) signals through plant protein kinases. *Annual Review of Plant Biology*, **55**, 263–288.

Higo, K., Ugawa, Y., Iwamoto, M., & Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Research*, **27**(1), 297–300.

Huang, J., Si, W., Deng, Q., Li, P., & Yang, S. (2014). Rapid evolution of avirulence genes in rice blast fungus Magnaporthe oryzae. *BMC Genetics*, **15**(1), 45.

Ito, T., Nakata, M., Fukazawa, J., Ishida, S., & Takahashi, Y. (2010). Alteration of Substrate Specificity: The Variable N-Terminal Domain of Tobacco Ca(2+)-Dependent Protein Kinase Is Important for Substrate Recognition. *The Plant Cell*, **22**(5), 1592–1604.

Jiang, M., & Zhang, J. (2002). Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *Journal of Experimental Botany*, **53**(379), 2401–2410.

Joo, J., Lee, Y., & Song, S. (2014). Rice CatA, CatB, and CatC are involved in environmental stress response, root growth, and photorespiration, respectively. *Journal of Plant Biology*, **57**(6), 375–382.

Kankanala, P., Czymmek, K., & Valent, B. (2007). Roles for Rice Membrane Dynamics and Plasmodesmata during Biotrophic Invasion by the Blast Fungus. *The Plant Cell*, **19**(2), 706–724.

Kissoudis, C., van de Wiel, C., Visser, R. G. F., & van der Linden, G. (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in Plant Science*, **5**, 207.

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., Yoshioka, H. (2007). Calcium-Dependent Protein Kinases Regulate the Production of Reactive Oxygen Species by Potato NADPH Oxidase. *The Plant Cell*, **19**(3), 1065–1080.

Kumar, M., Lee, S.-C., Kim, J.-Y., Kim, S.-J., Aye, S. S., & Kim, S.-R. (2014). Over-expression of dehydrin gene, OsDhn1, improves drought and salt stress tolerance through scavenging of reactive oxygen species in rice (Oryza sativa L.). *Journal of Plant Biology*, **57**(6), 383–393.

Lichtenthaler, H. K., & Buschmann, C. (2001). Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. In *Current Protocols in Food Analytical Chemistry*. John Wiley & Sons, Inc.

Lu, S. X., & Hrabak, E. M. (2002). An Arabidopsis Calcium-Dependent Protein Kinase Is Associated with the Endoplasmic Reticulum. *Plant Physiology*, **128**(3), 1008–1021.

Ludwig, A. A., Romeis, T., & Jones, J. D. G. (2004). CDPK-mediated signalling pathways: Specificity and cross-talk. In *Journal of Experimental Botany*, **55**, 181–188.

Mackill, D. J., Ismail, A. M., Pamplona, A. M., Darlene, L., Carandang, J. J., & Septiningsih, E. M. (2010). Stress Tolerant Rice Varieties for Adaptation to a Changing Climate. *Crop, Environment & Bioinformatics*, **7**, 250–259.

Martín, M. L., & Busconi, L. (2000). Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation. *Plant Journal*, **24**(4), 429–435.

Miller, G. A. ., Suzuki, N., Ciftci-Yilmaz, S., & Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell & Environment*, **33**(4), 453–467.

Mittler, R., & Blumwald, E. (2015). The Roles of ROS and ABA in Systemic Acquired Acclimation. *The Plant Cell Online*, **27**, 64-70.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., Van Breusegem, F. (2011). ROS signaling: The new wave? *Trends in Plant Science*, **16**(6), 300–309.

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H., Shinozaki, K., Michael, A. J., Tohge, T., Yamazaki, M., Saito, K. (2014). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *Plant Journal*, **77**(3), 367–379.

Narusaka, Y., Nakashima, K., Shinwari, Z. K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K., Yamaguchi-Shinozaki, K. (2003). Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. *Plant Journal*, **34**(2), 137–148.

Ohta, H., Shida, K., Peng, Y.-L., Furusawa, I., Shishiyama, J., Aibara, S., & Morita, Y. (1991). A Lipoxygenase Pathway Is Activated in Rice after Infection with the Rice Blast Fungus Magnaporthe grisea. *Plant Physiology*, **97**(1), 94–98.

Osakabe, Y., Osakabe, K., Shinozaki, K., & Tran, L.-S. P. (2014). Response of plants to water stress. *Frontiers in Plant Science*, **5**, 86.

Prestridge, D. S. (1991). SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. *Computer Applications in the Biosciences : CABIOS*, **7**(2), 203–206.

Ray, S. (2013). Calcium-Dependent Protein Kinase: A Tool for Plants to Crack the Calcium Code. In G. K. Pandey (Ed.), *Plant Stress 6* (pp. 43–59). Global Science Books.

Ray, S., Agarwal, P., Arora, R., Kapoor, S., & Tyagi, A. K. (2007). Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (Oryza sativa L. ssp. indica). *Molecular Genetics and Genomics*, **278**(5), 493–505.

Reddy, A. S. N., Ali, G. S., Celesnik, H., & Day, I. S. (2011). Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *The Plant Cell*, **23**(6), 2010–2032.

Rejeb, I., Pastor, V., & Mauch-Mani, B. (2014). Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. *Plants*, **3**(4), 458–475.

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., & Yao, X. (2008). CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Engineering, Design and Selection*, **21**(11), 639–644.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., & Izui, K. (2000). Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant Journal*, **23**(3), 319–327.

Sallaud, C., Meynard, D., van Boxtel, J., Gay, C., Bès, M., Brizard, J. P., Larmande, P., Ortega, D., Raynal, M., Portefaix, M., Ouwerkerk, P. B. F., Rueb, S., Delseny, M., Guiderdoni, E. (2003). Highly efficient production and characterization of T-DNA plants for rice (Oryza sativa L.) functional genomics. *Theoretical and Applied Genetics*, **106**(8), 1396–1408.

Schulz, P., Herde, M., & Romeis, T. (2013). Calcium-Dependent Protein Kinases: Hubs in Plant Stress Signaling and Development. *Plant Physiology*, **163**(2), 523–530.

Schwach, F., Vaistij, F. E., Jones, L., & Baulcombe, D. C. (2005). An RNA-Dependent RNA polymerase Prevents Meristem Invasion by Potato Virus X and Is Required for the Activity But Not the Production of a Systemic Silencing Signal. *Plant Physiology*, **138** (4), 1842–1852.

Sofo, A., Scopa, A., Nuzzaci, M., & Vitti, A. (2015). Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses. *International Journal of Molecular Sciences*, **16**(6), 13561–13578.

Spiteller, G. (2003). The relationship between changes in the cell wall, lipid peroxidation, proliferation, senescence and cell death. *Physiologia Plantarum*, **119**(1), 5–18.

Torres, M. A. (2010). ROS in biotic interactions. *Physiologia Plantarum*, **138**(4), 414–429.

Tripathy, J. N., Zhang, J., Robin, S., Nguyen, T. T., & Nguyen, H. T. (2000). QTLs for cell-membrane stability mapped in rice (Oryza sativa L.) under drought stress. *Theoretical and Applied Genetics*, **100**(8), 1197–1202.

Velikova, V., Yordanov, I., & Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Science*, **151**(1), 59–66.

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., & Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant Journal*, **45**(4), 523–539.

Wang, D., Qin, Y., Han, J., Zhang, L., Xu, X., Liu, X., Liu, X., Wang, C., Liu, X. (2014). Expression analysis of innate immunity related genes in the true/field blast resistance gene-mediated defence response. *Biotechnology & Biotechnological Equipment*, **28**(6), 999–1007.

Wei, S., Hu, W., Deng, X., Zhang, Y., Liu, X., Zhao, X., Luo, Q., Jin, Z., Lin, Y., Zhou, S., Sun, T., Wang, L., Yang, G., He, G. (2014). A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. *BMC Plant Biology*, **14**(1), 133.

Witte, C. P., Keinath, N., Dubiella, U., Demoulière, R., Seal, A., & Romeis, T. (2010). Tobacco Calcium-dependent Protein Kinases Are Differentially Phosphorylated in Vivo as Part of a Kinase Cascade That Regulates Stress Response. *The Journal of Biological Chemistry*, **285**(13), 9740–9748.

Wong-ekkabut, J., Xu, Z., Triampo, W., Tang, I.-M., Tieleman, D. P., & Monticelli, L. (2007). Effect of Lipid Peroxidation on the Properties of Lipid Bilayers: A Molecular Dynamics Study. *Biophysical Journal*, **93**(12), 4225–4236.

Xing, T., Wang, X.-J., Malik, K., & Miki, B. L. (2001). Ectopic Expression of an Arabidopsis Calmodulin-Like Domain Protein Kinase-Enhanced NADPH Oxidase Activity and Oxidative Burst in Tomato Protoplasts. *Molecular Plant-Microbe Interactions*, **14**(10), 1261–1264.

Yang, L., Ding, J., Zhang, C., Jia, J., Weng, H., Liu, W., & Zhang, D. (2005). Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR. *Plant Cell Reports*, **23**(10-11), 759–763.

Ye, N., Zhu, G., Liu, Y., Li, Y., & Zhang, J. (2011). ABA controls H 2O 2 accumulation through the induction of OsCATB in rice leaves under water stress. *Plant and Cell Physiology*, **52**(4), 689–698.

Yin, X., Huang, L., Zhang, X., Wang, M., Xu, G., & Xia, X. (2015). OsCML4 improves drought tolerance through scavenging of reactive oxygen species in rice. *Journal of Plant Biology*, **58**(1), 68–73.

Zou, J.-J., Li, X.-D., Ratnasekera, D., Wang, C., Liu, W.-X., Song, L.-F., Zhang, W.-Z., Wu, W.-H. (2015). Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE8 and CATALASE3 Function in Abscisic Acid-Mediated Signaling and H2O2 Homeostasis in Stomatal Guard Cells under Drought Stress. *The Plant Cell*, **27** (5), 1445–1460.

GENERAL DISCUSSION

This thesis contributes to a better understanding of the signaling network mediating plant adaptive responses to adverse environmental conditions. These results are relevant as they apply to rice, an economic and socially important crop worldwide, and also the model plant species for other agronomic important cereals. Rice crops are constantly affected by diseases and adverse environmental conditions that result in 30-60% yield losses worldwide every year, threatening global food security (Dhlamini et al., 2005). The blast disease caused by the fungus *M. oryzae* is the most devastating and widespread rice disease, and a serious constraint for rice grain production. Additionally, drought is one of the major abiotic stresses that affects rice crop yield in more than 30% of the world rice cultivating area. Our studies focused on the signaling mediating the responses of rice plants to *M. oryzae* fungal infection and drought stress. Clearly, new insights into the mechanisms and events that operate during the natural rice defense response to blast infection and the adaptation to drought stress could offer new possibilities in designing novel and efficient strategies for rice crop improvement.

The stress responses of rice plants have been extensively studied over the last decades, although research has so far been limited to responses to individual stresses. Such studies have identified components and elucidated mechanisms that mediate the perception of the stress, transmit and amplify the signal and provide the physiological, cellular, and molecular adaptive responses. These components can be used to improve, by genetic engineering or molecular breeding, rice tolerance to stress or resistance to pathogens. Of special interest are the signaling components, such as transcription factors or protein kinases, that control multiple outcomes offering a more robust adaptive response (Sing *et al*., 2012; Helliwell *et al*., 2013; Delteil *et al*., 2010; Singh *et al*., 2012). However, rice, like all higher plants, is a complex organism, in which many signaling processes are integrated in a network controlling the balance between growth, development, reproduction and interaction with the environment. For that reason, it is important to consider potential side effects on rice yield, tolerance to other abiotic stresses and defense against other pathogens with different life styles, when modifying a component of a signaling pathway. In this context, the studies reported in this thesis mainly focused on the signaling components of the rice

defense response to blast fungal infection, also considering their contribution to drought stress tolerance and to the plant performance.

Numerous studies have described that calcium is the main messenger in plant signaling pathways. Nearly all stimuli that a plant cell can perceive cause an increase in intracellular calcium concentration (Reddy *et al*., 2011). Different calcium sensors are able to detect calcium fluctuations as a signal but only CPKs can transmit the signal into protein phosphorylation by themselves (Kudla *et al*., 2010; Gao *et al*., 2014; Boudsocq and Sheen, 2013; Valmonte *et al*., 2014; Romeis and Herde, 2014). This ability to sense changes in calcium concentration and translate them into substrate phosphorylation, makes CPKs unique proteins and ideal candidates to act as signaling components. In this sense, several CPKs have been associated to the rice stress responses. These studies have been done mainly at the transcriptional level, showing the induction of specific *OsCPK* genes in response to different stress inducers (Ray *et al*., 2007; Wan *et al*., 2007; Ye *et al*., 2009; Das and Pandey, 2010). Only few functional characterizations are found in the literature, and most of them relate to a single stress response (Saijo *et al*., 2000; Abbasi *et al*., 2004; Asano *et al*., 2011; Wei *et al*., 2014; Fu *et al*., 2014). The OsCPK12 is the only one that has been characterized in the response to two different stresses, being a positive modulator of salt stress tolerance but a negative modulator of blast disease resistance (Asano *et al*., 2012). In this thesis, two OsCPK proteins, namely OsCPK4 and OsCPK10, are identified as signaling components playing a positive role in both blast disease resistance and drought tolerance in rice plants. To our knowledge, these are the first rice CPKs reported to modulate in a positive way two different stresses.

This thesis work demonstrates that OsCPK4 modulates the accumulation of the defense-mediating hormone SA, as well as the SA-mediated defense responses including callose deposition, ROS production, and defense related gene expression. We show that the overexpression of the *OsCPK4* gene enhances the resistance to blast disease in rice plants by potentiating their defense response and preventing the fungal penetration. Interestingly, the constitutive accumulation of OsCPK4 leads to an increased accumulation of glucosylated SA without compromising rice productivity,

and without interfering with the defense against other rice pathogens with different life styles. Moreover, our group reported that OsCPK4 modulates salt and drought tolerance by preventing lipid peroxidation (Campo *et al*., 2014). High levels of SA are known to inhibit lipid peroxidation (Dinis *et al*., 1994; Lapenna *et al*., 2009), suggesting that might be also promoting the salt and drought tolerance depicted by *OsCPK4-Ox* plants through protection of cell membrane integrity upon high salinity and desiccation conditions.

This thesis also demonstrates that OsCPK10 promotes both blast disease resistance and drought tolerance. The constitutive accumulation of OsCPK10HA confers rice with an improved tolerance to oxidative stress by increasing their antioxidant capacity. This improved ROS detoxifying capacity leads to a reduction in lipid peroxidation and preservation of the cellular membrane integrity upon desiccation, resulting in drought stress tolerance. These studies identify OsCPK4 and OsCPK10 as convergence components between both biotic and abiotic stress responses.

Plant signaling pathways are integrated in an elaborate network with frequent crosstalks, which might function with some responses running in parallel, some prioritized over others, antagonistically or synergistically (Fujita *et al*., 2006). The fine-tunning of the signaling network is critical for plant survival (López *et al*., 2010). Understanding how biotic and abiotic stresses coordinate and the identification of master regulators of stress signaling are important issues. The modulation of crosstalk points is a promising strategy for plant stress improvement (Balderas-Hernandez, 2013). Omics data analysis has revealed a convergence of signaling pathways for biotic and abiotic stress adaptation (Kissoudis *et al*., 2014; Bostock *et al*., 2015), where the major components of the regulatory networks are ROS signaling, plant hormones, changes in redox status and inorganic ion fluxes as Ca^{2+} (Kissoudis *et al*., 2014). Given that OsCPK4 and OsCPK10 are calcium sensors, are connected to hormones, and are related to ROS, we propose here that these two proteins might be functioning in the interaction between biotic and abiotic stress signaling pathways.

Our studies show clear connections between OsCPK4 and OsCPK10 and the SA and ABA hormones. These hormones are key players of biotic and abiotic stress signaling in

plants (Bostock *et al*., 2014). The connection of OsCPK4 with SA is clearly established in this work, in which we show how OsCPK4 contributes to the accumulation of SAG, the glucosylated form of SA. Although in rice plants SAG has been proposed to have *per se* a role in activating defenses for induced resistance (Umemura *et al.*, 2009), SAG is considered a likely storage form of physiologically active free SA, which is accumulated in the vacuole to serve as a source of free SA when required (Dean *et al.*, 2005; Seo *et al.*, 1995). The accumulation of hormone conjugates in the vacuole to allow fast and intensified release of the active metabolites when needed is considered as a priming mechanism (Conrath, 2009; Pastor *et al.*, 2013). Priming refers to the physiological state that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells (Conrath, 2009). *OsCPK4-Ox* plants show a priming state that allows them to trigger a fast and strong response upon pathogen infection, but they do not show constitutive activation of defense responses. Strategies based on priming have emerged as promising means to improve disease resistance and stress tolerance without affecting productivity (Beckers and Conrath, 2007). The other important hormone connected with OsCPK4 and OsCPK10 is ABA, both genes being induced in response to ABA treatment. This hormone has a crucial role in the fine-tunning of stress responses by controlling the switch in priority between the responses to biotic or abiotic stress (Atkinson, 2015). The resistance to *M. oryzae* in rice is mediated by the balance between ABA and SA. ABA interacts antagonistically with SA-signaling pathway conferring susceptibility to the fungus (Jiang *et al*., 2010). Since ABA is the most important hormone in drought stress (Verslues *et al*., 2006; Peleg *et al*., 2011; Ye *et al*., 2012), and ABA-mediated abiotic stress signaling potentially takes precedence over biotic stress signaling, it seems that for the plant water stress more significantly threatens survival than pathogen attack (Fujita *et al*., 2006). Given that both *OsCPK*s are transcriptionally activated by ABA and that they are related to drought stress and pathogen attack, these two OsCPK proteins could be mediating the stress signaling crosstalk. A better understanding of the role of these OsCPKs in the interaction between signaling pathways could be derived by studying the response of OsCPK overexpressing plants upon exposure to concurrent stresses.

ROS are also common signals produced in response to biotic and abiotic stresses that trigger a variety of downstream responses. Many examples can be found in the

literature that relate the CPKs to ROS, both in ROS generation (Kobayashi *et al*., 2007; Bulgarov *et al*., 2011; Dubiella *et al*., 2013) and in ROS detoxification (Asano *et al*., 2012; Ding *et al*., 2013). In this thesis, the two characterized OsCPKs are associated to ROS. On the one side, OsCPK4 contributes to the fast and increased production of ROS during plant defense response to the blast fungus *M. oryzae*. On the other side, OsCPK10 mediates a higher ROS detoxifying activity. This capacity is associated to the increased Catalase A accumulation during desiccation that leads to a reduction in lipid peroxidation and preservation of membrane integrity. It would be interesting to analyze the ROS content in simultaneous stresses and at different phases of *M. oryzae* infection to better characterize the role of these OsCPKs regulating ROS levels.

All together, our studies demonstrate that the OsCPK4 and OsCPK10 proteins act as points of convergence between biotic and abiotic stress responses, and suggest that they are potential targets to improve at the same time rice blast disease resistance and drought tolerance.

CONCLUSIONS

1. The *OsCPK4*, *OsCPK5, OsCPK10* and *OsCPK13* genes are induced by *M. oryzae* elicitors, and the *OsCPK4* and *OsCPK10* genes are also induced by the fungal infection, which suggests their involvement in the *M. oryzae* rice defense response.

2. A natural variation on the expression levels of the defense-related *OsCPK* genes is observed among different rice varieties and wild species, this variation could not be associated to known pathogen resistant-susceptible phenotypes.

3. The overexpression of *OsCPK4* enhances resistance to blast disease in rice plants by potentiating their defense response and preventing fungal penetration.

4. OsCPK4 modulates the accumulation of the defense-mediating hormone SA, as well as the SA-mediated defense responses including callose deposition, production of ROS, and defense related gene expression.

5. The constitutive accumulation of OsCPK4 leads to an increased accumulation of glucosilated SA without compromising rice productivity.

6. The *OsCPK10* gene is not only induced during the *M. oryzae* defense response but also during the drought stress response and in response to ABA treatment.

7. The overexpression of *OsCPK10* confers both blast disease resistance and drought tolerance in rice plants.

8. The constitutive accumulation of OsCPK10HA conferred the rice plants with an improved tolerance to oxidative stress by increasing their antioxidant capacity

9. OsCPK10HA modulates antioxidant activity of rice plants upon desiccation by increasing the accumulation of the Catalase A, reducing lipid peroxidation and preserving membrane integrity that results in drought stress tolerance.

10. OsCPK4 and OsCPK10 are convergence components of both biotic and abiotic signaling pathways that mediate tolerance to multiple stresses in the rice plants.

BIBLIOGRAPHY

Abbasi, F., Onodera, H., Toki, S., Tanaka, H., & Komatsu, S. (2004). OsCDPKI3, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. *Plant Molecular Biology*, **55**(4), 541–552.

Agarwal, P. K., Shukla, P. S., Gupta, K., & Jha, B. (2013). Bioengineering for salinity tolerance in plants: State of the art. *Molecular Biotechnology*, **54**(1), 102–123.

Aguilar, M., Grau, M., & Contreras, J. (1997). Effect of pre-seeding nitrogen fertilization on rice yield components under water high salinity conditions in Southern Spain. In *International sympossium on rice quality. Nottingham, UK* (pp. 24–27).

Ahangar, M. A., Najeeb, S., Rather, A. G., Bhat, Z. A., Parray, G. A., & Sanghara, G. S. (2012). Evaluation of fungicides and rice genotypes for the management of Bakanae. *Oryza*, **2**, 121–126.

Ahn, S., & Tanksley, S. D. (1993). Comparative linkage maps of the rice and maize genomes. *Porceedings of the National Academy of Sciences*, ***90***, 7980–7984.

Aimar, D., Calafat, M., & Andrade, a M. (2011). Drought Tolerance and Stress Hormones : From Model Organisms to Forage Crops. In H. Vasanthaiah (Ed.), *Plants and Environment* (p. 272). InTech.

Amatulli, M. T., Spadaro, D., Gullino, M. L., & Garibaldi, A. (2010). Molecular identification of Fusarium spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity. *Plant Pathology*, **59**(5), 839–844.

Aroca, R. (Ed.). (2012). *Plant Responses to Drought Stress: From Morphological to Molecular Features*. Springer Science & Business Media, 2012.

Asano, T., Hakata, M., Nakamura, H., Aoki, N., Komatsu, S., Ichikawa, H., Hirochika, H., Ohsugi, R. (2011). Functional characterisation of OsCPK21, a calcium-dependent protein kinase that confers salt tolerance in rice. *Plant Molecular Biology*, **75**(1), 179–191.

Asano, T., Hayashi, N., Kobayashi, M., Aoki, N., Miyao, A., Mitsuhara, I., Ichikawa, H., Komatsu, S., Hirochika, H., Kikuchi, S., Ohsugi, R. (2012). A rice calcium-dependent protein kinase OsCPK12 oppositely modulates salt-stress tolerance and blast disease resistance. *Plant Journal*, **69**(1), 26–36.

Asano, T., Tanaka, N., Yang, G., Hayashi, N., & Komatsu, S. (2005). Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: Comprehensive analysis of the CDPKs gene family in rice. *Plant and Cell Physiology*, **46**(2), 356–366.

Atkinson, N. (2015). The response of plants to simultaneous Biotic and Abiotic stress. In R. Mahalingam (Ed.), *Combined stresses in plants* (pp. 181–201). Springer.

Bagnaresi, P., Biselli, C., Orrù, L., Urso, S., Crispino, L., Abbruscato, P., Piffanelli, P., Lupotto, E., Cattivelli, L., Valè, G. (2012). Comparative Transcriptome Profiling of the Early Response to Magnaporthe oryzae in Durable Resistant vs Susceptible Rice (Oryza sativa L.) Genotypes. *PLoS ONE*, **7**(12).

Balderas-Hernández, V. E., Alvarado-Rodríguez, M., & Fraire-Velázquez, S. (2013). Conserved versatile master regulators in signalling pathways in response to stress in plants. *AoB Plants*, **5**,

Barna, B., Fodor, J., Harrach, B. D., Pogány, M., & Király, Z. (2012). The Janus face of reactive oxygen species in resistance and susceptibility of plants to necrotrophic and biotrophic pathogens. *Plant Physiology and Biochemistry*, **59**, 37–43.

Barrios Perez, I., & Brown, P. J. (2014). The Role of ROS Signaling in Cross-Tolerance: From Model to Crop. *Frontiers in Plant Science*. **5**, 754

Batistič, O., & Kudla, J. (2012). Analysis of calcium signaling pathways in plants. *Biochimica et Biophysica Acta (BBA) - General Subjects*, **1820**(8), 1283–1293.

Baxter, A., Mittler, R., & Suzuki, N. (2014). ROS as key players in plant stress signalling. *Journal of Experimental Botany*, **65**(5), 1229–1240.

Beckers, G. J. M., & Conrath, U. (2007). Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biology*, **10**(4), 425–31.

Boatwright, J. L., & Pajerowska-Mukhtar, K. (2013). Salicylic acid: An old hormone up to new tricks. *Molecular Plant Pathology*, **14**(6), 623–634.

Boller, T. (2009). Innate Immunity in Plants : An Arms Race. *Science*, ***742***(5928), 742–4.

Boller, T., & Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology*, **60**, 379–406.

Bostock, R. M., Pye, M. F., & Roubtsova, T. V. (2014). Predisposition in Plant Disease: Exploiting the Nexus in Abiotic and Biotic Stress Perception and Response. *Annual Review of Phytopathology*, **52**(1), 517–549.

Boter, M., Ruíz-Rivero, O., Abdeen, A., & Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and Arabidopsis. *Genes & Development*, **18** (13), 1577–1591.

Boudsocq, M., & Sheen, J. (2013). CDPKs in immune and stress signaling. *Trends in Plant Science*, **18**(1), 30–40.

Boyer, J. S. (1982). Plant productivity and environment. *Science*, **218**(4571), 443–448.

Browse, J. (2009). Jasmonate passes muster: a receptor and targets for the defense hormone. *Annual Review of Plant Biology*, **60**, 183–205.

Bulgakov, V. P., Gorpenchenko, T. Y., Shkryl, Y. N., Veremeichik, G. N., Mischenko, N. P., Avramenko, T. V, Fedoreyev, S.A., Zhuravlev, Y. N. (2011). CDPK-driven changes in the intracellular ROS level and plant secondary metabolism. *Bioengineered Bugs*, **2**(6), 327–330.

Campo, S., Baldrich, P., Messeguer, J., Lalanne, E., Coca, M., & San Segundo, B. (2014). Overexpression of a Calcium-Dependent Protein Kinase Confers Salt and Drought Tolerance in Rice by Preventing Membrane Lipid Peroxidation. *Plant Physiology*, **165**(2), 688–704.

Campos-Soriano, L., Gómez-Ariza, J., Bonfante, P., & San Segundo, B. (2011). A rice calcium-dependent protein kinase is expressed in cortical root cells during the presymbiotic phase of the arbuscular mycorrhizal symbiosis. *BMC Plant Biology*, **11**(1), 90.

Cao, H., Bowling, S., Gordon,. S., & Dong, X. (1994). Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. *The Plant Cell*, **6**(11), 1583–1592.

Chae, L., Sudat, S., Dudoit, S., Zhu, T., & Luan, S. (2009). Diverse transcriptional programs associated with environmental stress and hormones in the arabidopsis receptor-like kinase gene family. *Molecular Plant*, **2**(1), 84–107.

Chen, J., Xue, B., Xia, X., & Yin, W. (2013). A novel calcium-dependent protein kinase gene from Populus euphratica, confers both drought and cold stress tolerance. *Biochemical and Biophysical Research Communications*, **441**(3), 630–636.

Cheng, S., Willmann, M. R., Chen, H., & Sheen, J. (2002). Update on Calcium Signaling Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family 1. *Plant Physiology*, **129**, 469–485.

Chinnusamy, V., Schumaker, K., & Zhu, J. K. (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Journal of Experimental Botany*, **55**(395), 225–236.

Choudhury, S., Panda, P., Sahoo, L., & Panda, S. K. (2013). Reactive oxygen species signaling in plants under abiotic stress. *Plant Signaling & Behavior*, **8**(4).

Chugh, V., Kaur, N., & Gupta, A. K. (2011). Evaluation of oxidative stress tolerance in maize (Zea mays L.) seedlings in response to drought. *Indian Journal of Biochemistry & Biophysics*, **48**(1), 47–53.

Conrath, U. (2009). *Priming of Induced Plant Defense Responses*. *Advances in Botanical Research* (1st ed., Vol. 51). Elsevier Ltd.

Cruz de Carvalho, M. H. (2008). Drought stress and reactive oxygen species. *Plant Signaling & Behavior*, **3**(3), 156–165.

Dai, X., Xu, Y., Ma, Q., Xu, W., Wang, T., Xue, Y., & Chong, K. (2007). Overexpression of an R1R2R3 MYB Gene, OsMYB3R-2, Increases Tolerance to Freezing, Drought, and Salt Stress in Transgenic Arabidopsis. *Plant Physiology*, **143**(4), 1739–1751.

Das, R., & Pandey, G. K. (2010). Expressional analysis and role of calcium regulated kinases in abiotic stress signaling. *Current Genomics*, **11**(1), 2–13.

De Vleesschauwer, D., Gheysen, G., & Höfte, M. (2013). Hormone defense networking in rice: Tales from a different world. *Trends in Plant Science*, **18**(10), 555–565.

Dean, J., Mohammed, L., & Fitzpatrick, T. (2005). The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. *Planta*, **221**(2), 287–296.

Del Rio, L. (2015). ROS and RNS in plant physiology: an overview. *Journal of Experimental Botany*, **66**(10), 2827–2837.

Desikan, R., Neill, S. J., & Hancock, J. T. (2000). Hydrogen peroxide-induced gene expression in Arabidopsis thaliana. *Free Radical Biology & Medicine*, **28**(5), 773–778.

Desjardins, A. E., Plattner, R. D., & Nelson, P. E. (1997). Production of fumonisin B1 and moniliformin by Gibberella fujikuroi from rice from various geographic areas. *Applied and Environmental Microbiology*, **63**(5), 1838–1842.

Dhlamini, Z., Spillane, C., Moss, J. P., Ruane, J., Urquia, N., & Sonnino, A. (2005). Analysis of the FAO-BioDeC data on genetically modified (GM) crop varieties. In FAO (Ed.), *Status of reserach and application of crop biotechnologies in developing countries* (pp. 19–41). Rome.

Ding, Y., Cao, J., Ni, L., Zhu, Y., Zhang, A., Tan, M., & Jiang, M. (2013). ZmCPK11 is involved in abscisic acid-induced antioxidant defence and functions upstream of ZmMPK5 in abscisic acid signalling in maize. *Journal of Experimental Botany*, **64**(4), 871–884.

Dinis, T., Madeira, V., & Almeida, L. (1994). Action of phenolic derivatives (acetaminophen, salicylate,.pdf. *Archives of Biochemistry and Biophysics*, **315**(1), 161–169.

Dodd, A. N., Kudla, J., & Sanders, D. (2010). The language of calcium signaling. *Annual Review of Plant Biology*, **61**, 593–620.

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.-P., Schulze, W. X., Romeis, T. (2013). Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proceedings of*

the National Academy of Sciences of the United States of America, **110**(21), 8744–9.

Durrant, W. E., & Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology*, **42**, 185–209.

Fang, Y., You, J., Xie, K., Xie, W., & Xiong, L. (2008). Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. *Molecular Genetics and Genomics*, **280**(6), 547–563.

Flors, V., Ton, J., Van Doorn, R., Jakab, G., García-Agustín, P., & Mauch-Mani, B. (2008). Interplay between JA, SA and ABA signalling during basal and induced resistance against Pseudomonas syringae and Alternaria brassicicola. *Plant Journal*, **54**(1), 81–92.

Fu, L., Yu, X., & An, C. (2014). OsCPK20 positively regulates Arabidopsis resistance against Pseudomonas syringae pv. tomato and rice resistance against Magnaporthe grisea. *Acta Physiologiae Plantarum*, **36**(2), 273–282.

Fu, Z. Q., & Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annual Review of Plant Biology*, **64**, 839–63.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Current Opinion in Plant Biology*. **9**(4), 436-442

Gao, X., Cox Jr., K., & He, P. (2014). Functions of calcium-dependent protein kinases in plant innate immunity. *Plants*, **3**(1), 160–176.

Garris, A. J., Tai, T. H., Coburn, J., Kresovich, S., & McCouch, S. (2005). Genetic structure and diversity in Oryza sativa L. *Genetics*, **169**(3), 1631–1638.

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., & Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays*, **28**(11), 1091–1101.

Gianessi, L. P. (2014). *Importance of Pesticides for Growing Rice in South and South East Asia*. *CropLife Foundation*. 30-33

Gish, L., & Clark, S. E. (2011). The RLK/Pelle family of kinases. *Plant Journal*, **66**(1), 117–127.

Goff, S. A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., … Briggs, S. (2002). A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. japonica). *Science*, **296** (5565), 92–100.

Gridley, H. E., & Jones, M. P. (2002). Development of New Rice for Africa (NERICA) and participatory varietal selection. In J. R. Witcombe, L. B. Parr, & G. N. Atlin (Eds.), *Breding rainfed rice for drought-prone environments: integrating convetional and participatory plant breeding in South and Shouteast Asia. Proceedings of a DFID plant scieces research programme / IRRI conference, 12-15 March, IRRI, Los Baños, Laguna, Phil* (pp. 23–28). IRRI.

Guo, Z., Ou, W., Lu, S., & Zhong, Q. (2006). Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. *Plant Physiology and Biochemistry*, **44**(11–12), 828–836.

Wang, X., & Valent, B. (Eds.). (2009). *Advances in Genetics, Genomics and control of Rice Blast Disease* (1st ed.). Springer Netherlands. .

Halwart, M., & Gupta, M. V. (2004). The Rice Field Ecosystem. In M. Halwart & M. V Gupta (Eds.), *Culture of Fish in Rice Fields* (pp. 5–11). FAO and The WorldFish Center.

Ham, J. H., Melanson, R. A., & Rush, M. C. (2011). Burkholderia glumae: next major pathogen of rice? *Molecular Plant Pathology*, **12**(4), 329–339.

Harper, J. F., Breton, G., & Harmon, A. (2004). Decoding Ca(2+) signals through plant protein kinases. *Annual Review of Plant Biology*, **55**, 263–288.

Hasegawa, P. M., Bressan, R. A., Zhu, J.-K., & Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology*, **51**, 463–499.

He, Z., Wang, Z. Y., Li, J., Zhu, Q., Lamb, C., Ronald, P., & Chory, J. (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science*, **288**(5475), 2360–2363.

Helliwell, E. E., Wang, Q., & Yang, Y. (2013). Transgenic rice with inducible ethylene production exhibits broad-spectrum disease resistance to the fungal pathogens Magnaporthe oryzae and Rhizoctonia solani. *Plant Biotechnology Journal*, **11**(1), 33–42.

Herrera-vásquez, A., Salinas, P., & Holuigue, L. (2015). Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. *Frontiers in Plant Science*, **6**, 1–9.

Ho, S. L., Huang, L. F., Lu, C. A., He, S. L., Wang, C. C., Yu, S. P., Chen, J., Yu, S. M. (2013). Sugar starvation- and GA-inducible calcium-dependent protein kinase 1 feedback regulates GA biosynthesis and activates a 14-3-3 protein to confer drought tolerance in rice seedlings. *Plant Molecular Biology*, **81**(4-5), 347–361.

Horváth, E., Szalai, G., & Janda, T. (2007). Induction of abiotic stress tolerance by salicylic acid signaling. *Journal of Plant Growth Regulation*, **26**(3), 290–300.

Hossain, M. A., Cho, J. Il, Han, M., Ahn, C. H., Jeon, J. S., An, G., & Park, P. B. (2010). The ABRE-binding bZIP transcription factor OsABF2 is a positive regulator of abiotic stress and ABA signaling in rice. *Journal of Plant Physiology*, **167**(17), 1512–1520.

Howard, R. J., & Valent, B. (1996). Breaking and entering: host penetration by the fungal rice blast pathogen Magnaporthe grisea. *Annual Review of Microbiology*, **50**, 491–512.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q., & Xiong, L. (2006). Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proceedings of the National Academy of Sciences*, **103**(35), 12987–12992.

Hulbert, S. H., Richter, T. E., Axtell, J. D., & Bennetzen, J. L. (1990). Genetic mapping and characterization of sorghum and related crops by means of maize DNA probes. *Proceedings of the National Academy of Sciences of the United States of America*, **87**(11), 4251–4255.

Ichimura, K., Shinozaki, K., Tena, G., Sheen, J., Henry, Y., Champion, A., Kreis, M., Zhang, S., Hirt, H., Wilson, C., Heberle-Bors, E., Ellis, B. E., Morris, P. C., Innes, R. V., Ecker, J. R., Sheel, D., Kleesig, D. R., Machida, Y., Mundy, J., Ohashi, Y., Walker, J. C. (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trends in Plant Science*, **7**(7), 301–308.

Ismail, A. M., Heuer, S., Thomson, M. J., & Wissuwa, M. (2007). Genetic and genomic approaches to develop rice germplasm for problem soils. *Plant Molecular Biology*, **65**(4), 547–570.

Izawa, T., & Shimamoto, K. (1996). Becoming a model plant: The importance of rice to plant science. *Trends in Plant Science*, **1**(3), 95–99.

Jarzyniak, K. M., & Jasinski, M. (2014). Membrane transporters and drought resistance – a complex issue. *Frontiers in Plant Science,* **5***, 687*

Jiang, C.-J., Shimono, M., Sugano, S., Kojima, M., Yazawa, K., Yoshida, R., Inoue, H., Hayashi, N., Sakakibara, H., Takatsuji, H. (2010). Abscisic Acid Interacts Antagonistically with Salicylic Acid Signaling Pathway in Rice–Magnaporthe grisea Interaction. *Molecular Plant-Microbe Interactions*, **23**(6), 791–798.

Jones, J. D. G., & Dangl, J. L. (2006). The plant immune system. *Nature*, **444**(7117), 323–329.

Jones, M. P., Mande, S., & Aluko, K. (1997). Diversity and Potential of Oryza glaberrima Steud in Upland Rice Breeding. *Breeding Science*, **47**, 395–398.

Jwa, N.-S., Agrawal, G. K., Tamogami, S., Yonekura, M., Han, O., Iwahashi, H., & Rakwal, R. (2006). Role of defense/stress-related marker genes, proteins and secondary metabolites in defining rice self-defense mechanisms. *Plant Physiology and Biochemistry : PPB / Societe Francaise de Physiologie Vegetale*, **44**(5-6), 261–273.

Kagaya, Y., Hobo, T., Murata, M., Ban, A., & Hattori, T. (2002). Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. *The Plant Cell*, **14**(12), 3177–3189.

Kar, R. K. (2011). Plant responses to water stress: Role of reactive oxygen species. *Plant Signaling & Behavior*, **6**(11), 1741-1745.

Khush, G., Bennet, J., Datta, S., Brar, D., & Li, Z. (1998). Advances in Rice Genetics and Biotechnology. In *19th Session of the International Rice Commission. Cairo, Egypt*.

Kim, T.-W., & Wang, Z.-Y. (2010). Brassinosteroid signal transduction from receptor kinases to transcription factors. *Annual Review of Plant Biology*, **61**, 681–704.

Kissoudis, C., van de Wiel, C., Visser, R. G. F., & van der Linden, G. (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in Plant Science*, **5**, 207.

Klimecka, M., & Muszyńska, G. (2007). Structure and functions of plant calcium-dependent protein kinases. *Acta Biochimica Polonica*, **54**(2), 219–233.

Knepper. (2010). From Perception to Activation: The Molecular-Genetic and Biochemical Landscape of Disease Resistance Signaling in Plants. *The Arabidopsis Book*. **8**

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., Yoshioka, H. (2007). Calcium-Dependent Protein Kinases Regulate the Production of Reactive Oxygen Species by Potato NADPH Oxidase. *The Plant Cell*, **19**(3), 1065–1080.

Koga, H., Dohi, K., & Mori, M. (2004). Abscisic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of Magnaporthe grisea. *Physiological and Molecular Plant Pathology*, **65**(1), 3–9.

Köhler, F. E. (1897). *Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte : Atlas zur Pharmacopoea germanica*. (Gera-Untermhaus, Ed.).

Kong, X., Lv, W., Jiang, S., Zhang, D., Cai, G., Pan, J., & Li, D. (2013). Genome-wide identification and expression analysis of calcium-dependent protein kinase in maize. *BMC Genomics*, **14**, 433.

Kovach, M. J., Sweeney, M. T., & McCouch, S. R. (2007). New insights into the history of rice domestication. *Trends in Genetics*, **23**(11), 578–587.

Kramer, P. J., & Boyer, J. S. (1995). *Water relations of Plants and Soils*. San Diego, California: Academic Press, Inc.

Kudla, J., Batistič, O., & Hashimoto, K. (2010). Calcium Signals: The Lead Currency of Plant Information Processing. *The Plant Cell*, **22**(3), 541–563.

Kumar, M., Lee, S.-C., Kim, J.-Y., Kim, S.-J., Aye, S. S., & Kim, S.-R. (2014). Over-expression of dehydrin gene, OsDhn1, improves drought and salt stress tolerance through scavenging of reactive oxygen species in rice (Oryza sativa L.). *Journal of Plant Biology*, **57**(6), 383–393.

Lapenna, D., Ciofani, G., Pierdomenico, S. D., Neri, M., Cuccurullo, C., Giamberardino, M. A., & Cuccurullo, F. (2009). Inhibitory activity of salicylic acid on lipoxygenase-dependent lipid peroxidation. *Biochimica et Biophysica Acta*, **1790**(1), 25–30.

Levitt, J. (1972). *Responses of Plants to Environmental Stresses*. New York: Academic Press, Inc.

Li, A. L., Zhu, Y. F., Tan, X. M., Wang, X., Wei, B., Guo, H. Z., Zhang, Z. L., Chen, X. B., Zhao, G. Y., Kong, X. Y., Jia, J. Z., Mao, L. (2008). Evolutionary and functional study of the CDPK gene family in wheat (Triticum aestivum L.). *Plant Molecular Biology*, **66**(4), 429–443.

Li, Q., Chen, F., Sun, L., Zhang, Z., Yang, Y., & He, Z. (2006). Expression profiling of rice genes in early defense responses to blast and bacterial blight pathogens using cDNA microarray. *Physiological and Molecular Plant Pathology*, **68**(1-3), 51–60.

Liese, A., & Romeis, T. (2013). Biochemical regulation of in vivo function of plant calcium-dependent protein kinases (CDPK). *Biochimica et Biophysica Acta - Molecular Cell Research*, **1833**(7), 1582–1589.

Liu, D., Chen, X., Liu, J., Ye, J., & Guo, Z. (2012). The rice ERF transcription factor OsERF922 negatively regulates resistance to Magnaporthe oryzae and salt tolerance. *Journal of Experimental Botany*, **63**(10), 3899–3912.

M. A. Ahangar, S . Najeeb, A. G. Rather, Z. A. Bhat, G. A. Parray, G. S. Sanghara., S. C., & Kashap, F. A. A. and H. A. (2012). Evaluation of fungicides and rice genotypes for the management of Bakanae. *Oryza*, **49**-2, 121–126.

Mackill, D. J., Ismail, A. M., Pamplona, A. M., Darlene, L., Carandang, J. J., & Septiningsih, E. M. (2010). Stress Tolerant Rice Varieties for Adaptation to a Changing Climate. *Crop, Environment & Bioinformatics*, **7**, 250–259.

Meng, X., & Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annual Review of Phytopathology*, **51**, 245–66.

Mittler, R., & Blumwald, E. (2015). The Roles of ROS and ABA in Systemic Acquired Acclimation. *The Plant Cell Online*, **27**, 64–70.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., Van Breusegem, F. (2011). ROS signaling: The new wave? *Trends in Plant Science*, **16**(6), 300–309.

Mohanty, S. (2013). Trends in global rice consumption. *Rice Today*, 44–45.

Moore, G., Devos, K. M., Wang, Z., & Gale, M. D. (1995). Cereal genome evolution. Grasses, line up and form a circle. *Current Biology*, **5**(7), 737–739.

Moore, J. W., Loake, G. J., & Spoel, S. H. (2011). Transcription Dynamics in Plant Immunity. *The Plant Cell*, **23**(8), 2809–2820.

Moormann, F. R., & Breemen, N. Van. (1978). *Rice : Soil, Water, Land*. (I. R. R. Institute, Ed.). Los Baños, Laguna; Philippines.

Mou, Z., Fan, W., & Dong, X. (2003). Inducers of Plant Systemic Acquired Resistance Regulate NPR1 Function through Redox Changes. *Cell*, **113**(7), 935–944.

Moustafa, K., AbuQamar, S., Jarrar, M., Al-Rajab, A. J., & Trémouillaux-Guiller, J. (2014). MAPK cascades and major abiotic stresses. *Plant Cell Reports*, **33**(8), 1217–1225.

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., Matsuda, F., Kojima, M., Sakakibara, H., Shinozaki, K., Michael, A. J., Tohge, T., Yamazaki, M., Saito, K. (2014). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *Plant Journal*, **77**(3), 367–379.

Nakano, T., Suzuki, K., Fujimura, T., & Shinshi, H. (2006). Genome-Wide Analysis of the ERF Gene Family. *Plant Physiology*, ***140***, 411–432.

Nakashima, K., Tran, L.-S. P., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., Ito, Y., Hayashi, N., Shinozaki, K., Yamaguchi-Shinozaki, K. (2007). Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. *The Plant Journal*, **51**(4), 617–630.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., Yoshida, S. (2003). Brassinosteriod functions in a broad range of disease resistance in tobacco and rice. *The Plant Journal*, **33**, 887–898.

Nguyen, N. V. (2005). *Global climate changes and rice food security*. (FAO, Ed.)*International Rice Commission Newsletter*. Rome.

Nuruzzaman, M., Manimekalai, R., Sharoni, A. M., Satoh, K., Kondoh, H., Ooka, H., & Kikuchi, S. (2010). Genome-wide analysis of NAC transcription factor family in rice. *Gene*, **465**(1-2), 30–44.

Osakabe, Y., Osakabe, K., Shinozaki, K., & Tran, L.-S. P. (2014). Response of plants to water stress. *Frontiers in Plant Science*, **5**, 86.

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., & Tran, L. S. P. (2013). Sensing the environment: Key roles of membrane-localized kinases in plant perception and response to abiotic stress. *Journal of Experimental Botany*, **64**(2), 445–458.

Ou, S. H. (1985). *Rice Diseases* (2nd ed.). CAB International Mycological Institute.

Pandey, P., Srivastava, R. K., & Dubey, R. S. (2013). Salicylic acid alleviates aluminum toxicity in rice seedlings better than magnesium and calcium by reducing aluminum uptake, suppressing oxidative damage and increasing antioxidative defense. *Ecotoxicology*, **22**(4), 656–670.

Park, M.-R., Yun, K.-Y., Mohanty, B., Herath, V., Xu, F., Wijaya, E., Bajic, V. B., Yun, S-J., De Los Reyes, B. G. (2010). Supra-optimal expression of the cold-regulated OsMyb4 transcription factor in transgenic rice changes the complexity of transcriptional network with major effects on stress tolerance and panicle development. *Plant, Cell & Environment*, **33**(12), 2209–2230.

Pastor, V., Luna, E., Mauch-Mani, B., Ton, J., & Flors, V. (2013). Primed plants do not forget. *Environmental and Experimental Botany*, **94**, 46–56.

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Métraux, J-P., Raskin, L., (1995). Salicylic Acid in Rice. *Plant Physiology*, **108**, 633–639.

Peleg, Z., & Blumwald, E. (2011). Hormone balance and abiotic stress tolerance in crop plants. *Current Opinion in Plant Biology*, **14**(3), 290–295.

Perez-Nadales, E., Almeida Nogueira, M. F., Baldin, C., Castanheira, S., El Ghalid, M., Grund, E., Lengeler, K., Marchegiani, E., Mehrotra, P. V., Moretti, M., Naik, V., Oses-Ruiz, M., Oskarsson, T., Schäfer, K., Wasserstrom, L., Brakhage, A., Gow, N., Kahmann, R., Lebrun, M. H., Pérez-Martin, J., Di Pietro, A., Talbot, N. J., toquin, V., Walther, A Wendland, J. (2014). Fungal model systems and the elucidation of pathogenicity determinants. *Fungal Genetics and Biology*, **70**, 42–67.

Pritchard, L., & Birch, P. R. J. (2014). The zigzag model of plant-microbe interactions: is it time to move on? *Molecular Plant Pathology*, **15**(9), 865–870.

Pu, X. M., Zhou, J. N., Lin, B. R., & Shen, H. F. (2012). First Report of Bacterial Foot Rot of Rice Caused by a Dickeya zeae in China. *Plant Disease*, **96**(12), 1818.

Qiu, D., Xiao, J., Ding, X., Xiong, M., Cai, M., Cao, Y., Li, X., Xu, C., Wang, S. (2007). OsWRKY13 mediates rice disease resistance by regulating defense-related genes in salicylate- and jasmonate-dependent signaling. *Molecular Plant-Microbe Interactions*, **20**(5), 492–499.

Ray, S., Agarwal, P., Arora, R., Kapoor, S., & Tyagi, A. K. (2007). Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (Oryza sativa L. ssp. indica). *Molecular Genetics and Genomics*, ***278***(5), 493–505.

Reddy, A. S. N., Ali, G. S., Celesnik, H., & Day, I. S. (2011). Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *The Plant Cell*, **23**(6), 2010–2032.

Reguera, M., Peleg, Z., & Blumwald, E. (2012). Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, **1819**(2), 186–194.

Rejeb, I., Pastor, V., & Mauch-Mani, B. (2014). Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. *Plants*, **3**(4), 458–475.

Reyna, N. S., & Yang, Y. (2006). Molecular analysis of the rice MAP kinase gene family in relation to Magnaporthe grisea infection. *Molecular Plant-Microbe Interactions*, **19**(5), 530–540.

Rice, I., & Sequencing, G. (2005). The map-based sequence of the rice genome. *Nature*, **436**(7052), 793–800.

Riemann, M., Haga, K., Shimizu, T., Okada, K., Ando, S., Mochizuki, S., Nishizawa, Y., Yamanouchi, U., Nick, P., Yano, M., Minami, E., Takano, M., Yamane, H., Iino, M. (2013). Identification of rice Allene Oxide Cyclase mutants and the function of jasmonate for defence against Magnaporthe oryzae. *Plant Journal*, **74**(2), 226–238.

Rodriguez, M. C. S., Petersen, M., & Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. *Annual Review of Plant Biology*, **61**, 621–649.

Romeis, T. (2000). Protein kinases in the plant defense response. *Current Opinion in Plant Biology*, **32**(4), 407–414.

Romeis, T., & Herde, M. (2014). From local to global: CDPKs in systemic defense signaling upon microbial and herbivore attack. *Current Opinion in Plant Biology*, **20**, 1–10.

RoyChoudhury, A., Roy, C., & Sengupta, D. N. (2007). Transgenic tobacco plants overexpressing the heterologous lea gene Rab16A from rice during high salt and water deficit display enhanced tolerance to salinity stress. *Plant Cell Reports*, **26**, 1839–1859.

Ryu, H. S., Han, M., Lee, S. K., Cho, J. Il, Ryoo, N., Heu, S., Lee, Y. H., Boo, S. H., Wang, G. L., Hahn, T. R., Jeon, J. S. (2006). A comprehensive expression analysis of the WRKY gene superfamily in rice plants during defense response. *Plant Cell Reports*, **25**(8), 836–847.

Saijo, Y., Kinoshita, N., Ishiyama, K., Hata, S., Kyozuka, J., Hayakawa, T., Nakamura, T., Shimamoto, K., Yamaya, T., Izui, K. (2001). A Ca(2+)-dependent protein kinase that endows rice plants with cold- and salt-stress tolerance functions in vascular bundles. *Plant & Cell Physiology*, **42**(11), 1228–1233.

Sang, T., & Ge, S. (2007). Genetics and phylogenetics of rice domestication. *Current Opinion in Genetics and Development*, ***17***, 533–538.

Schulz, P., Herde, M., & Romeis, T. (2013). Calcium-Dependent Protein Kinases: Hubs in Plant Stress Signaling and Development. *Plant Physiology*, **163**(2), 523–530.

Second, G. (1982). Origin of the genic diversity of cultivated rice (Oryza spp.): study of the polymorphism scored at 40 isozyme loci. *The Japanese Journal of Genetics*, **57**(1), 25–57.

Semon, M., Nielsen, R., Jones, M. P., & McCouch, S. R. (2005). The population structure of African cultivated rice Oryza glaberrima (Steud.): Evidence for elevated levels of linkage disequilibrium caused by admixture with O. sativa and ecological adaptation. *Genetics*, **169**(3), 1639–1647.

Seo, E., Choi, D., & Choi. (2015). Functional studies of transcription factors involved in plant defenses in the genomics era. *Briefings in Functional Genomics*, 1–8.

Seo, S., Ishizuka, K., & Ohashi, Y. (1995). Induction of Salicylic Acid β-Glucosidase in Tobacco Leaves by Exogenous Salicylic Acid. *Plant and Cell Physiology*, **36**(3), 447–453.

Serraj, R., McNally, K. L., Slamet-Loedin, I., Kohli, A., Haefele, S. M., Atlin, G., & Kumar, A. (2011). Drought Resistance Improvement in Rice: An Integrated Genetic and Resource Management Strategy. *Plant Production Science*, **14**(1), 1–14.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, **2012**, 1–26.

Shimamoto, K., & Kyozuka, J. (2002). Rice as a model for comparative genomics of plants. *Annual Review of Plant Biology*, **53**, 399–419.

Shinozaki, K., & Yamaguchi-Shinozaki, K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, **58**(2), 221–227.

Shiu, S., & Bleecker, A. B. (2003). Expansion of the Receptor-Like Kinase / Pelle Gene Family and Receptor-Like Proteins in Arabidopsis. *Plant Physiology*, **132**, 530–543.

Singh, M. P., Lee, F. N., Counce, P. a, & Gibbons, J. H. (2004). Mediation of partial resistance to rice blast through anaerobic induction of ethylene. *Phytopathology*, **94**(8), 819–825.

Singh, P., Bawankar, R., Gothandam, K. M., Subashkumar, R., Vivekanandhan, G., Thayumanvan, T., & Babu, S. (2012). "Master switch" genes for disease resistance in rice: lessons learnt and lessons to learn. *Research in Biotechnology*. **3**(1).

Singh, S., Modi, M. K., Gill, S. S., & Tuteja, N. (2012). Rice: Genetic Engineering Approaches for Abiotic Stress Tolerance – Retrospects and Prospects. In *Improving Crop Productivity in Sustainable Agriculture* (pp. 201–236). Wiley-VCH Verlag GmbH & Co. KGaA.

Skamnioti, P., & Gurr, S. J. (2009). Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology*, **27**(3), 141–50.

Solh, M. (2005). *Rice is Life in 2004 and beyond*. (FAO, Ed.) *International Rice Commission Newsletter* (Vol. 54). Rome.

Sparks, A., Nelson, A., Castilla, N. (2012). Where rice pests and diseases do the most damage. *Rice Today*, **11**(4), 26–27.

Stone, J. M., & Walker, J. C. (1995). Plant protein kinase families and signal transduction. *Plant Physiology*, **108**(2), 451–457.

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E., & Mittler, R. (2014). Abiotic and biotic stress combinations. *New Phytologist*, **203**(1), 32–43.

Sweeney, M., & McCouch, S. (2007). The complex history of the domestication of rice. *Annals of Botany*, **100**, 951–957.

Takasaki, H., Maruyama, K., Kidokoro, S., Ito, Y., Fujita, Y., Shinozaki, K., Yamaguchi-Shinozachi, K., Nakashima, K., (2010). The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. *Molecular Genetics and Genomics*, **284**(3), 173–183.

Tena, G., Asai, T., Chiu, W.-L., & Sheen, J. (2001). Plant mitogen-activated protein kinase signaling cascades. *Current Opinion in Plant Biology*, **4**(5), 392–400.

Tena, G., Boudsocq, M., & Sheen, J. (2011). Protein kinase signaling networks in plant innate immunity. *Current Opinion in Plant Biology*, **14**(5), 519–529.

Todaka, D., Nakashima, K., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. *Rice*, **5**(1), 6.

Torres, M. A. (2010). ROS in biotic interactions. *Physiologia Plantarum*, **138**(4), 414–429.

Umemura, K., Satou, J., Iwata, M., Uozumi, N., Koga, J., Kawano, T., Koshiba, T., Anzai, H., Mitomi, M. (2009). Contribution of salicylic acid glucosyltransferase, OsSGT1,

to chemically induced disease resistance in rice plants. *The Plant Journal*, **57**(3), 463–472.

Valmonte, G. R., Arthur, K., Higgins, C. M., & Macdiarmid, R. M. (2014). Calcium-dependent protein kinases in plants: Evolution, expression and function. *Plant and Cell Physiology*, **55**(3), 551–569.

Van Nguyen, N., & Ferrero, A. (2006). Meeting the challenges of global rice production. *Paddy and Water Environment*, **4**(1), 1–9.

Vaughan, D. a., Morishima, H., & Kadowaki, K. (2003). Diversity in the Oryza genus. *Current Opinion in Plant Biology*, **6**(2), 139–146.

Venu, R. C., Jia, Y., Gowda, M., Jia, M. H., Jantasuriyarat, C., Stahlberg, E., Li, H., Rhineheart, A., Boddhireddy, P., Singh, P., Rutger, N., Kudrna, D., Wing, R., Nelson, J. C., Wang, G. L. (2007). RL-SAGE and microarray analysis of the rice transcriptome after Rhizoctonia solani infection. *Molecular Genetics and Genomics*, **278**(4), 421–431.

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., & Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant Journal*, **45**(4), 523–539.

Wan, B., Lin, Y., & Mou, T. (2007). Expression of rice Ca2+-dependent protein kinases (CDPKs) genes under different environmental stresses. *FEBS Letters*, **581**(6), 1179–1189.

Wassmann, R., Jagadish, S. V. K., Heuer, S., Ismail, a., Redona, E., Serraj, R., Singh, R. K., Howell, G., Pathak, H., Sumfleth, K. (2009). *Chapter 2 Climate Change Affecting Rice Production. The Physiological and Agronomic Basis for Possible Adaptation Strategies*. *Advances in Agronomy* (Vol. 101).

Wei, S., Hu, W., Deng, X., Zhang, Y., Liu, X., Zhao, X., Luo, Q., Jin, Z., Lin, Y., Zhou, S., Sun, T., Wang, L., Yang, G., He, G. (2014). A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. *BMC Plant Biology*, **14**(1), 133.

Wernimont, A. K., Artz, J. D., Finerty, P., Lin, Y.-H., Amani, M., Allali-Hassani, A., Senisterra, G., Vedadi, M., Tempel, W., Mackenzie, M., Chau, I., Lourido, S., Sibley, L. D., Hui, R. (2010). Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. *Nature Structural & Molecular Biology*, **17**(5), 596–601.

Wulff, E. G., Sørensen, J. L., Lübeck, M., Nielsen, K. F., Thrane, U., & Torp, J. (2010). Fusarium spp. associated with rice Bakanae: Ecology, genetic diversity, pathogenicity and toxigenicity. *Environmental Microbiology*, **12**(3), 649–657.

Wurzinger, B., Mair, A., Pfister, B., & Teige, M. (2011). Cross-talk of calcium-dependent protein kinase and MAP kinase signaling. *Plant Signaling & Behavior*, **6**(1), 8–12.

Xiang, Y., Tang, N., Du, H., Ye, H., & Xiong, L. (2008). Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant Physiology*, **148**(4), 1938–1952.

Xie, K., Chen, J., Wang, Q., & Yang, Y. (2014). Direct Phosphorylation and Activation of a Mitogen-Activated Protein Kinase by a Calcium-Dependent Protein Kinase in Rice. *The Plant Cell*, **26**, 1–14.

Yang, D. L., Yang, Y., & He, Z. (2013). Roles of plant hormones and their interplay in rice immunity. *Molecular Plant*, **6**(3), 675–685.

Yang, X., Yang, Y.-N., Xue, L.-J., Zou, M.-J., Liu, J.-Y., Chen, F., & Xue, H.-W. (2011). Rice ABI5-Like1 regulates abscisic acid and auxin responses by affecting the expression of ABRE-containing genes. *Plant Physiology*, **156**(3), 1397–1409.

Yang, Y., Qi, M., & Mei, C. (2004). Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. *Plant Journal*, **40**(6), 909–919.

Ye, N., Jia, L., & Zhang, J. (2012). ABA signal in rice under stress conditions. *Rice*, **5**(1), 1.

Ye, S., Wang, L., Xie, W., Wan, B., Li, X., & Lin, Y. (2009). Expression profile of calcium-dependent protein kinase (CDPKs) genes during the whole lifespan and under phytohormone treatment conditions in rice (Oryza sativa L. ssp. indica). *Plant Molecular Biology*, **70**(3), 311–325.

Yu, J., Hu, S., Wang, J., Wong, G. K.-S., Li, S., Liu, B., … Yang, H. (2002). A Draft Sequence of the Rice Genome (Oryza sativa L. ssp. indica). *Science*, **296** (5565), 79–92.

Zeigler, R. S., & Savary, S. (2010). The Role of Plant Pathology in Food Safety and Food Security. *Plant Pathology in the 21st Century*, 3–9.

Zhang, Q., Chen, Q., Wang, S., Hong, Y., & Wang, Z. (2014). Rice and cold stress: methods for its evaluation and summary of cold tolerance-related quantitative trait loci. *Rice*, **7**(1), 24.

Zheng GX, Lv B, W. R. and N. H. (1993). Study on screening methods for resistance to bakanae disease of rice. *Acta Phytopatholgica Sinica*, **20**, 289–293.

Zhou, J.-M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., & Klessig, D. F. (2000). NPR1 Differentially Interacts with Members of the TGA/OBF Family of Transcription Factors That Bind an Element of the PR-1 Gene Required for Induction by Salicylic Acid. *Molecular Plant-Microbe Interactions*, **13**(2), 191–202.

Zuo, R., Hu, R., Chai, G., Xu, M., Qi, G., Kong, Y., & Zhou, G. (2013). Genome-wide identification, classification, and expression analysis of CDPK and its closely related gene families in poplar (Populus trichocarpa). *Molecular Biology Reports*, **40**(3), 2645–2662.

Mireia Bundó Barberà

Barcelona, 2015

UAB

Universitat Autònoma
de Barcelona