Fear of the dark

How light signalling modulates the JA-mediated wounding response in rice

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

Fakultät für Chemie und Biowissenschaften

Karlsruher Institut für Technologie (KIT) - Universitätsbereich

genehmigte

DISSERTATION

von

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aus

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Tag der mündlichen Prüfung: 13.02.2015

“When one tugs at a single thing in nature, he finds it attached to the rest of the world.”

John Muir

Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Dissertation, abgesehen von der Verwendung der angegebenen Hilfsmittel, selbstständig verfasst zu haben. Alle Stellen, die dem Wortlaut oder Inhalt nach anderen Arbeiten entnommen sind, wurden durch Angabe der Quellen als Entlehnungen gekennzeichnet.

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Karlsruhe, den 16.12.2014

Rita Erika Brendel

Die vorliegende Arbeit wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Lehrstuhl I für molekulare Zellbiologie, im Zeitraum von Mai 2011 bis Dezember 2014 angefertigt.

Teile dieser Arbeit sind veröffentlicht in:

Brendel, R., Svyatyna, K., Jikumaru, Y., Reichelt, M., Mithöfer, A., Takano, M., Kamiya, Y., Nick, P. & Riemann, M. (2014). Effects of light and wounding on jasmonates in rice phyAphyC mutants. *Plants* **3**, 143-159.

Svyatyna, K., Jikumaru, Y., Brendel, R., Reichelt, M., Mithöfer, A., Takano, M., Kamiya, Y., Nick, P. & Riemann, M. (2013). Light induces jasmonate-isoleucine conjugation via OsJAR1-dependent and -independent pathways in rice. *Plant Cell Environ*.

Danksagung

An erster Stelle möchte ich mich bei Prof. Peter Nick dafür bedanken, dass er mir die Anfertigung meiner Dissertation an seinem Lehrstuhl ermöglicht hat.

Prof. Natalia Requena danke ich herzlichst für die Betreuung dieser Arbeit als Korreferentin.

Besonderer Dank gebührt Dr. Michael Riemann, der meine Arbeit betreut hat, jederzeit ein offenes Ohr für Fragen und Probleme hatte, und mich während der gesamten Arbeit durch seine wissenschaftliche Kompetenz mit neuen Ideen und Lösungsvorschlägen unterstützt hat.

Des Weiteren möchte ich mich bei meinen Kooperationspartnern am MPI:CE in Jena bedanken. Aus der Arbeitsgruppe von Prof. Wilhelm Boland sind Dr. Axel Mithöfer und Andrea Lehr zu nennen, die mich bei den Phytohormonanalysen unterstützt haben und im Allgemeinen danke ich der Arbeitsgruppe Boland für die Bereitstellung des MecWorm. Auch Dr. Michael Reichelt, aus der Abteilung Biochemie, der die LC-MSMS Analysen durchgeführt hat, gebührt Dank.

Ich danke herzlichst Ernst Heene für seinen Einsatz bei der Beseitigung von technischen Problemen, für das Notlösungen finden und das Bewältigen von Hindernissen unterschiedlichster Art.

Sybille Wörner, die mir mit ihrer Laborerfahrung immer in jeder Situation Rat und Hilfestellungen gegeben hat und sich ohnehin einfach nur wunderbar um das gesamte Labor kümmert, gebührt ebenfalls großer Dank.

Ich danke allen gegenwärtigen und ehemaligen Auszubildenden der Botanik 1, die stets einen großen Beitrag zum reibungslosen Ablauf des Laboralltags beisteuern und immer hilfsbereit sind. Insbesondere zu nennen ist Lukas Geschwender, der einen Teil der 24 Stunden Experimente übernommen und mich damit unheimlich entlastet hat!

Allen anderen Doktoranden und Kollegen des Botanischen Institutes einen herzlichen Dank für die schöne Zeit und eine angenehme Arbeitsatmosphäre.

Meinen Bachelor Studentinnen Lisa Heilemann, Svenja Wehrle, Ilona Ihle und Hyeon-Yeong Kim danke ich für die Unterstützung meiner Forschung durch zahlreiche Vorversuche und ergänzende Experimente.

Der Landesgraduiertenförderung Baden-Württemberg gebührt großer Dank für die finanzielle Förderung des Großteils dieser Promotionsarbeit und dem KHYS danke ich für die Förderung eines Konferenzaufenthaltes im Zuge des Weiterqualifizierungsstipendiums.

Den besten Freundinnen, die man sich wünschen kann, Marina Lehmann, Katharina Nicola und Andrea Rickmann, gebührt ebenfalls Dank. Ihr seid für mich wie Schwestern und ich danke Euch von ganzem Herzen für alles!

Für sowohl technische als auch emotionale Unterstützung danke ich Florian Firnkorn. Ohne Dich wäre so einiges anders gelaufen in den letzten Monaten und ich bin mehr als glücklich, Dich an meiner Seite zu haben.

Größter Dank gebührt allerdings meinen Eltern, die mich über die gesamte Zeit des Studiums und der Dissertation vorbehaltlos unterstützt haben und immer für mich da sind. Ich kann mich glücklich schätzen, Euch zu haben!

Zusammenfassung

Als sessile Lebewesen können Pflanzen unvorteilhaften Lebensbedingungen oder Angreifern nicht einfach ausweichen, sondern müssen sich stattdessen an ihre Umwelt anpassen und gegen Herbivorie oder Pathogenbefall verteidigen. Aus diesem Grund ist es lebensnotwendig, dass Pflanzen die Vielzahl von Signalen, denen sie permanent ausgesetzt sind, wahrnehmen und verarbeiten können, und dass eine angemessene Antwort auf die jeweilige Situation erfolgt.

Diese Reaktionen sind einem komplexen Signalnetzwerk unterworfen, welches sicherstellt, dass die jeweils passende Antwort auf die verschiedenen Umwelteinflüsse zeitnah stattfindet. Die Menge an unterschiedlichen Komponenten und Signalwegen, die in dieses Netzwerk involviert sind, ermöglicht der Pflanze ihre Reaktionen der jeweiligen Situation anzupassen.

Diese Dissertation konzentriert sich auf zwei wichtige externe Signale, denen eine Pflanze ausgesetzt sein kann: Licht und Verwundungsstress. Während die Wahrnehmung von Lichtverhältnissen das Wachstum und die Entwicklung der Pflanze über einen lange Zeitraum beeinflusst, ist Verwundungsstress ein unmittelbar erfolgendes Ereignis, auf das die Pflanze unverzüglich reagieren muss, da der Verlust von Blattmaterial eine konkrete Bedrohung für die pflanzliche Lebensgrundlage darstellt. Aus neueren Studien haben sich Hinweise darauf ergeben, dass sich die Signalwege von Licht und Verteidigung gegenseitig beeinflussen können. Der mechanistische Hintergrund dieser Interaktion stellt ein interessantes Forschungsthema dar und deshalb wurden in dieser Arbeit zwei der beteiligten Komponenten untersucht: das Phytohormon Jasmonat und die Familie der Phytochrome, pflanzliche Photorezeptoren.

Als Modellpflanze für diese Studie wurde Reis ausgewählt, da Reis nicht nur eines der wichtigsten Grundnahrungsmittel der Weltbevölkerung ist, sondern auch Mutanten in den untersuchten Signalwegen vorhanden sind. Diese Mutanten und ihre zugehörigen Wildtypen wurden auf ihre Reaktion hinsichtlich mechanischer Verwundung untersucht, wobei Hormonmessungen und Genexpressionsanalysen durchgeführt wurden.

Die Ergebnisse dieser Doktorarbeit zeigen, dass Licht während der Verwundungsantwort der Pflanze, über Phytochrome, die Jasmonat-Antwort beeinflussen kann. Während über Phytochrome die Produktion von Jasmonat gehemmt wird, wird die Antwort auf transkriptioneller Ebene durch Licht verstärkt. Dementsprechend führt ein Mangel an Licht oder aktivem Phytochrom dazu, dass die Pflanze eine schwächere Expression von stressinduzierten Antwortgenen zeigt. Dieser Umstand weist darauf hin, dass Phytochrome die jasmonatabhängige Reaktion auf Verwundung modulieren können und eine korrekte Jasmonat-Antwort nur in Gegenwart von aktivem Phytochrom erfolgt. Diese Ergebnisse decken sich mit Studien, die eine Verminderung der pflanzlichen Verteidigung bei Dunkelheit oder Beschattung belegen.

Folglich sind Licht und der Signalweg von Jasmonat während der Verwundungsantwort auf mehreren Ebenen miteinander verbunden und können sich gegenseitig beeinflussen. Außerdem scheint diese Interaktion negative Auswirkungen auf die pflanzliche Verteidigung zu haben, sofern unvorteilhafte Lichtverhältnisse herrschen. Dies dient möglicherweise der ausbalancierten Verteilung von Ressourcen auf Wachstum und Verteidigung, zwei energieträchtige Prozesse in der Pflanze.

Abstract

Due to their sessile lifestyle, plants are not able to escape from unfavourable conditions or potential aggressors, but have to adapt to their environment and defend themselves against herbivores and pathogens. Therefore it is crucial that the variety of signals, which plants are permanently exposed to, are perceived and integrated and that an appropriate response is exhibited by the plant. These responses are subject to a complex signalling network which ensures, that the correct response to different challenges is exhibited in a timely manner. The multitude of involved compounds and pathways in this signalling network enables the plant to tailor the response adequately to the respective situation it is confronted with.

In this study, we focus on the integration of two major external signals a plant has to react to, light conditions and stress imposed by wounding. While the perception of light conditions influences the growth and development of a plant on a long time scale, wounding stress is an immediate event, to which the plant has to respond in a fast manner, as the loss of leaf material threatens the basis of its life. Recent studies have given solid evidence, that the signalling pathways of light and defence are able to influence each other. The mechanistic background of this interaction represents an intriguing research topic and thus, we wanted to investigate the signalling pathways of two central players which might potentially be involved: the plant hormone jasmonate (JA) and the plant photoreceptor family of phytochromes (phy).

We chose rice as a model plant for our studies, as it is not only an important staple food but also as mutants are available in the pathways we were interested in. We subjected the plants to mechanical wounding treatments and examined the phytohormone levels and gene expression changes in response to wounding.

The results retrieved in this study indicate, that during the wounding response light can exert influence via phy on different parts of the JA-pathway. While hormone production is repressed via a phy-mediated mechanism, the transcriptional response to JA is enhanced by light. Thus, in absence of light or active phy, the plant is not able to exhibit a transcriptional response as strong as in the light. This implies, that phytochromes modulate JA-signalling and that the JA-response to wounding is only executed properly in presence of active phy. These results are in line with recent findings reporting an attenuation of plant defence responses to herbivory and pathogens in the dark.

Thus, light and hormone signalling are linked on more than one level and can mutually influence each other during the response to wounding. Moreover, this interaction appears to have negative effects on plant defence under certain light conditions like shading or darkness, an effect which possibly serves the regulation of resource allocation to growth and defence.

Content

Abbreviations

13-HPOT	13-hydroperoxyoctadecatrienoic acid
ABA	Abscisic acid
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
FR	Far-red
GA	Gibberellic acid
IAA	Indole-3-acetic acid
Ile	Isoleucin
JA	Jasmonate
JA-Ile	(+)-7-iso-jasmonoyl-L-isoleucine
JAR1	JASMONATE RESISTANT 1
JAZ	JASMONATE-ZIM DOMAIN
MeJA	Methyl-Jasmonate
OPDA	cis-(+)-12-oxophytodienoic acid
phy	Phytochrome
R	Red
RT-qPCR	Reverse transkription quantitative PCR
SAS	Shade avoidance syndrome

1 Introduction

Due to their sessile lifestyle, plants are confronted with certain challenges, which animals do not have to face. When an animal is challenged by either inhospitable living conditions or the attack of a life-threatening predator, it is able to either fight or to flee from the dangerous situation.

As running away from unfavourable conditions or potential aggressors is not an option for plants, they have to adapt to their environment and defend themselves against aggressors instead. Therefore it is crucial that the variety of signals, which plants are permanently exposed to, are perceived and integrated and that an appropriate response is exhibited by the plant. External signals plants have to perceive and respond to range from abiotic factors like gravity, temperature or light conditions to biotic stresses like the attack by pathogens or herbivorous animals.

Nevertheless, plants are not defencelessly exposed to their environment, but have evolved a multitude of strategies to respond to a variety of conditions. These responses are subject to a complex signalling network which ensures, that the correct reaction to different challenges is exhibited in a timely manner. The multitude of involved compounds and pathways in this signalling network enables the plant to tailor the response adequately to the respective situation it is confronted with.

In this study, we focus on the integration of two major external signals a plant has to react to, light conditions and stress imposed by wounding. While the perception of light conditions influences the growth and development of a plant on a long time scale, wounding stress is an immediate event, to which the plant has to respond in a fast manner, as the loss of leaf material threatens the basis of its life.

While at first view a connection between light and wounding responses is not obvious, observations of ecological research have given solid evidence, that both signalling pathways are able to influence each other (Ballare, 2014; Hua, 2013; Kazan & Manners, 2011; Morker & Roberts, 2011). These studies support a direct influence of light conditions on the defence of plants to herbivores or pathogens. The mechanistic background of this interaction represents an intriguing research topic and thus, we wanted to investigate the signalling pathways potentially involved.

For our studies we chose rice (*Oryza sativa*) as a model plant. It not only has a high relevance as staple food and model organism for cereals, but there are numerous mutants in different signalling pathways available, which makes it an excellent tool for our research.

1.1 Light as a signal in plants

One of the most important environmental signals a plant has to be able to perceive is light.

Without light, plants cannot assimilate carbon via photosynthesis, which makes the recognition of light conditions one of the most essential skills for a plant. As seasons change and the day length varies, light conditions are subjected to variations in quantity as well as quality. To sense these changes, plants have evolved an array of photoreceptors, which enables them to detect their surrounding conditions and consequently react to them in an adequate manner. In response to the information gained by light perception, plants control and adjust different processes like for example germination, growth or flowering time in accordance to the prevalent situation (Smith, 2000).

1.1.1 Light perception via photoreceptors

To date, three types of photoreceptors are known in plants: phototropins and cryptochromes, which are sensitive to blue light (Briggs & Christie, 2002; Christie, 2007; Lin & Shalitin, 2003; Sancar, 2003), and phytochromes, which perceive red (R) and far-red (FR) light (Furuya, 1993; Nagy & Schäfer, 2002; Quail, 1991; Smith, 1995). Recently, two other types of photoreceptors have been discovered, one which perceives UV-B light, "UVR8" (Rizzini *et al.*, 2011), and one, which is apparently sensitive for blue-light and has been named "ZEITLUPE" (Kim *et al.*, 2007).

In this study, we focus on the phytochrome (phy) photoreceptor family, the members of which have been shown to be the only red/far-red light receptors in rice plants (Takano *et al.*, 2009). Phys are photoconvertible pigments, which exist in two distinct conformations, P_r and P_{fr}. Both forms can be interconverted by absorption of the respective light wavelength (Butler *et al.*, 1959). The P_r form of phy absorbs red light (600-700 nm) and is physiologically inactive (Fankhauser, 2001). Upon irradiation with red light, P_r is converted to P_{fr}, which is the physiologically active form of phy. This conformation change is reversible, upon absorption of FR light (700-800 nm), P_{fr} is converted to P_r again. This photoreversibility makes phytochromes "switches", which can be activated or inactivated by the respective light conditions and therefore can turn downstream signalling on or off.

Rice only contains three phys, phyA, phyB and phyC (Takano *et al.*, 2005), which makes the research on phytochrome function less complex in rice compared to *Arabidopsis* plants, which have five phy genes. Thus, the action of the different phys has been extensively studied in germinating rice seedlings. In general there are two different types of phys, which are labile and stable in light, respectively. In rice, only phyB is stable in the light, while phyA and phyC are labile in continuous light and degraded after activation, a process which is called photodestruction (Takano *et al.*, 2005). Additionally, plants respond differently

according to the energy of the irradiation they receive, and therefore these responses have been classified into three different modes of action: very-low-fluence response (VLFR), low-fluence response (LFR), and far-red dependent high-irradiance response (FR-HIR) (Kneissl *et al.*, 2008). Therefore, while phyA and phyC are mostly responsible for the VFLR and HIR responses, the LFR response is preferentially mediated via phyB.

In rice, phy single, double and triple mutants have been generated, which makes rice a convenient tool to study phytochrome action. For our study, we chose the double mutants *phyAphyC* and *phyBphyC*. The examination of the *phyAphyB* and *phyAphyBphyC* mutant plants was not possible in our experimental setup. These mutants exhibited an altered phenotype in the developmental stage our experiments were carried out, which made it impossible to use them in our wounding assay in a way comparable to the other genotypes.

1.2 The response to light signals can be modulated by the plant hormone jasmonate

When a signal from the environment causes a response in a plant, there are two basic steps involved: perception of the signal, and signal transduction from the site of perception to the site of action. The idea of mobile messengers, which can transduce signals in the plant and evoke responses far from their source, similar to the hormones in animals, dates back to the 19th century. Interestingly, the function due to which auxin, one of the first plant hormones known, was discovered, is connected to a classical light response of plants: phototropism.

Phototropism is the bending of plant parts towards a light source (Whippo & Hangarter, 2006) and can for example be observed in coleoptiles of grass seedlings. In grass plants, the first leaves that emerge after germination are ensheathed in a coleoptile, a sheltering organ which is very sensitive to light. When illuminated from one side, the coleoptile bends towards the light source, which is caused by asymmetrical growth on the illuminated and shaded sides of the coleoptile. Darwin and Darwin, who examined the growth of grass seedlings, first suggested a transmissible and possibly chemical signal, which was produced in the coleoptile tip and then transferred to the growth zone some millimetres below the coleoptile (Darwin & Darwin, 1880). Later, Went could prove this theory true and was able to show, that the signal has to be a chemical compound (Went, 1926), which again later was identified to be indolyl-3-acetic-acid, auxin (Kögl *et al.*, 1934; Thimann, 1935). The model, explaining phototropic responses, is called the Cholodny-Went hypothesis (Whippo & Hangarter, 2006): In response to light, auxin is produced in the coleoptile tip and transported downwards. Simultaneously, a lateral gradient of auxin is established, so that more auxin accumulates on the shaded side of the coleoptile. Subsequently, the cells on this side elongate faster and the tip bends toward the light source.

Thus, the impact light can have on the production or redistribution of phytohormones has been known for decades. In the last years a variety of evidence has been found, that an

influence of light signalling on other phytohormones, like e.g. gibberellic acid (GA) and jasmonic acid (JA) (Feng *et al.*, 2008; Riemann *et al.*, 2003) exists.

1.2.1 *Hebiba*: JA is required for correct light signal transduction in rice seedlings

In rice seedlings, the coleoptile protects the young leaves on their way through the soil, splitting open as soon as it perceives light. Subsequently leaves unfold and develop their photosynthesis apparatus. This is part of a developmental process which is called photomorphogenesis. In the course of the search for rice mutants defective in photomorphogenesis, the response of rice coleoptiles to light was analysed for phenotypic screening. In this way, a mutant was isolated, which exhibited a "red-light blind" phenotype: the coleoptile was elongated when seedlings were grown under red light, mimicking the phenotype of a dark grown wild type (see Figure 1A). The mutant was called *hebiba*, which is Japanese for "snake-leaf", as the adult plants show a phenotype with elongated, "snake-like" leaves (Figure 1B).

Hebiba turned out not to be a photoreceptor mutant, as initially assumed, but to be deficient in the phytohormone jasmonate (Riemann *et al.*, 2003). An analysis of plant hormone levels in *hebiba* and wild type coleoptiles showed, that the wild type induced JA accumulation after red light irradiation, while *hebiba* did not. Further analysis showed, that *hebiba* was not able to produce neither JA nor its precursor OPDA after light irradiation or after wounding, while these treatments triggered elevation of JA levels in the wild type (Riemann *et al.*, 2003). Via map-based cloning, the cause of this JA-deficiency could be detected: *hebiba* contains a deletion in chromosome 3, which expands over 170 kb and includes the *OsAOC* gene (see Figure 2).

Thus, *hebiba* is a mutant deficient in the biosynthesis of JA, exhibiting an impaired photomorphogenesis, which implies, that JA is required for the correct transduction of the light signal.

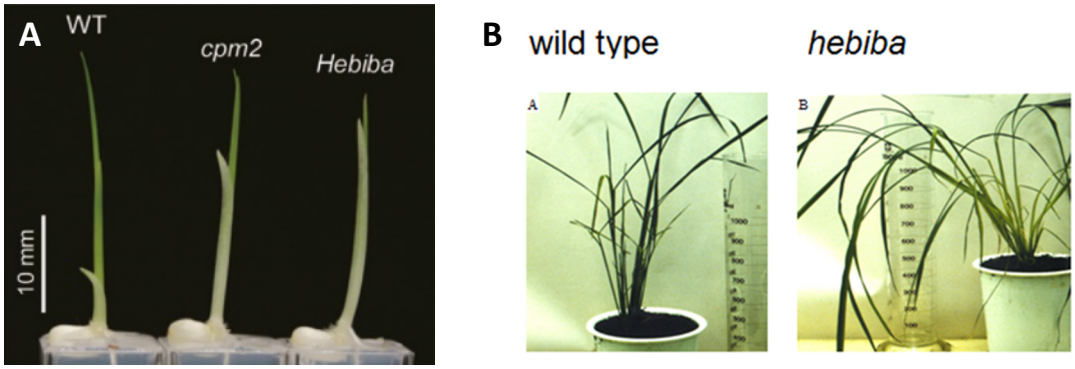


***Figure 1: Phenotypes of JA-deficient mutants* cpm2 *and* hebiba.**
A: Final coleoptile length of red light grown seedlings of wild type, *cpm2* and *hebiba*. (Figure taken from Riemann *et al.*, 2013)
B: Adult plants of wild type and *hebiba*. (Figure adapted from Riemann *et al.*, 2003)

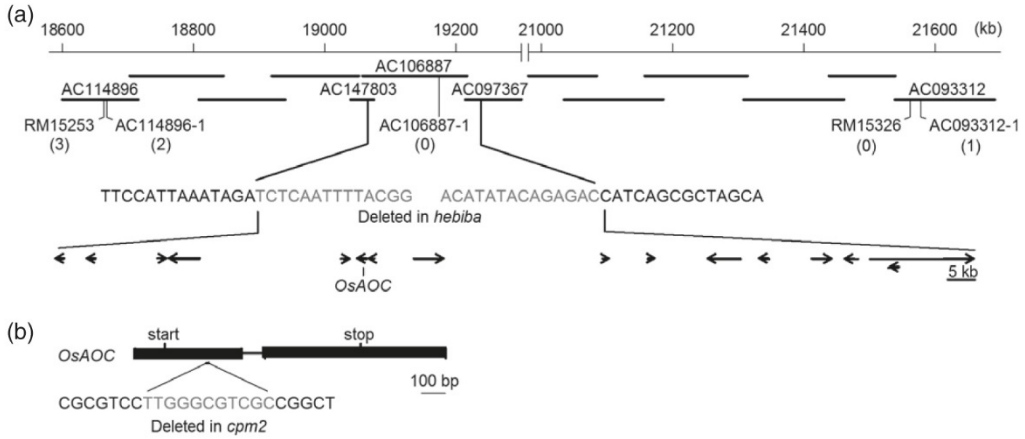


Figure 2: Loci responsible for the mutations in* hebiba *(a) and* cpm2 *(b), discovered via map-based cloning.
A: *Hebiba* has a deletion of 170 kb on chromosome 3, located between the markers AC147803 and AC097367. The entire genomic sequence of *OsAOC* is included in this region.
B: In *cpm2*, a small deletion of 11 bp in the first exon of the *OsAOC* gene is leading to a frameshift.
(Figure taken from Riemann *et al.*, 2013)

Besides *hebiba*, other mutants with a similar photomorphogenesis phenotype and an impaired JA-biosynthesis pathway were found. On the one hand *coleoptile morphogenesis 2* (*cpm2*), in which only a small part of 11 bp in the gene for *OsAOC* is deleted, which leads to a frameshift and therefore to a different mRNA sequence (Riemann *et al.*, 2013, Figure 2). Secondly, *kamakubi*, in which the promotor and coding region of the *OsAOS1* gene are missing due to a deletion of 7000 bp on chromosome 3 (unpublished data, see also chapter 2.1, page 14). These mutants represent convenient tools for studies on the JA pathway and thus were chosen for our research on the JA-mediated wounding response.

Thus, not only the phytohormone auxin, which has been known for decades to mediate light responses in the plant, but also jasmonate apparently is required for correct light signal transduction. Interestingly, jasmonate is a so-called “stress hormone” (see chapter 1.3 for detailed information), mediating the plant response to different biotic stresses like wounding or pathogen infestation. This indicates, that the responses to two very diverse external signals, light and wounding stress, are modulated by the same plant hormone: Jasmonate. Consequently the question arises, if a mutual influence between both signals exists and if jasmonate is involved in this potential interaction.

1.3 Plant hormones, signalling molecules mediating a multitude of plant processes

Plant hormones, also called phytohormones, are low molecular signalling molecules that are functional in very low concentrations. In contrast to hormones in the animal kingdom, plants do not have special glands for hormone production, rather in many cases all plant cells are able to produce phytohormones (Davies, 2004). Additionally, in plants the source of the

hormone and the site of its action are not as strictly divided as is the case in classical hormones of animals and the function of the hormone is strongly dependent on the responsivity and competence of the targeted tissue. Thus, plant hormones can excite a variety of responses and fulfil many different tasks depending on the site of their action, the type of process they are operating in and also on the presence and concentration of other phytohormones. Additionally, often two or three phytohormones are involved in the same process, e.g. developmental growth, without acting redundantly (Jaillais & Chory, 2010). Between the different plant hormones, an extensive crosstalk with synergistic and antagonistic interactions exists, which still needs to be completely elucidated (Depuydt & Hardtke, 2011; Robert-Seilaniantz *et al.*, 2011).

Plant hormones mediate a variety of different processes like developmental growth or responses to biotic and abiotic stresses (Davies, 2004). This dissertation focuses on the function of the phytohormone jasmonate in the response to wounding stress.

1.3.1 Jasmonates - biosynthesis, signalling and catabolism

Jasmonates are a group of phytohormones, derived from the oxylipin pathway. Jasmonic acid (JA), is the final product of the biosynthesis pathway, but is presumably not an active compound. Several different derivatives of JA have been detected in plant, of which in particular the conjugate with the amino acid isoleucin (Ile) can induce JA-signalling. The first jasmonate described was the volatile compound methyl jasmonate (MeJA), as a component of the essential oil of jasmine (Demole *et al.*, 1962). The physiological function of JA and MeJA was discovered years later in the 1980s by two different research groups, who reported senescence promotion (Ueda & Kato, 1980) and inhibition of growth (Dathe *et al.*, 1981) as functions of JA.

However, one of the major roles of jasmonates is the orchestration of a variety of different biotic and abiotic stress responses like salt stress, pathogen infestation or herbivore attack (Farmer & Ryan, 1990; Ismail *et al.*, 2012; Reymond & Farmer, 1998). Thus, JA is often described as a “stress hormone”. Nevertheless, JA also fulfils a variety of roles apart from mediating stress responses, for example in developmental processes like tendril coiling (Falkenstein *et al.*, 1991), fertility and sex determination (Acosta *et al.*, 2009; Ishiguro *et al.*, 2001; Krajncic *et al.*, 2006; Li *et al.*, 2004; Riemann *et al.*, 2013), as well as in the modulation of light signalling in seedlings (Riemann *et al.*, 2003).

Biosynthesis

The biosynthesis of JA occurs in two compartments, the chloroplasts and the peroxisomes (Vick & Zimmerman, 1984; Wasternack & Hause, 2013, see also Figure 3). In the chloroplasts, the starting substrate α-linolenic acid is released from the galactolipides of chloroplast membranes by a phospholipase D (PLD) and oxygenated by a 13-lipoxygenase (13-LOX) to 13-hydroperoxyoctadecatrienoic acid (13-HPOT) (Lyons *et al.*, 2013; Wasternack & Hause,

2013). Subsequently, the conversion of 13-HPOT into the intermediate product *cis*-(+)-12-oxophytodienoic acid (OPDA) is catalysed in two successive steps by the enzymes ALLENE OXIDE SYNTHETASE (AOS) and ALLENE OXIDE CYCLASE (AOC) (Schaller & Stintzi, 2009). OPDA is then transferred to the peroxisome by an unknown mechanism. In the peroxisome, a reduction of OPDA by OPDA REDUCTASE3 (OPR3) is followed by 3 β-oxidation steps, leading to the comparatively unstable (+)-7-*iso*-JA which epimerises to the more stable (-)-JA (Wasternack, 2007).

JA is no longer thought to be the biologically active signalling molecule of the JA hormone response (Thines *et al.*, 2007) and is rather acting as a prohormone, which can be modified by different kinds of actions like methylation, hydroxylation or conjugation to amino acids (Koo & Howe, 2012). The most important modification is the conjugation of JA to the amino acid isoleucin by the enzyme JAR1, forming the bioactive (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile, Figure 3), which is generally believed to be the main signalling compound in the JA pathway responses (Staswick & Tiryaki, 2004; Thines *et al.*, 2007).

Signalling

Similar to the signalling pathways of other phytohormones (e.g. auxin, see Figure 4), the signal response to JA is based on the derepression of transcription factors and involves degradation of repressive proteins by the 26S-proteasome. The repressors acting in JA-signalling are called JASMONATE-ZIM DOMAIN proteins (JAZ) (Chini *et al.*, 2007; Thines *et al.*, 2007) and are part of a bigger protein family, the TIFY proteins (Vanholme *et al.*, 2007; Ye *et al.*, 2009). In rice, the family of JAZ proteins contains 15 known members (Ye *et al.*, 2009). It is believed that JAZs act to some extent redundantly but also mediate different aspects of the JA response, depending on the prevalent situation.

In the absence of JA-Ile, the JAZ-proteins are interacting via their Jas domain with JA-responsive transcription factors like MYC2 and suppress the expression of JA-responsive genes (Figure 3). In this process also the co-repressors TOPLESS (TPL) and “Novel Interactor of JAZ” (NINJA) are involved, TPL being connected to JAZ via NINJA (Pauwels & Goossens, 2011). After induction of the JA-pathway, JA-Ile is produced and binds to its receptor CORONATINE INSENSITIVE 1 (COI1), which is able to recognize and bind JAZ proteins in presence of JA-Ile (Fonseca *et al.*, 2009; Xie *et al.*, 1998; Yan *et al.*, 2009). COI1 is part of the SCF^{COI1} complex, a SKP-CULLIN-F-box complex. After binding to SFC^{COI1}, JAZ proteins are ubiquitinated and degraded by the 26S-proteasome. Subsequently, the JA-responsive transcription factors are liberated from repression of JAZ and the expression of JA-responsive genes is initiated. Among those genes are JA-biosynthesis genes like *OsAOS* and *OsAOC* as well as the *JAZ* genes themselves. Thus, a positive (biosynthesis) and a negative (repressors) feedback loop are established, fine tuning the JA-response (Svyatyna & Riemann, 2012).

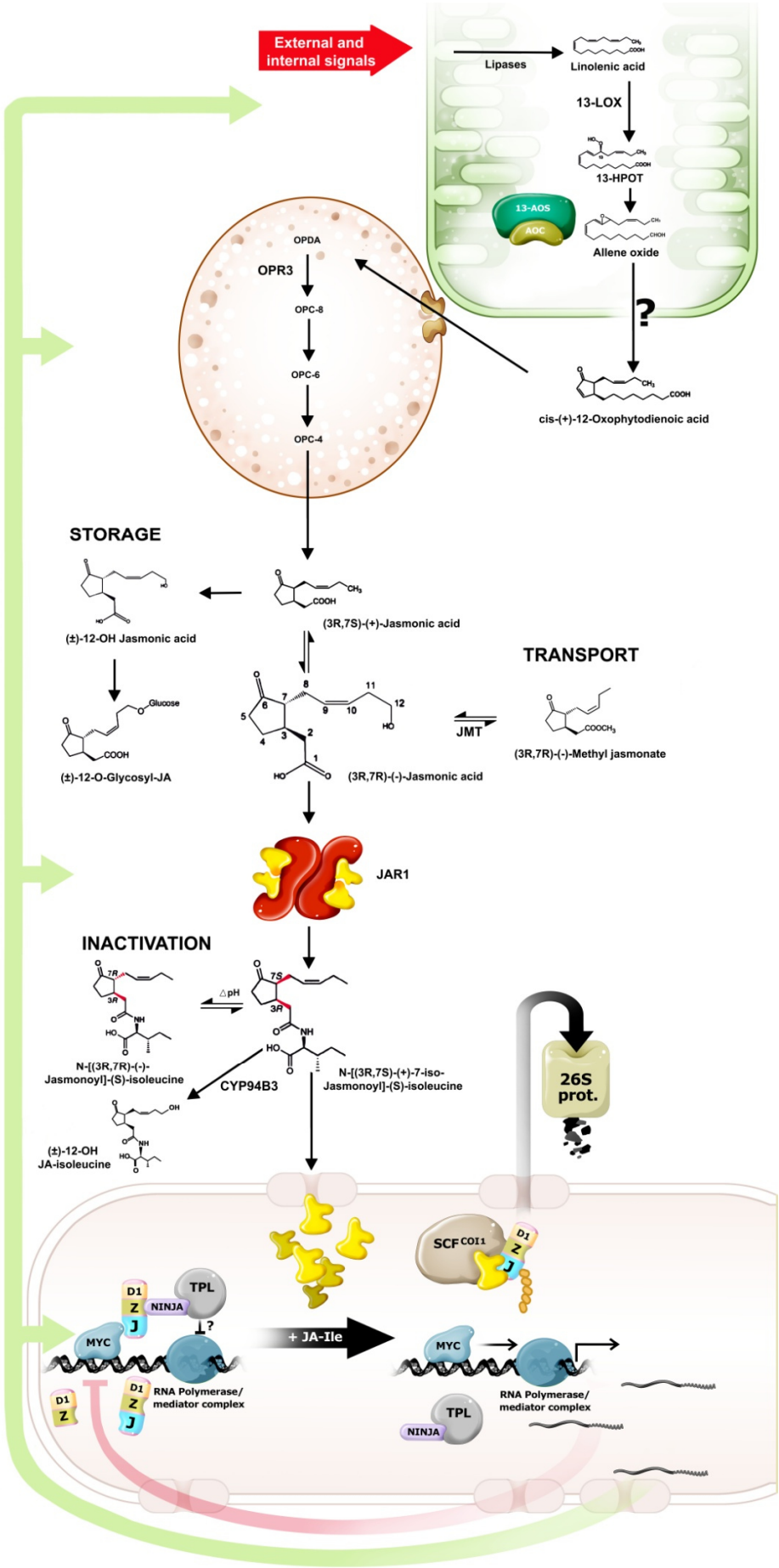


Figure 3: Jasmonate biosynthesis and signalling

In response to external elicitation, linolenic acid is cleaved from the chloroplast plasmamembrane and subsequently converted to 12-OPDA. After transfer to the peroxisome, 12-OPDA is converted to (+)-7-*iso*-JA by one reduction and three oxidation steps. The unstable (+)-7-*iso*-JA epimerizes to (-)-JA.

JA can be modified by different reactions, one of which is the conjugation to isoleucin, forming the bioactive JA-Ile.

In presence of JA-Ile, JAZ proteins are recruited to the SCF^{COI1} complex and ubiquitinated and subsequently degraded via the 26S-proteasome. This leads to the release of JAZ-repression of JA-responsive transcription factors (e.g. MYC) and thus JA-responsive gene expression is initialized. Among the induced genes are JA-biosynthesis genes as well as JAZ-genes themselves. This results in a positive and a negative feedback loop.

(see text for more details, Figure adapted from Svyatyna & Riemann, 2012)

The transcription factor MYC2 appears to be the master switch for JA-signalling (Kazan & Manners, 2013) but nevertheless, MYC2-independent pathways are also known to exist (Wasternack & Hause, 2013). Thus, the variety of homologous JAZ repressors as well as the different JA-responsive transcription factors enable the plant to tailor its response to different stresses very specifically.

Catabolism

After the elucidation of the JA-biosynthesis and the core signalling, research has focussed on JA and JA-Ile catabolism. The action of a signalling compound is dependent on its availability and thus directly connected to the homeostasis of production and degradation. Recently, two cytochrome P450 hydroxylases have been identified to catabolize JA-Ile to 12-OH-JA-Ile and subsequently to 12-COOH-JA-Ile (Heitz *et al.*, 2012; Koo & Howe, 2012). These compounds are less active than JA-Ile and are thought to be inactivated metabolites of JA-Ile. This indicates, that ω-oxidation by P450s might be the main pathway for catabolism of JA-Ile, serving the purpose of attenuation of JA-signalling.

1.3.2 Interactions of JA with other phytohormones

As a variety of processes in the plant are subjected to the regulation of phytohormones and as often more than one phytohormone is involved in the regulation of a single process, it can be expected that the signalling pathways of plant hormones intersect at certain points and influence each other. These interactions have been a key aspect of research in the recent years (Lyons *et al.*, 2013; Pieterse *et al.*, 2012; Robert-Seilaniantz *et al.*, 2011) and are sometimes referred to as “crosstalk” (Jaillais & Chory, 2010). As the interactions of plant hormones are seldom crosstalk in the sense of the biological definition, which is specified by signalling pathways sharing one component (Mundy *et al.*, 2006), the terminology “interaction” is more accurate.

Considering a multitude of studies reporting mutual influence of different phytohormones, there appears to be a whole network of connections, whose mechanistic backgrounds still have to be elucidated. Thus, when analysing the action of a single plant hormone in a physiological event, the influence of other hormones has to be kept in mind.

Similarities between hormone signalling pathways have provided evidence on one possible mechanistic background of phytohormone interactions. The signalling pathways of auxin (indole-3-acetic acid, IAA), gibberellic acid (GA) and JA are very similar: in presence of the respective hormone, a negative regulator of the response is recruited to a SCF complex and subsequently degraded via the 26S-proteasome (see Figure 4).

Thus, the parallelism of these pathways implies, that certain compounds of the different hormonal pathways could interact directly. An example for a protein functional in several hormonal signalling pathways, is the co-repressor Topless (TPL), which is a component of

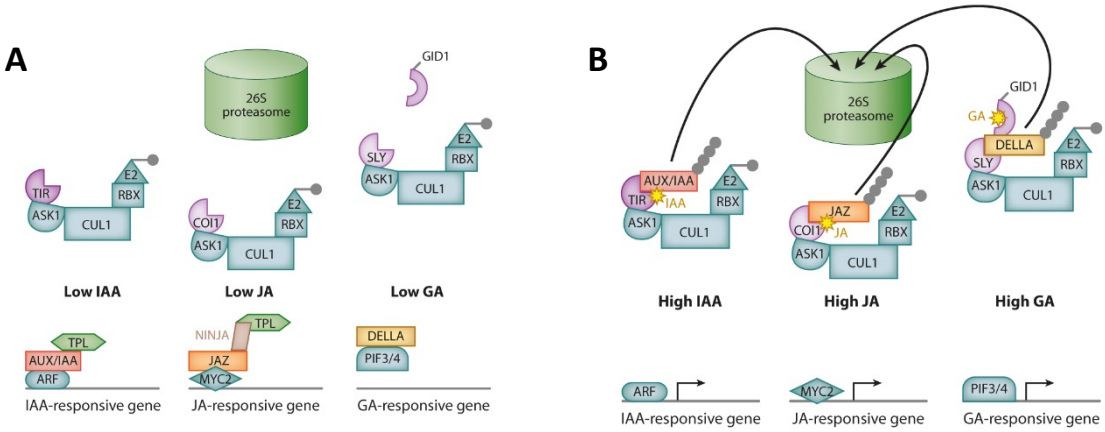


Figure 4: Similarities between the signalling pathways of IAA, JA and GA. Inactive (A) and active (B) states.
A: under non-induced conditions, the expression of responsive genes is repressed via binding of the respective repressor proteins (AUX/IAA, JAZ or DELLA) to the positively regulating transcription factor (ARF, MYC2 or PIF3/4).
B: Upon induction of the hormone pathway, active hormones mediate the binding of the repressor to an SCF complex, and subsequently the repressors are ubiquitinated and degraded by the 26S-proteasome. Consequently, the hormone responsive transcription factors are released from repression and gene expression is induced.
(IAA: Auxin, JA: Jasmonic acid, GA: Gibberellic acid, TPL: Topless, ARF: Auxin response factor, MYC2: bHLHzip transcription factor MYC2, PIF: Phytochrome interacting factor; Figure taken from Robert-Seilaniantz *et al.*, 2011)

IAA, and JA signalling and has recently even been hypothesized to be linked to ABA signalling (Causier *et al.*, 2012; Pauwels *et al.*, 2010). Additionally, the DELLA proteins, which are the equivalent to JAZ in the GA signalling pathway, have been shown to directly interact with JAZ proteins, thus providing an interface between these both hormonal pathways (Hou *et al.*, 2010). Thus, competition for shared proteins could be one mechanism, by which hormones can influence each other.

Moreover, is has been suggested, that because of the similarity of these pathways, proteins might even take over moonlighting functions, acting in pathways they are not genuinely designed for (Robert-Seilaniantz *et al.*, 2011).

This mechanism is only one example of the connection points of plant hormone signalling. Many interactions still remain elusive and considerable efforts will be required to elucidate the whole hormone signalling network with all its components.

1.4 Light signalling and plant defence: the growth vs. defence dilemma

The plant hormone jasmonate is involved in light signalling as well as in plant defence, which hints at a potential interaction of both signalling pathways. Interestingly, it has been reported by a variety of ecological studies that the defence response of plants to pathogens and herbivores actually can be modulated by light conditions (Ballare, 2014; Cerrudo *et al.*, 2012; de Wit *et al.*, 2013; Moreno *et al.*, 2009).

Depending on the severity of an herbivore attack, the loss of leaf material can be life threatening for a plant. Therefore, plants have evolved a variety of constitutive and induced defences to fend off attackers. Constitutive defences are permanently exhibited by the plant and for example include mechanical barriers like trichomes (Levin, 1973) or toxic chemical compounds like alkaloids (Wittstock & Gershenzon, 2002). Induced defences in contrast, are only expressed in direct response to an attack, which is advantageous, as it limits the energy the plant invests to situations, when defence really is necessary. Examples for induced defences are the accumulation of secondary metabolites which are toxic or repellent (Kessler & Baldwin, 2002), as well as the emission of volatile organic compounds, to warn adjacent plants and attract natural enemies of the herbivore (Baldwin, 2010; Dicke, 2009).

As one can easily deduce from these descriptions, defence responses of any kind are costly for the plant as they require a reprogramming of metabolic pathways as well as changes in gene expression. The general limitations for these responses are therefore resources like sugars, chemical compounds and precursors of metabolites. The same, however, also applies for growth processes, which need a lot of energy and metabolites, too. This poses a challenge for the plant, as resources have to be divided to diverse processes and a balance has to be kept as both, growth and defence are essential. This problem has been called the "dilemma" of plants (Herms & Mattson, 1992).

Research in the last decades has focused on this dilemma, which is extremely pronounced if shade-intolerant plants are not grown under optimal light conditions. When growing in the shade of bigger plants or in dense populations, plants can sense their competitive neighbours through the light quality they receive and exhibit the so called shade avoidance syndrome (SAS). The SAS is characterized by stem elongation, reduced lateral branching and increased leaf angles (Ballare, 1999; Izaguirre *et al.*, 2006), which indicates, that these plants try to "outgrow" their neighbours to gain more sunlight.

One signal known to induce such a response is FR light, respectively the amount of FR light in proportion to the total amount of light. Under canopies or in dense populations, the amount of R light is low, as it already has been absorbed by leaves, while at the same time, the amount of FR light is elevated, as is passes through and is reflected by green tissues . Therefore, the ratio of R to FR light is higher in full sunlight (R:FR>1) as compared to under a canopy (R:FR <1, Kazan & Manners, 2011). It has been reported, that plants can sense competition via the R:FR ratio and respond to this signal by exhibition of SAS long before they are actually in the shade (Ballare *et al.*, 1990). Additionally, under experimental conditions it could be shown, that the supplementation with FR enriched light, simulating the conditions under a canopy, is sufficient to trigger SAS (Izaguirre *et al.*, 2006; Moreno *et al.*, 2009). As regulators of the SAS exhibition, phytochromes have been established. Especially phyB appears to be the major player mediating the response of plants to shading (Ballare, 2009).

Plants exhibiting SAS, irrespective of induction by natural competition or artificial FR-light supplementation, have been found to show reduced defences and immunity (Izaguirre *et al.*,

2006; Moreno *et al.*, 2009). Thus, apparently unfavourable light conditions, which force the plant to invest energy into growth, lead to a deficiency in the expression of defence responses.

These observations are directly related to the "dilemma" of plants and therefore, it has long been hypothesized, that this dilemma is the reason for the antagonistic relationship of growth and defence (Ballare, 2009). Nevertheless, studies on the Arabidopsis *sav3* mutant, which initializes SAS responsive signalling, but does not exhibit SAS morphology due to an impaired auxin biosynthesis, have challenged this hypothesis (Moreno *et al.*, 2009). It could be shown, that in *sav3*, supplementation with FR light compromises defence against caterpillar feeding, even though there are no resources diverted to the growth response. Additionally, in the same study it was shown that FR-light reduced the JA-sensitivity of the plant. Thus, there appears to be a more complex regulation mechanism behind the light modulation of defence responses than just a problem of resource allocation. In our study we aimed to shed light on the mechanistic background of this interaction.

1.5 Scope of the dissertation

In developing rice seedlings, a connection between light and JA has been found, but there are no studies on this interaction in adult rice plants. Additionally, observations in other plant species on the influence light can have on JA-mediated defence responses, imply, that there actually might be an interaction of light and JA in the defence of rice.

To our knowledge, a shade avoidance response was not found in cultivated rice plants so far. However, crop plants usually are grown in high densities on the field, and thus it has been hypothesized, that by breeding most shade intolerance responses might have been reduced or even completely eliminated in these species (Kebrom & Brutnell, 2007). Nevertheless, as the expression of SAS morphology is not necessarily required for the negative influence of shading on defence, it is possible, that defence of rice plants might still be influenced by light conditions. We therefore chose to examine the response to wounding stress in rice plants in the vegetative state.

The existence of mutant plants deficient in JA-biosynthesis as well as mutant plants deficient in different phytochrome photoreceptors made rice an excellent tool for our studies.

We conducted mechanical wounding treatments, using a device called “MecWorm” (Mithöfer *et al.*, 2005), a machine which is able to mimic the continuous feeding of caterpillars (see chapter 2.3, page 15, for detailed information). We examined the levels of JA and its bioactive derivative JA-Ile as well as other phytohormones in response to mechanical wounding, to monitor the plant response on the level of hormone signalling. Additionally, the investigation of JA-responsive gene expression after wounding with MecWorm should give evidence on the impact of hormone level changes on the transcriptional level.

In this way, we were able to compare the wounding response in plants impaired in different parts of the JA- and photoperception pathways and thus shed light on the mechanistic background of this interaction.

2 Materials and Methods

2.1 Plant material

All seeds used for the experiments were propagated in the greenhouses of the Botanical Garden of the KIT (Karlsruhe Institute of Technology, Karlsruhe, Germany).

The *hebiba* und *coleoptile photomorphogenesis 2* (*cpm2*) mutant lines originate from γ-ray mutagenesis and are described in Riemann *et al.* (Riemann *et al.*, 2003; Riemann *et al.*, 2013). For these mutant lines the wild type background is *Oryza sativa* L. ssp. *japonica* cv. Nihonmasari, therefore, this cultivar was used as a control.

The *kamakubi* mutant line originates from the Tos17 mutant panel (Miyao *et al.*, 2003) and carries a deletion of 7000 bp on chromosome 3 which encompasses the entire coding and promoter region of *OsAOS1* (Accession number: AB116527; Haga & Iino, 2004). The mutant phenotype could be complemented by transformation with the wild type *OsAOS1* gene (Riemann, unpublished data).

The *phyAphyC* (phyA-2phyC-1 alleles) and *phyBphyC* (phyB-1phyC-1 and phyB-2phyC-1 alleles, respectively) double mutant lines were obtained by crossing of *phyA*, *phyB* and *phyC* single mutants, which had also been generated by Tos17-mediated mutagenesis. All these mutant lines are described in detail by Takano *et al.* (Takano *et al.*, 2005). The wild type background for the *phyAphyC* and *phyBphyC* lines, as well as the *kamakubi* and *osjar1-2* lines, is *Oryza sativa* L. ssp. *japonica* cv. Nipponbare, which was used as a control plant for these mutants.

2.2 Plant cultivation

Rice seeds were dehusked and surface sterilized before sowing. Seeds were shaken in 70% ethanol for 1 min and washed with ultrapure H_2O two times. Afterwards, the seeds were incubated in a solution of sodium-hypochlorite (5% w/v active chlorine, Carl Roth, Karlsruhe, Germany), shaking at 100 rpm for 20 min, and then washed with sterile ultrapure H_2O under sterile conditions. Subsequently, 20 to 25 seeds were sown into one magenta box (Sigma-Aldrich, Taufkirchen, Germany) onto 0.4% phytoagar (Duchefa, Haarlem, Netherlands) and raised at 25 °C under continuous white light (20 µmol/m^2s, light source: neon tube TLD 36W/25, Philips, Hamburg, Germany). After two weeks, seedlings were transferred to sand and raised for four additional weeks in a phytochamber (BBC York, Mannheim, Germany) under short-day conditions (10 h light at 28 °C, 280 µmol/m^2s, 14 h darkness at 22 °C). Once a week plants were fertilized (Wuxal, “TopN” and “Super” fertilizers, Manna, Ammerbuch-Pfäffingen, Germany).

2.3 Treatments

For our experiments, we chose a mechanical device called “MecWorm” (Mithöfer *et al.*, 2005) to conduct wounding treatments instead of using real herbivory. This choice was made in consideration of the complexity of the herbivory event.

The event of caterpillar feeding on a plant is constituted of two components, (i) continuous mechanical damage to the tissue and (ii) chemical elicitation by compounds from the caterpillar regurgitate (Bricchi *et al.*, 2010; Kessler & Baldwin, 2002). Especially the chemical component of herbivory is extremely variable, as every caterpillar species has its own concoction of spit and moreover some parts of the oral secretions are also comprised from plant tissue (Roda *et al.*, 2004; Vadassery *et al.*, 2012; Voelckel & Baldwin, 2004). Additionally, some of the constituents of the regurgitate have been shown to directly suppress the plants defence responses, which adds another level of influence (Consales *et al.*, 2012; Kahl *et al.*, 2000; Musser *et al.*, 2002).

Thus, herbivory is a very complex event with a multitude of different processes which are preceding simultaneously. We therefore decided to only examine the mechanical part of the wounding response, using the “MecWorm”, to impose wounding stress on our plants. MecWorm has been developed by the Max Planck Institute for Chemical Ecology (MPI:CE, Jena, Germany) in the group of Prof. Boland and is able to mimic the mechanical part of herbivory (Mithöfer *et al.*, 2005).

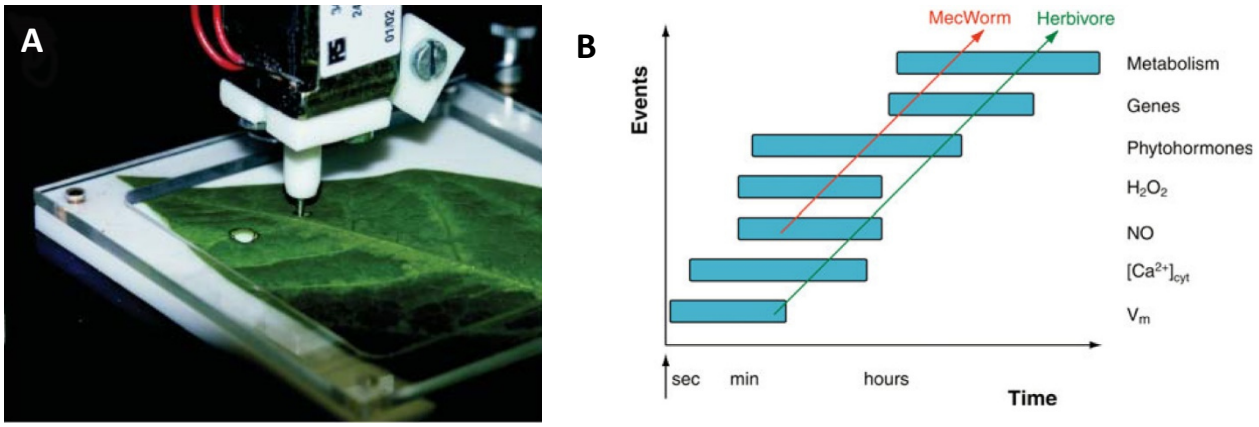


Figure 5: MecWorm, action and responses
A: MecWorm picking on a lima bean leaf. The electronically controlled needle is continuously wounding the leaf, following a predefined schedule. (Figure taken from Mithöfer *et al.*, 2005)
B: Time course of consecutive events in response to wounding via MecWorm or herbivory. Earliest events like membrane potential changes (V_m) and cytosolic Ca^{2+} changes cannot be induced by wounding with MecWorm. All other responses like induction of gene expression and changes in metabolism are similar between both wounding treatments (Figure taken from Bricchi *et al.*, 2010)

Comparative experiments with real herbivory showed, that apart from very early events like membrane potential changes and Ca^{2+} fluxes, MecWorm is able to induce all other plant responses like hormone accumulation and alteration gene expression (Bricchi *et al.*, 2010, see also Figure 5B).

In comparison to experiments with real herbivory, which are subjected to natural deviations in feeding behaviour, the damage inflicted by MecWorm can be defined and is therefore highly reproducible. This makes MecWorm the ideal tool for comparative studies on the wounding response of different mutant plants and wild types.

The youngest, fully expanded leaf of a six weeks old rice plant was treated for different time intervals. The duration of the wounding treatment was set for either 30 minutes (0.5 hours), 1 hour or 6 hours. For each treatment, two defined areas on the adaxial leaf surface, parallel to the middle vein of the leaf, were damaged (see Figure 6B).

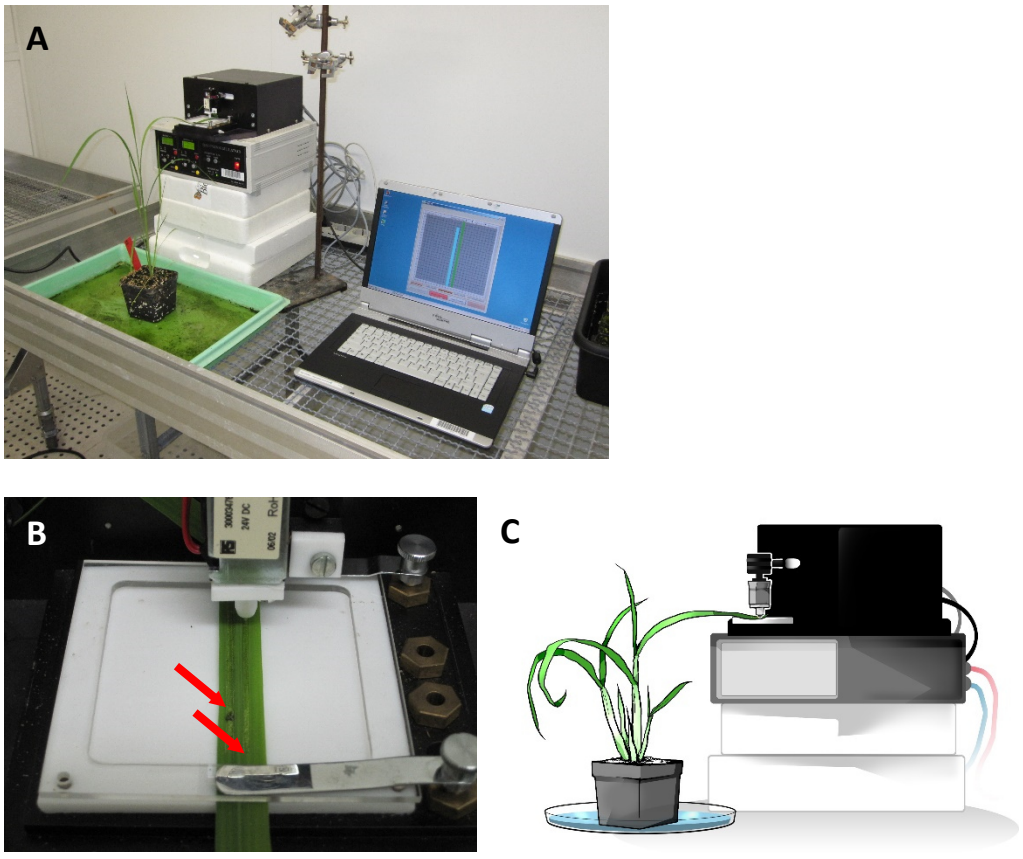


Figure 6: MecWorm, experimental setup
(A) Setup of regular MecWorm treatment in the phytochamber with 6 weeks old rice plant attached to the machine
(B) punching unit of MecWorm and damaged rice leaf, wounded areas are indicated by red arrows
(C) schematic picture of MecWorm with punching unit and rice plant (Picture designed by Michael Rühle)

The specifications of the wounding programmes used in these experiments are given in Table 1. Single punches of the MecWorm needle were applied at a frequency of 8.5 to 16 punches per minute to cover the predefined leaf area. Hence, depending on the programme, the interval between each punch was 2-5 seconds. For the 6 hours treatment, each of the programmed fields was treated twice to reach 6 hours of treatment, indicated by a "(2x)" in the respective column of Table 1.

As a control, the respective leaf of a non-treated plant of the same age, raised in the same phytochamber, was harvested and analysed.

Plant material was collected directly after the end of each MecWorm treatment and frozen in liquid nitrogen. The material was stored at −80 °C until further processing for gene expression analysis or hormone extraction.

Table 1: MecWorm treatment conditions

Treatment time	Duration of wounding treatment (hh:mm:ss)	Areas wounded	Pause time between single punches
0.5 hours	00:29:58	0.5 x 1.5 cm	4 s
		0.5 x 4.4 cm	2 s
1 hour	00:59:34	0,5 x 1.5 cm	5 s
		0.5 x 4.4 cm	5 s
6 hour	06:02:48	1.5 x 4.3 cm (2x)	5 s
		1.0 x 4.8 cm (2x)	5 s

2.3.1 Interaction of wounding treatments with the diurnal rhythm

Considering the hypothetical contribution of the day and night rhythm on hormone status and gene expression profiles, all experiments were conducted precisely according to a schedule and all repeats were performed in their designated time slots as shown in Table 2, page 18.

Experimental manipulation during the dark period was performed under conditions of photobiological darkness, i.e. all potential sources of stray light, including the LEDs of the MecWorm or the computer, were masked with dark tape, such that plants were only exposed to low-intensity green safelight for approximately 3 minutes during the harvest of plant material, which was immediately followed by shock-freezing in liquid nitrogen.

Table 2: Timing of MecWorm treatments according to one "phytochamber-day"

Green boxes indicate time intervals of the respective experiments. Material was harvested directly at the end of the respective time interval

Gene expression was analysed either at 10:30 a.m. (1 h), or at 4:30 p.m. (6 h)

Phytohormone content was analysed either at 10:00 a.m. (0.5 h), at 10:30 a.m. (1 h), or at 4:30 p.m. (6 h)

To probe for the potential influence of the diurnal rhythm, treatments of 1 h duration were conducted at the diurnal time indicated in the right column.

	Time	Gene	Phytohormones	Diurnal rhythm
night	00:00			
	00:30			
	01:00			1 h
	01:30			
	02:00			
	02:30			
	03:00			
	03:30			
	04:00			1 h
	04:30			
	05:00			
	05:30			
	06:00			
	06:30			
	07:00			1 h
	07:30			
	08:00			
dawn	08:30			
	09:00			
day	09:30		0,5 h	
	10:00	1 h	1 h	1 h
	10:30			
	11:00			
	11:30			
	12:00			
	12:30			
	13:00	6 h	6 h	1 h
	13:30			
	14:00			
	14:30			
	15:00			
	15:30			
	16:00			1 h
	16:30			
	17:00			
dusk	17:30			
	18:00			
night	18:30			
	19:00			1 h
	19:30			
	20:00			
	20:30			
	21:00			
	21:30			
	22:00			1 h
	22:30			
	23:00			
	23:30			

2.4 Gene expression analysis

2.4.1 RNA-extraction

Samples, frozen in liquid nitrogen, were homogenized into a powder (TissueLyser, Qiagen, Hilden, Germany). Subsequently RNA was extracted using the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany), a column-based extraction method, according to the manufacturer's instructions. In brief, homogenized plant material was lysed by adding a lysis buffer and vortexing it thoroughly. The cell debris was centrifuged down briefly and the supernatant was centrifuged on a column to remove genomic DNA. After addition of ethanol to the flowthrough of the column, this mixture was loaded onto another column for selective RNA-binding. After centrifugation and removal of the liquid flowthrough, contaminations by residual genomic DNA were removed by incubating the RNA on the column with RNAse-free DNAse (Qiagen, Hilden, Germany). After three subsequent washing-steps, RNA was eluted from the column using RNAse-free H_2O. Spectrophotometrical and electrophoretical quality control of RNA and cDNA-synthesis were performed directly after this step and RNA was afterwards stored at -20 °C.

2.4.2 cDNA synthesis

Only RNA with high quality and integrity, as verified via spectrophotometry and gel electrophoresis, was used for cDNA-synthesis with the DyNAmo cDNA-Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), using oligo(dT)-primers. For synthesis a fixed amount of 1µg RNA per sample was mixed with the kit components and reversely transcribed in a PCR according to the manufacturer's protocol. The cDNA samples were stored at -20 °C until further use.

2.4.3 qPCR

Preparation of SYBR Green I

10000x concentrated SYBR Green I (Invitrogen, Darmstadt, Germany) was diluted to 100x in molecular biology grade DMSO (Sigma-Aldrich, Taufkirchen, Germany) and stored at -80 °C. The working stock was further diluted to 2x in Tris 10mM, pH 8.0, molecular biology grade (AppliChem, Darmstadt, Germany), aliquoted and stored in the dark at -20 °C.

Setup and cycling conditions

The qPCR analysis was performed using the components and concentrations as shown exemplarily for a single reaction of 20 µl in Table 3 .

For each gene, a mastermix was prepared, containing all components excluding template cDNA. SYBR Green I was added as last component because it is light sensitive. Three technical replicates were analysed for each sample. For this purpose an aliquot of gene-mastermix was mixed with sample cDNA and distributed into three different wells of a 96-well plate.

Cycling conditions are specified in Table 4, qPCR was performed on a DNA Engine Opticon 2 System (Bio-Rad Laboratories Inc., Munich, Germany).

Table 3: qPCR single reaction

1x PCR setup	Component	Distributor
1 µl	cDNA template (1:10 diluted)	
4 µl	5x GoTaq buffer	Promega, Mannheim, Germany
0.4 µl	dNTPs (10mM)	NEB, Frankfurt/Main, Germany
0.1 µl	GoTaq polymerase	Promega, Mannheim, Germany
1 µl	$MgCl_2$ (50mM)	AppliChem, Darmstadt, Germany
0.8 µl	mixed forward and reverse primers (10 µM)	Sigma-Aldrich, Taufkirchen, Germany and Primerdesign Ltd., Southampton, UK
9.5 µl	2x SYBR Green I	see “Preparation of SYBR Green I”, page 19
3.2 µl	nuclease free H_2O	Qiagen, Hilden, Germany

Table 4: qPCR cycling conditions

Temperature	Time	
95°C	3 min	
95°C	15 s	40x
63°C	40 s	
Melting curve from 50.0 °C to 95.0 °C in 1.0°C increments		

Quality control of qPCR results and inter-run calibration

All primers were tested by running a standard dilution curve with 10-fold dilutions of pooled sample cDNA (Hellemans *et al.*, 2007). Determination of primer efficiency was carried out by calculating a linear regression of the cycle threshold (Ct)-values of these serial dilutions. Only primers with an efficiency between 90 and 120% and a correlation coefficient (r^2) of more than 0.99 were used in this study (D'Haene *et al.*, 2010).

For each primer on each plate, a no template control (NTC) was set up. In the NTC the sample cDNA was substituted with nuclease-free H_2O to monitor the potential formation of any PCR by-products. Positive signals in the NTC were classified as insignificant if the dissociation curve peak was at a different temperature as the sample product peak, or if the Ct value of the NTC-signal was at least 5 cycles later than the latest sample peak (e.g. sample peak at 25, NTC Peak at 32) (D'Haene *et al.*, 2010).

Moreover, an inter-run calibrator (IRC) was used to stabilize the data which were obtained in more than one run. IRC sample cDNA consisted of a mix of all used cDNAs and was prepared as a stock in sufficient quantity to cover the entire qPCR analysis. For each primer on each plate, an IRC sample was loaded for inter-run calibration (Hellemans *et al.*, 2007).

Samples with dissociation curve peaks deviating from the calculated product melting temperature, or samples with extremely variable Ct values within one technical repeat were excluded from the analysis.

qPCR data analysis

Analysis of the qPCR raw data was performed using the modified delta-delta Ct calculation method described in Hellemans *et al.* (Hellemans *et al.*, 2007). As recommended by different publications (Jain *et al.*, 2006; Vandesompele *et al.*, 2002), two housekeeping genes, Ubiquitin 5 (UBI5) and Elongation-factor 1α (EF1a), were used for normalisation.

For the details of the statistical analysis of the results please refer to paragraph 2.6 “Statistical Analysis” (page 23).

Primers

All primers used in this study are listed in Table 5.

Table 5: Primers used for qPCR

Gene name Accession	Primer Name	Sequence	Product size Product Tm Efficiency	source/citation
OsAOS1 Os03g0767000	OsAOS1.F	CGCCTCGGCATTGCA	95 bp 87 °C 104%	self-designed botany 1
	OsAOS1.R	AGCGTCGGGAACAGGATCT		
OsAOC Os03g0438100	AOCReTi.F3	TGCCTCAACAACTTCACCAACTA	142 bp 83 °C 93%	self-designed botany 1
	AOCReTi.R3	CACATGCCGCAATTAACACTAAA		
OsJAR1 Os05g0586200	OsJAR1.RT2.F	AGGAGGCATCAAAGTTCCTGG	109 bp 85 °C 116%	self-designed botany 1
	OsJAR1.RT2.R	CTCAGCTCCCAGAAGATCACG		
OsJAZ 5 (OsTIFY9) Os04g0395800	Os04g0395800	ACGATAAGGCAGAGGCTATAATG	123 bp 73.4 °C 119%	commercial (Primerdesign Ltd.)
		CGAGAGATTTAGTTCTTGTGAGTG		
OsJAZ 8 (OsTIFY10c) Os09g0439200	TIFY10c.F	GAAGGCTCAACAGCTGACCAT	69 bp 81°C 98%	Ye *et al.*, 2009
	TIFY10c.R	TTGGTGGACGGGAAGTTCTC		
Ef1a Os03g0177900	EFIα_ReTi3.F	GATGATTCCCACCAAGCCCA	134 bp 84.5 °C 105%	self-designed botany 1
	EFIα_ReTi3.R	CGGTTGGGTCCTTCTTCTCC		
UBQ5 Os01g0328400	UBQ5.ReTi.F	ACCACTTCGACCGCCACTACT	69 bp 86.5 °C 99%	Jain *et al.*, 2006
	UBQ5.ReTi.R	ACGCCTAAGCCTGCTGGTT		

2.5 Hormone analysis

Frozen sample material was ground roughly and fresh weight was measured very exactly using an analytical balance. Samples were stored at −80 °C until shipping on dry ice for hormone extraction and analysis. Extraction of hormones was conducted by the Max-Planck-Institute for Chemical Ecology (Department for Bioorganic Chemistry, MPI:CE, Jena, Germany) by a methanol-extraction method. Frozen plant material was homogenized into a powder and mixed with 1.5 ml methanol containing 60 ng of D_2-JA, D_4-SA, D_6-ABA and 12 ng of JA-$^{13}C_6$-Ile as internal standards. After shaking at 4°C for 30 minutes and centrifugation at 4°C and 13000 g for 20 minutes, the supernatant was separated from the sediment and

additional 0.5 ml of methanol (without internal standards) were added to the pellet. Subsequently, the incubation and centrifugation steps described above were repeated and the supernatant was added to the supernatant collected during the first extraction step. These combined extracts were dried in a vacuum centrifuge (Eppendorf Concentrator, Eppendorf, Hamburg, Germany) and resuspended with 500 µl methanol. After another centrifugation step at 4°C and 13000 g for 20 minutes, 400 µl of the supernatant were transferred to a 1.5 ml HPLC vial and kept at -20 °C until analysis.

Analysis of the extracts was conducted by the MPI:CE, Department for Biochemistry (Jena, Germany) using a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 µm, Agilent Technologies, Waldbronn, Germany) installed on an Agilent 1200 HPLC system (Agilent Technologies, Böblingen, Germany) coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) as described in more detail in Svyatyna *et al*. (Svyatyna *et al.*, 2013)

2.6 Statistical Analysis

Data obtained in this thesis were examined with two different statistical methods, Student's *t*-test and Tukey-Kramer test, with the same results. Confidence levels of Student's *t*-tests were depicted in the respective graphs, one asterisk indicating $p < 0.05$, two asterisks indicating $p < 0.01$. In all figures displayed in this thesis, error bars represent the standard error of the mean (SE), calculated with the formula: $SE_{\bar{x}} = \frac{s}{\sqrt{n}}$ (s= sample standard deviation, n=size of the sample).

3 Results

Research on the photomorphogenesis of rice has shown, that JA is a modulator of the light response in the seedling stage (Riemann *et al.*, 2003). Additionally, a multitude of studies have reported, that light conditions can influence a variety of stress induced JA-responses (Ballare, 2014; Kazan & Manners, 2011). Thus, a reciprocal interaction of light signalling and the JA-pathway has been suggested.

As rice is an economically important plant, and mutants impaired in the biosynthesis of JA and light perception are available, it is an ideal system to study interactions of both pathways.

During the development of rice seedlings, an interaction of light and JA exists, but this interaction could be limited to photomorphogenic development of young seedlings. In the study that reported this interaction, not only an induction of JA by light but also by wounding was shown (Riemann *et al.*, 2003). Thus, when two diverse signals apparently activate the same hormonal response pathway, the question arises, if one of those signals has an impact on the hormonal response of the other signal.

In this study we therefore wanted to address, to what extent JA is involved in the response to the attack of herbivores, and how light influences the wounding response of rice plants. Herbivore attack is usually a very severe event for plants and JA is known to control different responses during wound signalling. In general there are two aspects of an herbivore attack on a plant: (i) the plant is challenged by mechanical wounding when the herbivore is chewing the tissue, and (ii) chemicals targeted to attenuate defence mechanisms of the plant are released by the herbivore. To focus on one aspect of an herbivore attack, we chose to subject rice plants to mechanical wounding using the MecWorm system, which is highly reproducible and not subjected to natural variations like the feeding behaviour of caterpillars.

By comparing the gene expression profiles and hormone levels of mutant and wild type plants in response to wounding stress, we were able to gain valuable new insights on the mutual influence of light and JA in the stress response of rice.

3.1 JA-deficiency leads to a suppressed wounding response in rice plants

We treated rice plants in the vegetative state with MecWorm for different periods and analysed the expression of JA-responsive genes and the production of several plant hormones in these plants. A comparison of the regulation patterns of wild type and JA-deficient mutants should give information about the role of JA in the stress response and the consequence of the loss of this hormone.

3.1.1 The expression of JA-responsive genes is not induced by mechanical stress in JA-deficient mutants

To elucidate the different responses of wild type and JA-deficient mutants in the stress signal transduction, the expression of representative JA-response genes, two biosynthesis (*OsAOC* and *OsJAR1*) and two signalling genes (*OsJAZ5* and *OsJAZ8*), was examined. Therefore, 6 weeks old rice plants were continuously wounded for 1 hour or 6 hours with MecWorm. Treated leaves and control leaves from unwounded plants were harvested, RNA was extracted and gene expression was analysed via RT-qPCR. The results of the qPCR analysis are depicted in Figure 7 for the biosynthesis genes and in Figure 8 for the signalling genes.

In the wild type Nihonmasari, a relative expression change in the biosynthesis genes *OsAOC* and *OsJAR1* could be observed in samples treated with MecWorm for 1 hour.

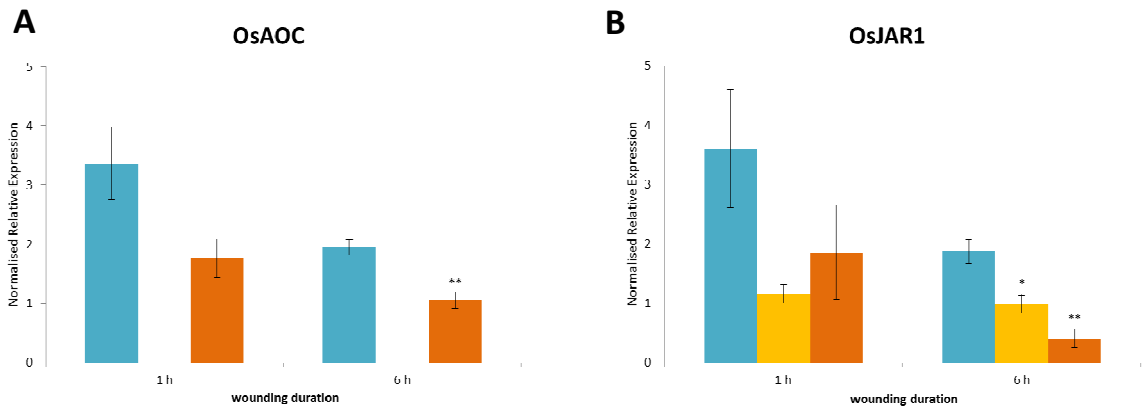


Figure 7: Transcript levels of the JA-biosynthesis genes* OsJAR1 *(A) and* OsAOC *(B), in wild type and JA-deficient mutant plants in response to wounding.
Plants of wild type (blue bar), *hebiba* (yellow bar) and *cpm2* (orange bar) were wounded with MecWorm 6 weeks after germination for the indicated time intervals. qPCR analysis was carried out using two standard genes, *eEF-1α* and *OsUBI5*, for normalization. The fold change induction for each gene was calculated relative to a corresponding untreated control plant. Data represent the average of three independent experiments, error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p>0.01$), respectively.

The expression of *OsAOC* as well as *OsJAR1* increased around 3-fold relative to the non-treated control leaf (Figure 7A, B). After 6 hours of continuous wounding both genes were induced around 2-fold in comparison to the control. However, due to the variation in the measured values at that time point, the observed induction is statistically not significant.

In both JA-deficient mutants tested, continuous wounding did not cause any increase in the JA-biosynthesis genes. As expected, expression of *OsAOC* was not detectable at all in *hebiba* (Figure 7A), as this gene is absent due to a deletion (see chapter 1.2.1, page 4). Because the *OsAOC* gene only has a deletion of 11 bp in the *cpm2* mutant, leading to a frameshift, the gene is still expressed in *cpm2*, but the gene product will not be functional. Nevertheless, an expression of *OsAOC* was detectable in *cpm2*, but the relative induction compared to the control plant was not significant (Figure 7A).

A similar observation could be made for both JA-deficient mutants with respect to *OsJAR1* expression. *OsJAR1* was expressed in all *hebiba* and *cpm2* samples, but the relative induction level was below 2-fold for all MecWorm treatments (Figure 7B). Thus the gene expression of *OsJAR1* was not induced by continuous wounding in those mutants.

Summarizing, both JA-biosynthesis genes were slightly induced after 1 hour of continuous wounding in the wild type, while the mutant plants showed no induction or, in the case of *OsAOC* in *hebiba*, no expression at all. Nevertheless, as the relative change in expression was not very pronounced in the wild type in the first place and the standard errors are high, no statistically significant difference between wild type and mutants could be found.

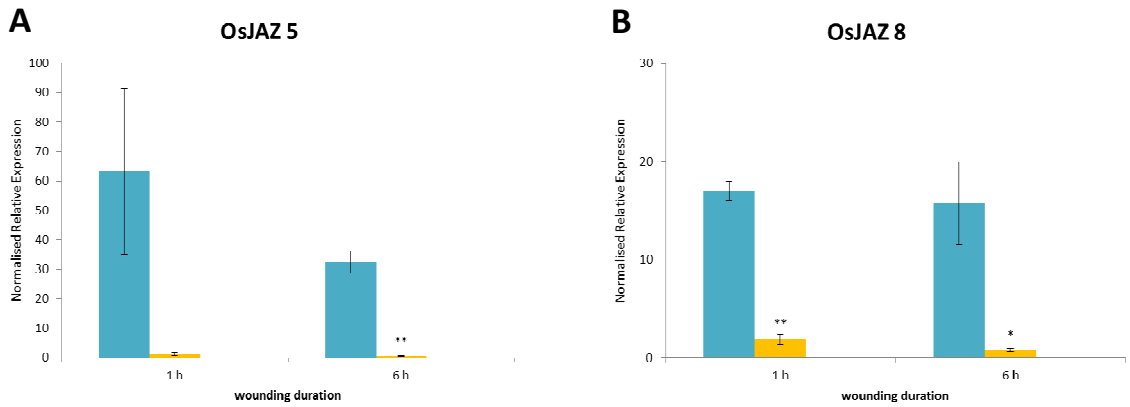


Figure 8: Transcript levels of the JA-signalling genes* OsJAZ5 *(A) and* OsJAZ8 *(B), in wild type and JA-deficient mutant plants in response to wounding.
Plants of wild type (blue bar), *hebiba* (yellow bar) and *cpm2* (not detected) were wounded with MecWorm 6 weeks after germination for the indicated time intervals. qPCR analysis was carried out using two standard genes, *eEF-1α* and *OsUBI5*, for normalization. The fold change induction for each gene was calculated relative to a corresponding untreated control plant. Data represent the average of three independent experiments, error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's t-test, $p < 0.05$ or $p < 0.01$), respectively.

In parallel to the JA-biosynthesis genes, we examined the JA-signalling genes *OsJAZ5* and *OsJAZ8* to elucidate the effect of the MecWorm treatment on the JA-signal response pathway.

In the wild type, both tested JAZ genes were strongly induced by wounding with MecWorm. Already one hour of continuous wounding caused an expression change of around 60-fold in *OsJAZ5* (Figure 8A) and 16-fold in *OsJAZ8* (Figure 8B). After 6 hours MecWorm treatment, the induction factor of *OsJAZ5* decreased by 50% to 30-fold (Figure 8A), while the induction level of *OsJAZ8* remained at 16-fold (Figure 8B).

In contrast to the high gene-expression levels in the treated wild type samples, *OsJAZ5* and *OsJAZ8* were not induced relative to the control plants in the JA-biosynthesis mutants. In *hebiba*, both representative JAZ genes were expressed, but the calculated expression change relative to the control was below 2.0 in all the samples tested (Figure 8). In *cpm2*, no expression of either JAZ gene could be detected at all, therefore in Figure 8 no bars are displayed for this mutant.

These results meet the expectations based on former studies in the JA-deficient mutants. JA and its bioactive form JA-Ile (see 3.1.2, page 28) are required to induce the expression of genes involved in the JA biosynthesis and signalling pathways in response to mechanical stress.

3.1.2 Mechanical wounding fails to induce JA-accumulation in *hebiba* and *cpm2*

To examine the ability of MecWorm to induce a stress response in wild type plants and to elucidate how the lack of JA in *cpm2* and *hebiba* influences the hormone levels in these mutants, we measured the amount of different phytohormones in rice plants exposed to mechanical stress. Therefore, 6 weeks after germination rice plants were continuously wounded for 30 minutes, 1 hour and 6 hours with MecWorm and the hormone content in treated and control leaves was measured via LC-MSMS.

In the wild type, 30 minutes of continuous wounding were sufficient to induce the production of JA and JA-Ile on a high level (Figure 9A and B). After 1 hour of MecWorm treatment, the amount of JA and JA-Ile was lower than after 30 minutes wounding, but this difference is not statistically significant and might rather be due to natural variation. Six hours of MecWorm treatment also induced the production of JA at a similar level as the shorter treatments (Figure 9A). Thus, independent of the duration time of the mechanical stress event the amount of JA production was always on a similar level in the wild type.

The amount of JA-Ile, in contrast, was significantly elevated after 6 hours of continuous wounding in the wild type, compared to shorter MecWorm treatments. While after 0.5 and 1 hours of wounding the JA-Ile-level was about 20-25 ng/gFW, the Nihonmasari plants accumulated around double the amount of JA-Ile after 6 hours of MecWorm treatment (Figure 9B).

In *cpm2* as well as *hebiba* there was no JA and consequently no JA-Ile produced, neither in the controls nor in the wounded plants (Figure 9A, B).

In summary, JA and JA-Ile were rapidly induced by mechanical wounding in the wild type, while the JA-deficient mutants only contained trace amounts of these hormones. The absence of bioactive JA-Ile in the mutant plants eventually affected the expression of the JA-response related genes. While in the wild type an induction of biosynthesis and signalling genes in response to continuous wounding could be observed, no such effect was noticed in *hebiba* and *cpm2*.

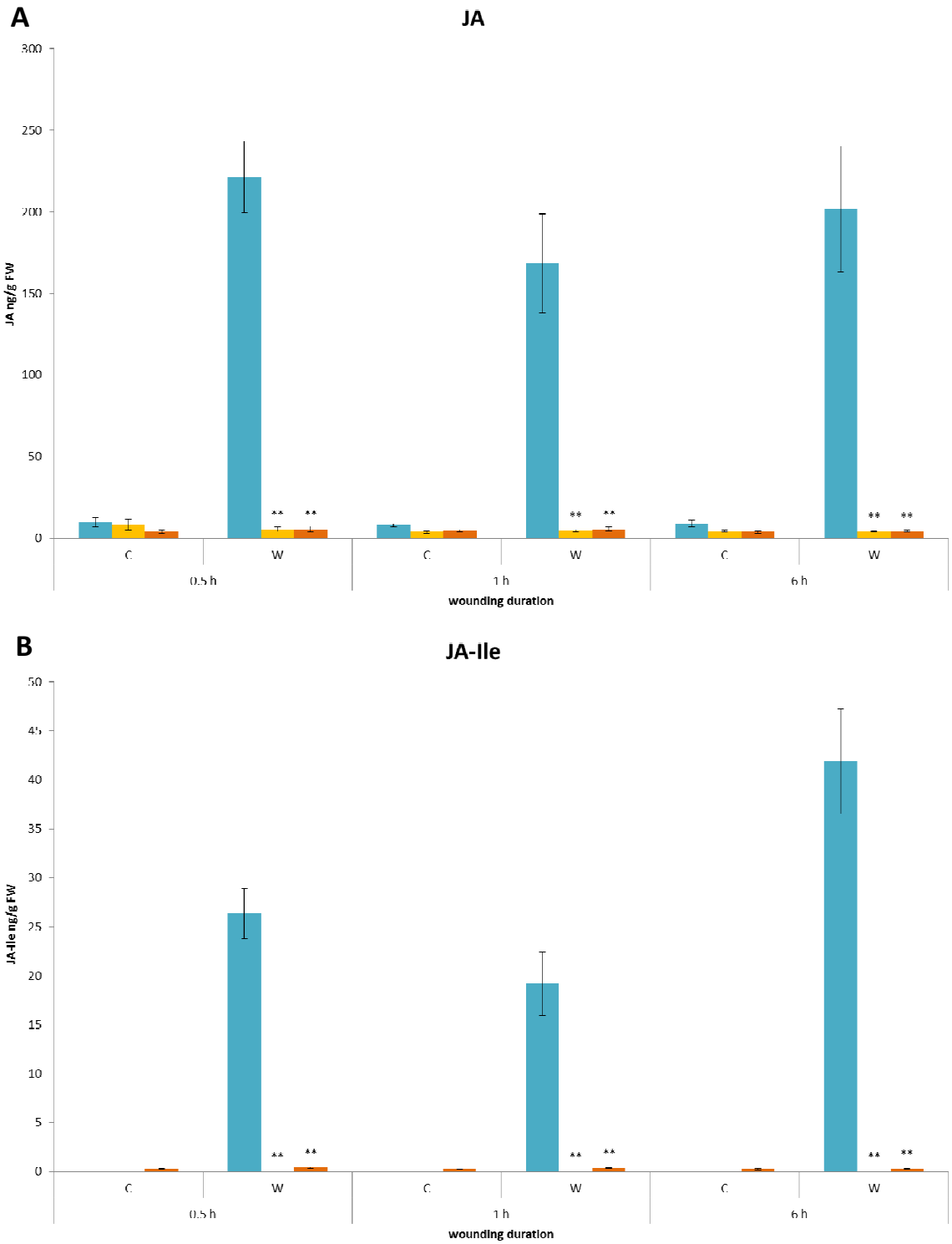


Figure 9: JA (A) and JA-Ile (B) levels of rice plants in response to wounding. Comparison of hebiba (yellow bar) and cpm2 (orange bar) to the wild type (blue bar)

Six weeks old rice plants were wounded with MecWorm for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of four independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p < 0.01$), respectively.

3.2 Partial loss of phytochrome function leads to an imbalance in hormone and gene regulation in response to wounding

After successfully defining conditions which induce JA-biosynthesis reproducibly, we were able to ask the question, whether light might have an impact on JA-dependent responses and thus affects the wounding response in rice. Therefore we examined the reaction of two different phytochrome double mutants, *phyAphyC* and *phyBphyC*, to mechanical stress applied by MecWorm. As a reference we included another JA-deficient mutant, *kamakubi*, with the same genetic background as the phytochrome mutants, into this study. This mutant is deleted in the gene encoding for OsAOS1.

3.2.1 Mutants lacking different photoreceptors accumulate more Jasmonates after wounding

Initially, the regulation of JA and JA-Ile in the photoreceptor mutants *phyAphyC* and *phyBphyC* in response to mechanical stress was analysed. Therefore rice plants were continuously wounded 6 weeks after germination for different time intervals (0.5 hours, 1 hour and 6 hours) with MecWorm and the content of JA and JA-Ile in treated and control leaves was measured via LC-MSMS.

Considerable induction of JA could be observed in the wild type as well as in both *phy*-mutants after 30 minutes of continuous wounding (Figure 10A). After 1 hour of MecWorm treatment, the amount of JA was induced 4- to 11-fold relative to the respective control, depending on the genotype. These levels were similar to those measured after 0.5 hours of continuous wounding and the differences in the average JA-amounts are statistically not significant. Thus, in general, 0.5 and 1 hour of continuous wounding caused the same reaction in the wild type and the *phy*-mutants.

Similarly, 6 hours of MecWorm treatment induced an increase in JA in comparison to the control leaves in the wild type as well as the *phy*-mutants. However, in Nipponbare the amount of JA was significantly lower by approximately 30% compared to the 0.5 and 1 hour treatments (Figure 10A) while in *phyAphyC* and *phyBphyC* JA-levels were equally high as those measured for the shorter wounding periods (Figure 10A). While the result for *phyAphyC* is statistically not significant due to a high standard error, *phyBphyC* produced almost 2-fold as much JA as the wild type after 6 hours of continuous wounding.

Like observed in *hebiba* and *cpm2*, in the JA-deficient *kamakubi* no induction of JA or JA-Ile was noticeable after any of the applied wounding treatments (Figure 10A, B). Hence, a second AOS of rice, OsAOS2, cannot complement the function of OsAOS1 in mechanical wounding.

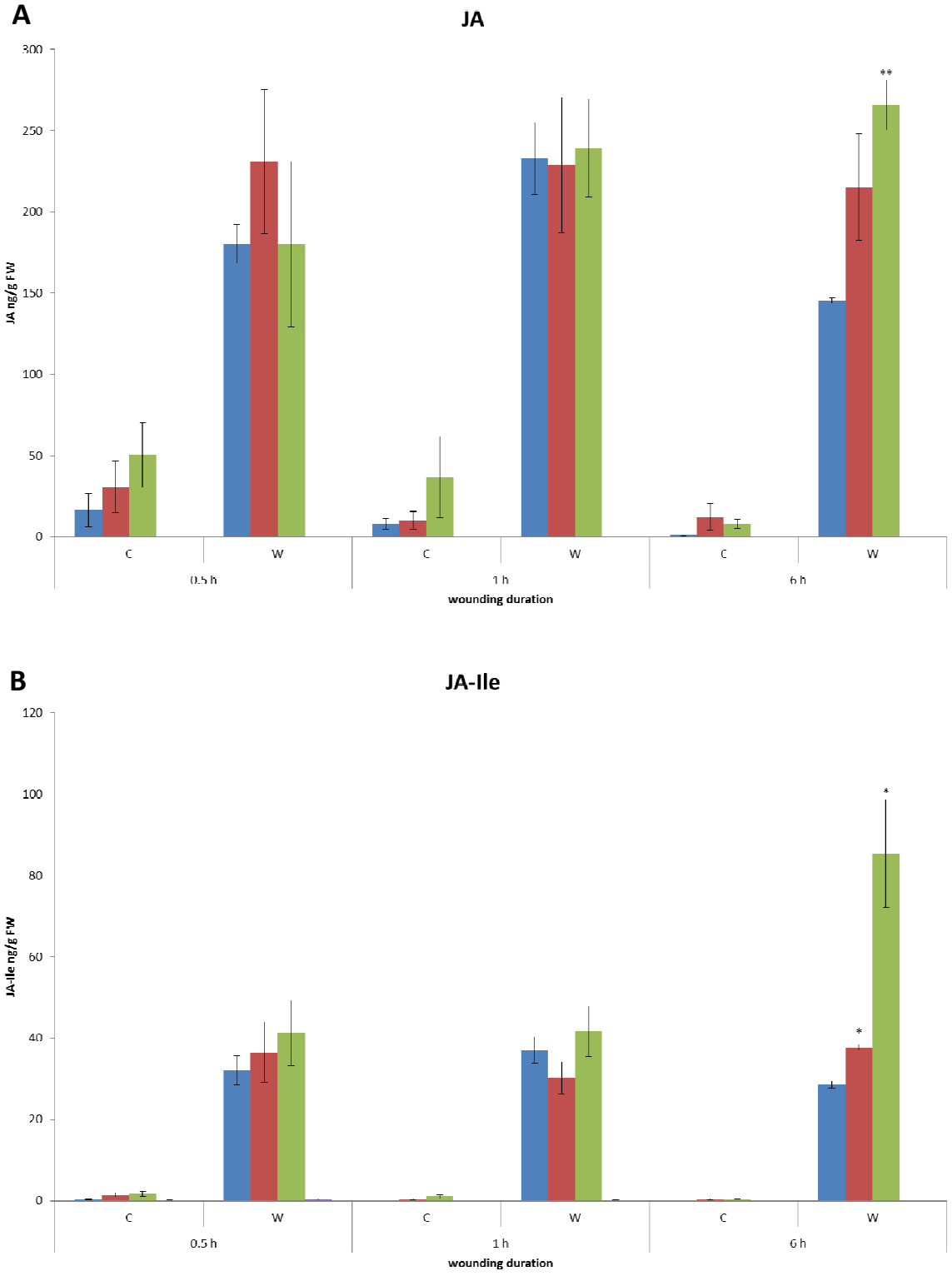


Figure 10: JA (A) and JA-Ile (B) levels of rice plants in response to wounding. Comparison of the wild type (blue bar) to* phyAphyC *(red bar),* phyBphyC *(green bar) and* kamakubi *(violet bar).
Six weeks old rice plants were wounded with MecWorm for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p < 0.01$ respectively).

The pattern of JA-Ile accumulation over time resembles that of the JA measurement. After 0.5 and 1 hour of MecWorm treatment the production of JA-Ile was induced with no significant differences between wild type, *phyAphyC* and *phyBphyC* (Figure 10B). Interestingly, after 6 hours of wounding, no decrease in the amount of JA-Ile was observed in the wild type compared to the shorter treatments, even though JA itself had declined by 30% (Figure 10A). Moreover *phyBphyC* produced almost 3-fold more JA-Ile than the wild type after 6 hours of continuous wounding and also the JA-Ile level of *phyAphyC* was significantly elevated compared to Nipponbare (Figure 10B).

Thus, the *phy*-mutants accumulated more JA and JA-Ile than the wild type after 6 hours of MecWorm treatment. Intriguingly, in *phyBphyC*, the amount of JA-Ile was twice as high after 6 hours of wounding as after the shorter treatments, while the amount of produced JA did not rise significantly with increasing wounding duration (Figure 10A, B). Thus, in *phyBphyC* after 6 hours of continuous wounding, more JA-Ile was produced from the available amount of JA than after 0.5 or 1 hour of treatment.

3.3 The JA-responsive gene expression is reduced in phyAphyC and phyBphyC in response to mechanical wounding

Subsequently, the expression of the same JA-inducible genes already tested in Nihonmasari, *hebiba* and *cpm2* (3.1.1, page 25) was examined in *phyAphyC*, *phyBphyC* and *kamakubi* and their corresponding wild type Nipponbare. Therefore rice plants were wounded with MecWorm 6 weeks after germination for different periods (0.5, 1 hour and 6 hours) and the gene expression analysis of this material was conducted by RT-qPCR.

The qPCR-analysis of the biosynthesis genes showed, that in the wild type, 1 hour of continuous wounding did not change the expression of *OsAOC* and *OsJAR1* compared to a non-treated control leaf (Figure 11A, B). After 6 hours of MecWorm treatment, however, *OsAOC* was significantly induced (3-fold, Figure 11A), while the calculated relative expression of *OsJAR1* was approximately 2-fold (Figure 11B) and due to the high standard error statistically not significant. Thus, the expression of both biosynthesis genes was stimulated only marginally by wounding stress, which is consistent with the observations made in the wild type Nihonmasari (Figure 7A, B).

In *phyAphyC* and *phyBphyC*, similar to the wild type, 1 hour of continuous wounding did not cause any significant expression change in comparison to the control, all calculated expression changes in *OsAOC* and *OsJAR1* were below 2-fold (Figure 11A, B). Likewise, 6 hours of MecWorm treatment did not significantly induce neither *OsAOC* nor *OsJAR1* (Figure 11B).

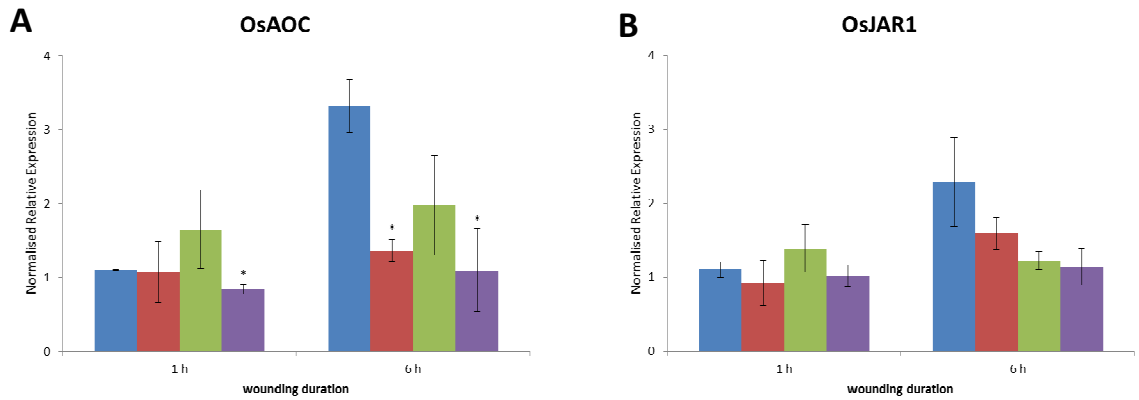


Figure 11: Transcript levels of the JA-biosynthesis genes* OsAOC *(A) and* OsJAR1 *(B), in wild type and* phy- *or JA-deficient mutant plants in response to wounding.
Plants of wild type (blue bar), *phyAphyC* (red bar), *phyBphyC* (green bar) and *kamakubi* (violet bar) were wounded with MecWorm 6 weeks after germination for the indicated time intervals. qPCR analysis was carried out using two standard genes, *eEF-1α* and *OsUBI5*, for normalization. The fold change induction for each gene was calculated relative to a corresponding untreated control plant. Data represent the average of three independent experiments, error bars indicate ±SE. Statistically significant differences to the wild type are indicated by an asterisk (Student's *t*-test, $p < 0.05$).

The JA-deficient mutant *kamakubi* did not show any response to the wounding treatment in both examined genes, all the relative expression change values ranged around 1.0 (Figure 11A, B)

In summary, a statistically significant difference was only observed in the response of the *OsAOC* gene after 6 hours of continuous wounding between the 3-fold induced wild type and the mutants *phyAphyC* and *kamakubi*, which did not show increased expression levels. In the *phyBphyC* mutant the induction of *OsAOC* in response to the MecWorm treatment was also not as high as in the wild type, but this difference could not be verified by the statistical analysis. The expression of *OsJAR1* was clearly not altered by the MecWorm treatment and was similar in all tested mutants and the wild type.

The JA-signalling genes *OsJAZ5* and *OsJAZ8*, like already observed in the wild type Nihonmasari (Figure 8A, B), were more strongly induced in response to mechanical wounding than the biosynthesis genes. In Nipponbare, after 1 hour of MecWorm treatment, the fold expression change compared to the control leaf was 18 for *OsJAZ5* and 2 for *OsJAZ8* (Figure 12A, B). These values increased extremely after 6 hours of continuous wounding, as then the induction of *OsJAZ5* was almost 300-fold (Figure 12A) and of *OsJAZ8* 25-fold (Figure 12B) relative to a non-treated control.

Similarly, the expression of those genes was also elevated by the MecWorm treatments in the *phy*-mutants. After 1 hour of continuous wounding, *OsJAZ5* was induced 5-fold in *phyAphyC* and 55-fold in *phyBphyC* (Figure 12A) compared to the respective control leaves.

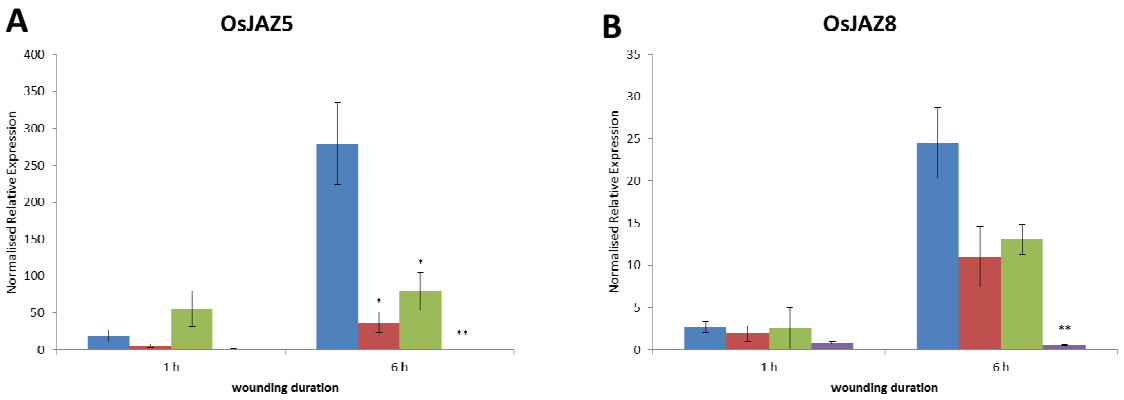


Figure 12: Transcript levels of the JA-signalling genes* OsJAZ5 *(A) and* OsJAZ8 *(B), in wild type and phy- or JA-deficient mutant plants in response to wounding.
Plants of wild type (blue bar), *phyAphyC* (red bar), *phyBphyC* (green bar) and *kamakubi* (violet bar) were wounded with MecWorm 6 weeks after germination for the indicated time intervals. qPCR analysis was carried out using two standard genes, *eEF-1α* and *OsUBI5*, for normalization. The fold change induction for each gene was calculated relative to a corresponding untreated control plant. Data represent the average of three independent experiments, error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p < 0.01$ respectively).

The expression of *OsJAZ8* was not clearly altered after 1 hour of wounding relative to the non-treated control (Figure 12B). However, after 6 hours of MecWorm treatment, *OsJAZ5* was induced strongly to about 40-fold in *phyAphyC* and even 80-fold in *phyBphyC* compared to the control (Figure 12A). Nevertheless, these expression changes are significantly smaller in the *phy*-mutants than in the wild type, *phyAphyC* only showing 14% and *phyBphyC* 28% of the induction level of Nipponbare. The gene expression of *OsJAZ8* was also increased about 10- to 13-fold in 6h MecWorm treated *phyAphyC* and *phyBphyC* (Figure 12B). Thus, a similar tendency as for *OsJAZ5* could be observed for *OsJAZ8*, the relative expression values of the *phy*-mutants were half as high as in the wild type, but this observation could not be confirmed by statistical methods.

The JA-deficient mutant *kamakubi,* did not respond to the MecWorm treatments by expression changes in *OsJAZ5* nor *OsJAZ8.* The expression of neither of both genes was induced under the tested conditions (Figure 12A, B).

In summary, in the wild type Nipponbare, as already observed in the wild type Nihonmasari, continuous wounding induced the JA-biosynthesis genes only slightly and the JA-signalling genes very strongly, especially after 6 hours of MecWorm treatment.

The photoreceptor mutants *phyAphyC* and *phyBphyC* also responded to mechanical stress with an enhanced gene expression, but less strongly in comparison to the wild type. The pattern of jasmonate accumulation and JA-dependent gene expression in *phyAphyC* and *phyBphyC,* however, was surprising: While both *phy*-mutants accumulated more JA-Ile than the wild type, the expression of the JA-responsive genes was reduced in comparison to the wild type. Hence, although more active signalling compound was present, the response to the signal was attenuated in these mutants.

The JA-deficient *kamakubi* was completely "silent" in its reaction to the wounding treatments, with respect to the tested JA-responsive genes. The comparison of the wild type Nipponbare with the JA-deficient mutant *kamakubi* gave similar results to those already obtained from the analysis of *hebiba* and *cpm2*. Therefore, a functional OsAOS1 enzyme is required to induce the JA-response after wounding.

3.4 Jasmonates are needed for wound-induced abscisic acid accumulation

As the different plant hormone pathways are linked on the level of both biosynthesis and signalling (see chapter 1.3.2, page 9), the following question emerged: Do the changed JA-levels, which could be observed in the different mutants, have an impact on other plant hormones?

Therefore, not only JA and its derivatives, but also other important hormones like salicylic acid or abscisic acid (ABA) were measured in MecWorm treated rice leaves. The analysis revealed that ABA is induced by mechanical wounding in the wild type (Figure 13). After 6 hours of continuous wounding, the amount of ABA was induced more that 2-fold in comparison to the control.

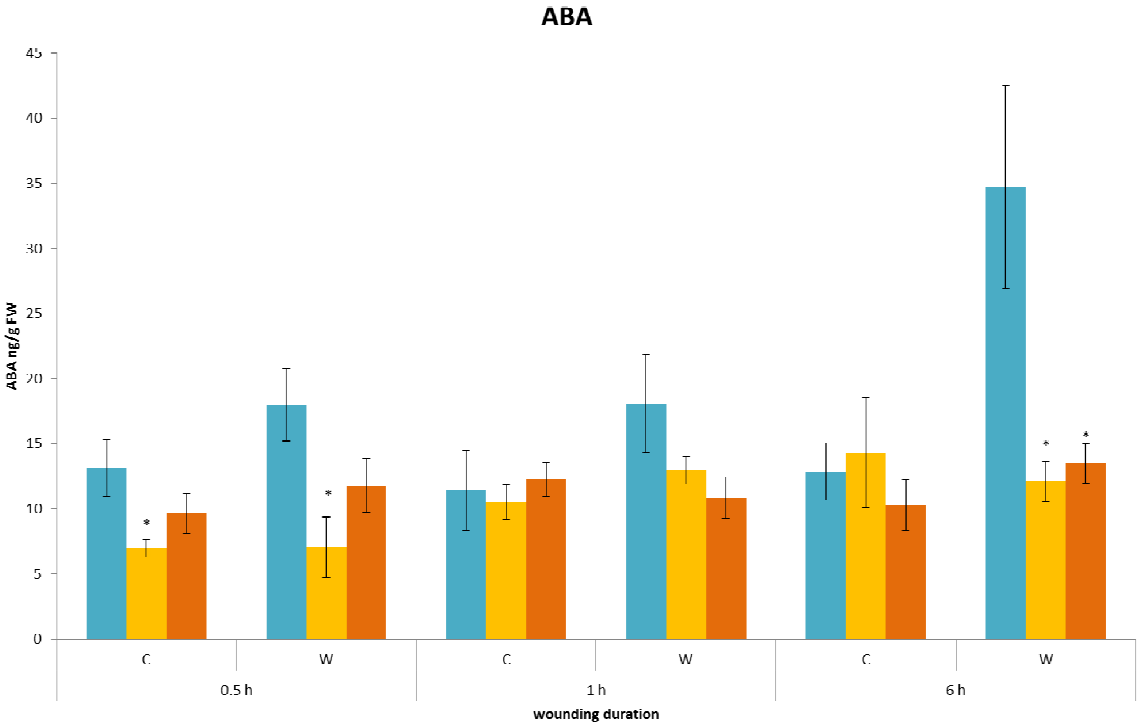


Figure 13: ABA-levels of rice plants in response to wounding. Comparison of JA-deficient mutants* hebiba *(yellow bar) and* cpm2 *(orange bar) to the wild type (blue bar).
Six weeks old rice plants were wounded with MecWorm for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by an asterisk (Student's *t*-test, $p < 0.05$).

In contrast, in both JA-deficient mutants, *hebiba* and *cpm2*, an induction of ABA by wounding could not be observed. Independent of the duration of the MecWorm treatment, the content of ABA was comparatively stable in all tested leaves relative to the respective control (Figure 13). Thus, the accumulation of ABA in response to mechanical wounding is dependent of JA.

Subsequently we measured the wound induced ABA levels in the photoreceptor mutants *phyAphyC* and *phyBphyC*, the JA-deficient mutant *kamakubi* and their corresponding wild type. In these genotypes, the induction of ABA by wounding was distinct and therefore, for a better visualisation of this time depended change in ABA content, Figure 14 shows the single genotypes, while the comparison of the mutants and the wild type is shown in Figure 15.

In the wild type, leaves treated for 1 and 6 hours by continuous wounding showed a higher ABA content in comparison to the control leaves (Figure 14A). Moreover, the total amount of ABA in wounded leaves increased with the duration time of the treatment, peaking in a 3-fold induction after 6 hours of continuous wounding.

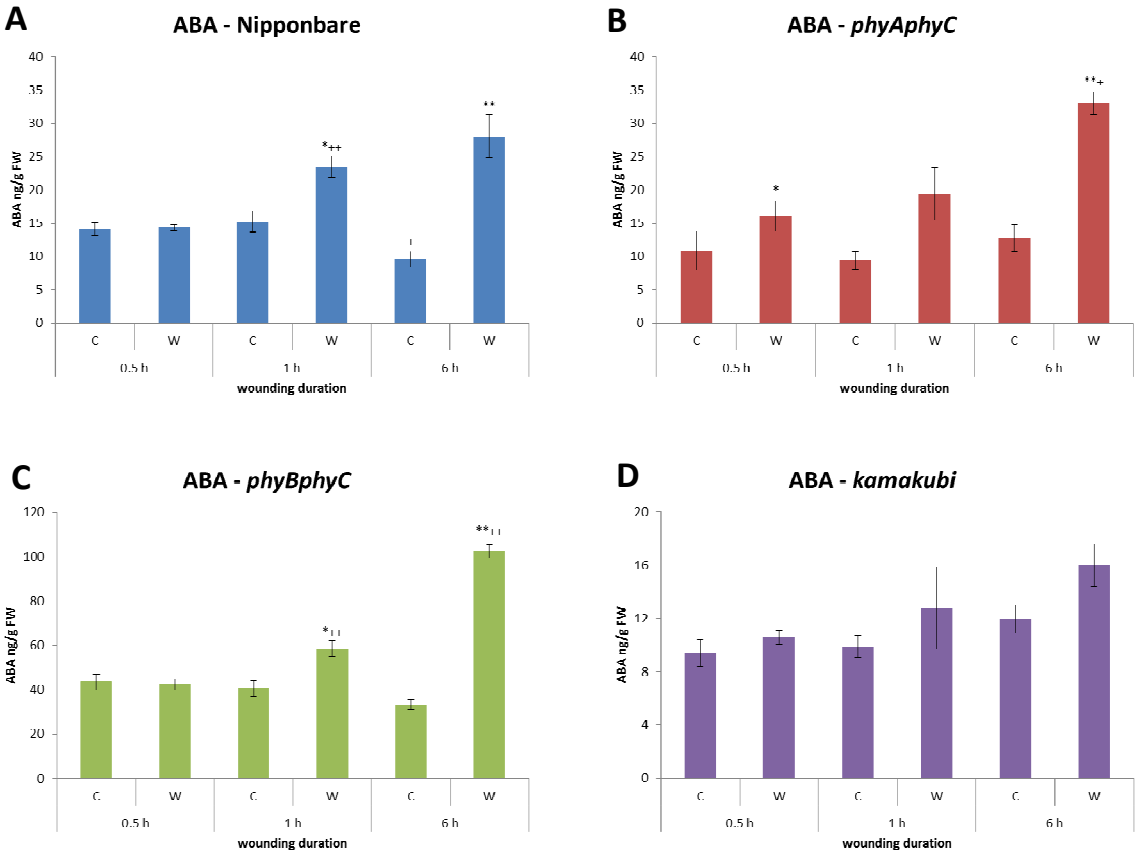


Figure 14: ABA-levels of rice plants in response to wounding.
Wild type Nipponbare (A), *phyAphyC* (B) *phyBphyC* (C) and *kamakubi* (D) were wounded with MecWorm 6 weeks after germination for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the respective control are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p < 0.01$ respectively). Statistically significant differences to the preceding time point are indicated by one or two crosses (Student's *t*-test, $p < 0.05$ or $p < 0.01$ respectively).

The *phy*-mutants showed a similar time dependent ABA accumulation to the one observed in the wild type. In *phyAphyC*, after 0.5 hours of continuous wounding a significantly induced ABA production could be observed (Figure 14B). After 6 hours of MecWorm treatment the ABA-level in *phyAphyC* was elevated to almost 3-fold in comparison to the control (Figure 14B). Like already noticed in the wild type, in *phyAphyC* the highest amount of ABA was measured in 6 hours treated leaves.

A similar observation was made for *phyBphyC*, although the increase of ABA due to wounding was a bit delayed and could only be noticed after 1 hour of MecWorm treatment (Figure 14C). Like in the wild type and the *phyAphyC* mutant, the content of ABA increased further with longer duration of the wounding. At 6 hours of MecWorm treatment, the ABA level was 3-fold higher relative to the respective control leaves (Figure 14C).

In summary, in the wild type as well as both *phy*-mutants, ABA was inducible by mechanical stress, the ABA level rising with increasing duration of the treatment. At 6 hours continuous wounding the amount of ABA was about three times higher than in the respective control leaves.

The *kamakubi* mutant, in contrast, did not show any significant ABA induction in response to wounding. Similar to *hebiba* and *cpm2*, the ABA-levels were quite stable and not significantly

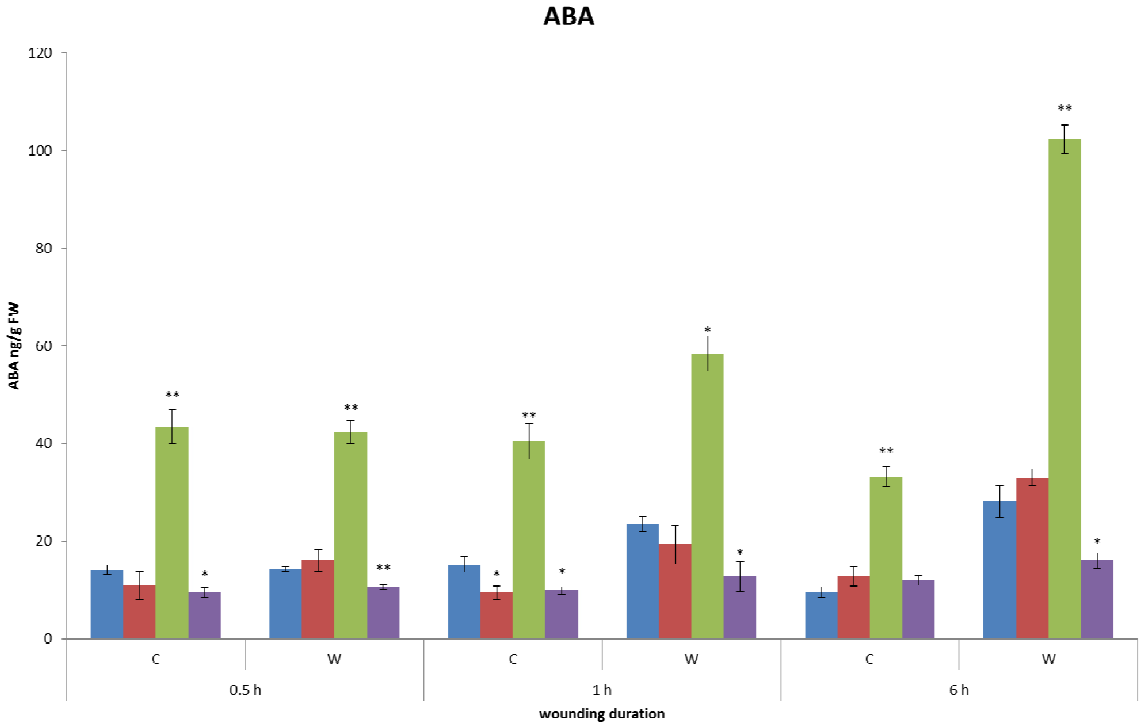


Figure 15: ABA-levels of rice plants in response to wounding. Comparison of phyAphyC (red bar), phyBphyC (green bar) and kamakubi (violet bar) to the wild type (blue bar)
Six weeks old rice plants were wounded with MecWorm for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's *t*-test, $p < 0.05$ or $p < 0.01$ respectively).

modified by the MecWorm treatment (Figure 14D). Moreover, the amount of ABA was generally lower in this mutant when compared to the wild type (Figure 15).

Furthermore, the comparison of all three mutants with the wild type (Figure 15) revealed that not only *kamakubi* produced a significantly lower amount of ABA relative to the wild type, but also that *phyBphyC* accumulated significantly higher ABA-levels. In the control plants of *phyBphyC* the average content of ABA was approximately three times higher than in the wild type controls (Figure 15). Despite this elevated basal level, the production of ABA could still be induced 3-fold by the MecWorm treatment.

Summarizing, the JA-deficient mutants did not increase their ABA-levels in response to mechanical stress, while the wild type as well as the *phy*-mutants accumulated more ABA the longer the wounding treatment was performed. Thus, the wound induced ABA accumulation is dependent on a functional biosynthesis of JA.

Another interesting observation is the high basal level of ABA caused by the lack of the phytochrome B, which implies, that this phytochrome is important for the correct phytohormone homeostasis of ABA.

3.5 Effect of the daytime on the hormone status and gene expression in rice plants

In the previous chapters we described the effect, that 6 hours of continuous wounding generally caused a stronger JA-response than shorter wounding periods. Therefore we hypothesized, that the intensity of the response of rice plants to wounding depends on the treatment length. However, the differences may also be caused by the experimental setup. As described in the methods section, the rice plants used for the experiments were raised under short day conditions in a phytochamber, therefore the changing light conditions have to be taken into account. The observed effect of a stronger JA-response after 6 hours of wounding might be due to the fact that the 6 hour treatments always ended shortly before the onset of dusk. Thus, the question arises, if the responsivity to mechanical wounding is dependent on daytime and light conditions or even subordinated to the plants circadian rhythm. To address this question, we decided to investigate the hormone induction caused by shorter wounding treatments at different times of the day. Therefore, we treated rice plants with MecWorm for 1 hour at different time points during a 24 h period in the same short day conditions used for the previous measurements and determined the phytohormone levels.

We compared the wounding response of one of the phy-mutants, *phyAphyC*, with the wild type, to examine if phytochrome deficiency affects the JA-response differently at different times of the day. Moreover, to detect any diurnal oscillations in JA-levels, we also looked into unwounded control samples of rice plants, harvested at different time points during the day.

3.5.1 The response intensity to short period wounding in rice is not dependent on the daytime

To examine the regulation of different plant hormones during one 24h day, rice plants were wounded for 1 hour with MecWorm at pre-set time points every four hours (see chapter 2.3.1, page 17). The hormone levels of the collected samples were analysed via LC-MSMS.

JA – potentially subordinated to diurnal oscillations

We analysed the amount of JA in 1 hour continuously wounded, as well as non-treated control samples, an overview over the results of this experiment is depicted in Figure 16A. As already observed in all the other MecWorm experiments, the 1 hour treatment with MecWorm was sufficient to induce a high accumulation of JA in the wild type as well as in *phyAphyC* (Figure 16A).

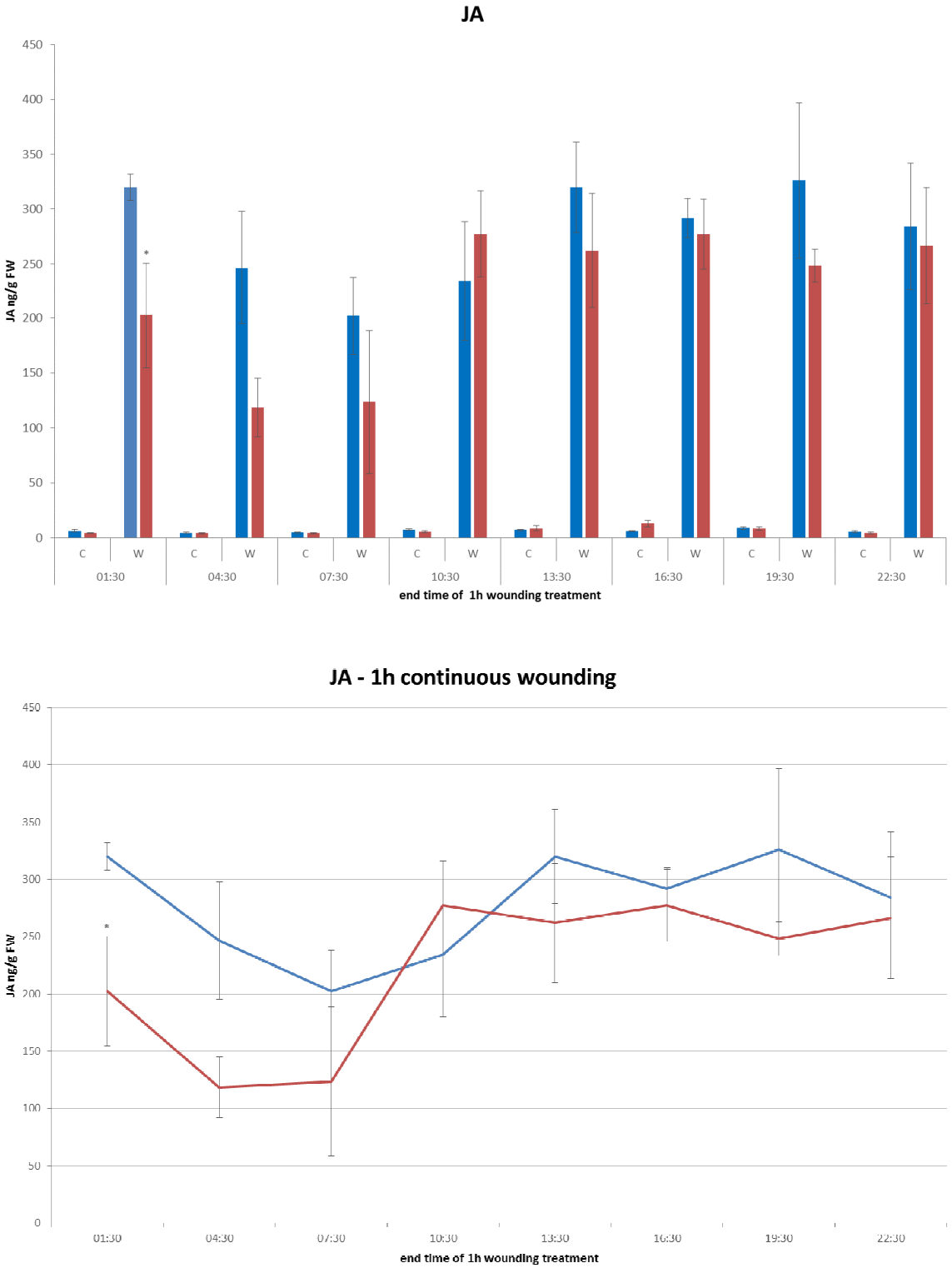


Figure 16: JA levels of rice plants in response to wounding. Comparison of* phyAphyC *(red bar), to the wild type (blue bar) showing treated samples opposed to control leaves (A). Detailed comparison of diurnal JA-level changes in treated samples of* phyAphyC *(red line) and the wild type (blue line) (B).
Six weeks old rice plants were wounded with MecWorm for 1 hour at different time points during the day, treatments ending at the indicated time points. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one asterisk (Student's t-test, $p < 0.5$).

When comparing the JA-levels of the treated samples of wild type and *phyAphyC* (Figure 16B), evidently no significant differences could be observed apart from a lower JA-production in *phyAphyC* at 01:30 a.m. harvesting time (Figure 16B). All other measured JA-levels were not significantly different between wild type and mutant, which is also due to the variation between the single independent experiments. Nevertheless, though not statistically significant, the JA levels of the wounded *phyAphyC* plants appeared generally lower than those of the wild type plants in the early morning before the onset of dawn.

Apart from the differences between wild type and photoreceptor mutant we also were interested in the diurnal regulation of the JA production in the genotypes themselves. In the non-treated control samples of wild type and *phyAphyC*, there were no diurnal oscillations of the JA content (see Supplemental Figure 1, page 67). However, looking at the JA production kinetics depicted in Figure 16B, there is a slight deviation visible in both analysed genotypes, with less JA produced in darkness and more produced during the day. Even though this observation could not be supported by statistical analysis because of variation, it still should be considered. The results suggest, that during the night, the amount of JA produced in response to 1h of continuous wounding decreases continuously and then rises abruptly after the onset of dawn, keeping a plateau level during the day and the beginning night.

JA-Ile – stable content throughout the whole day

The results of the JA-Ile content analysis of 1 hour MecWorm treated rice plants (Figure 17) were similar to those of the JA measurement. JA-Ile, as well as JA, was induced strongly in the wild type as well as the *phy*-mutant by continuous wounding, compared to non-treated plants (Figure 17A).

The average levels of JA-Ile after wounding were statistically mostly similar between the wild type and the *phy*-mutant when comparing them at the respective time points (Figure 17A). Nevertheless, *phyAphyC* generally accumulated less JA-Ile than the wild type, although with the exception of two time points in the afternoon, this observation could not be verified by statistical means. At 1:30 p.m. and 4:30 p.m., however, *phyAphyC* produced significantly less JA-Ile than the wild type (Figure 17A). This contrasts with the measured JA-levels, which were similar between wild type and *phyAphyC* at these time points.

Regarding the JA-Ile content in the single genotypes, there was no diurnal oscillation visible in the control samples of neither Nipponbare nor *phyAphyC*. All measured JA-Ile values were below 1.0 ng/g FW and resemble the level of JA-Ile in unwounded leaves at this developmental stage (see Supplemental Figure 1, page 67). Additionally the levels of JA-Ile after 1 hour of continuous wounding did not fluctuate at all and were stable during the course of the day (Figure 17B). In summary, a constant amount of JA-Ile was produced in response to the 1h wounding treatment in wild type and *phyAphyC*, independent of the light situation.

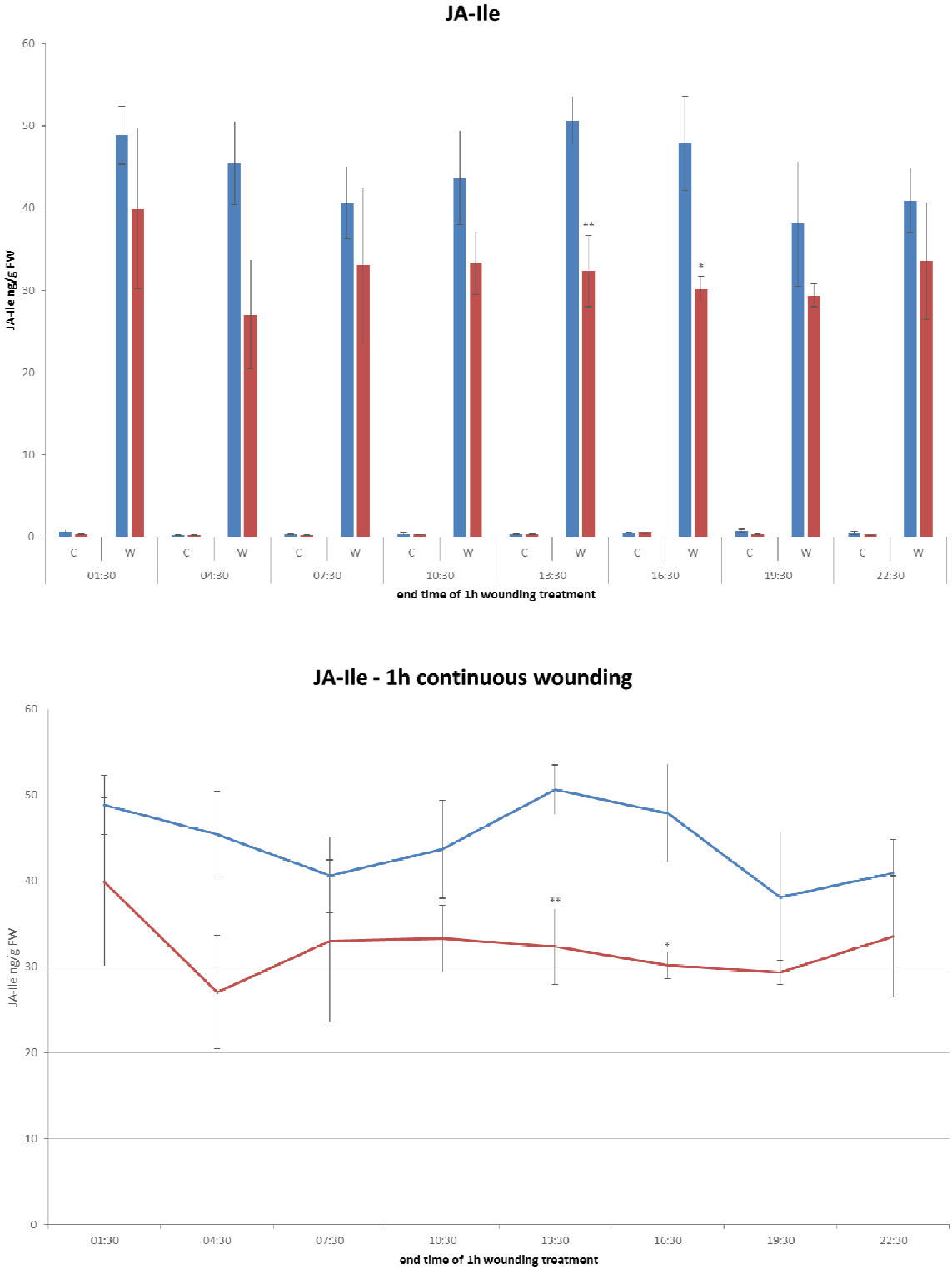


Figure 17: JA- Ile levels of rice plants in response to wounding. Comparison of* phyAphyC *(red bar), to the wild type (blue bar) showing treated samples opposed to control leaves (A). Detailed comparison of diurnal JA-Ile level changes in treated samples of* phyAphyC *(red line) and the wild type (blue line) (B).
Six weeks old rice plants were wounded with MecWorm for 1 hour at different time points during the day, treatments ending at the indicated time points. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. C = control plant, W = wounded plant. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's t-test, $p < 0.5$ or $p < 0.01$), respectively.

Ratio of JA to JA-Ile

As we observed less JA-Ile production in the afternoon in *phyAphyC* compared to the wild type, but not in the JA content, we calculated the ratio of JA-Ile to JA in the treated samples for each time point.

The ratios of JA-Ile to JA were generally quite similar between the wild type and *phyAphyC*, with the exception of the time point at 1:30 a.m., where *phyAphyC* showed a significantly higher rate (20%, Figure 18) compared to the wild type (15%, Figure 18). At 01:30 a.m., *phyAphyC* produced significantly less JA than the wild type (Figure 16A, B) but no difference in the amount of JA-Ile could be observed (Figure 17A, B).

Apart from the comparison of wild type and *phy*-mutants, we also examined the alteration in the ratio of JA-Ile and JA in the course of the day in the genotypes themselves. While in the wild type, no statistically significant differences in JA-Ile production were visible between the single time points, *phyAphyC* clearly showed a peak in the JA-Ile/JA ratio at 4:30 a.m. (24%, Figure 18), which was significantly higher than the ratios reached from 10:30 a.m. to 10:30 p.m.. We conclude that the JA-Ile production in *phyAphyC* can be modulated by the light situation or the relative time of the day, with higher JA-Ile conjugation during the dark phase and lower rates during the day. This effect was also slightly visible in the wild type, though statistically not significant due to variation and smaller amplitude in the ratios.

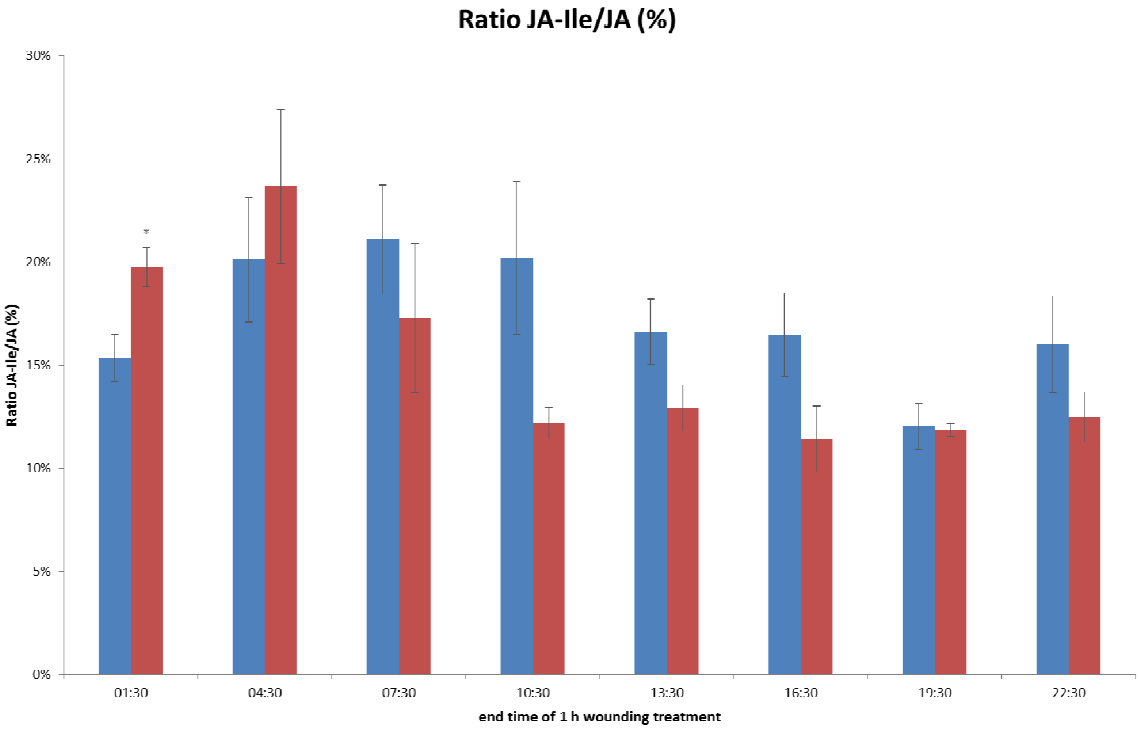


Figure 18: Ratio of JA-Ile to JA (%) in response to wounding. Comparison of phyAphyC (red bar), to the wild type (blue bar).
Six weeks old rice plants were wounded with MecWorm for 1 hour at different time points during the day, treatments ending at the indicated time points. Percentages were calculated from JA and JA-Ile levels and represent an average of at least 3 independent experiments. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one asterisk (Student's t-test, $p < 0.5$).

3.6 Summary

In this dissertation we wanted to elucidate the link between light and jasmonates during the response to mechanical wounding in rice plants. The experiments conducted with MecWorm in different JA-deficient and *phy*-deficient mutant lines resulted in the following intriguing main findings:

1. Mechanical wounding with MecWorm is sufficient to trigger a JA-mediated stress response in wild type rice plants.
2. The absence of JA in the mutants *hebiba*, *cpm2* and *kamakubi* leads to an attenuation of this response.
3. The JA-biosynthesis in response to mechanical wounding is exclusively dependent on the AOS1 enzyme. The second AOS in rice, AOS2, cannot compensate for the loss of AOS1 in the wounding response.
4. The mutants *phyAphyC* and *phyBphyC* accumulated more JA in response to long wounding events compared to the wild type, while simultaneously the gene expression of JA-responsive genes was reduced. Hence phytochromes seem to be negative regulators of JA-biosynthesis but enhancers of the JA response in rice.
5. Not only JA, but also ABA is induced by wounding with MecWorm, ABA levels appear to rise with increasing wounding period. This effect is JA-dependent, the JA-deficient mutants did not induce ABA accumulation after wounding.
6. The levels of JA and JA-Ile do not necessarily correlate. Thus, the availability of JA is not the only restricting factor for the synthesis of bioactive JA-Ile.

4 Discussion

Plants have to perceive and process a multitude of signals to adapt to their environment. The integration of this variety of signals is an interesting and challenging subject for research, as proper development and health of the plant are directly connected to its ability to correctly respond to the conditions it is exposed to.

In this study we were able to gain intriguing insights in the regulation of the wounding response in rice plants and retrieve useful information on the mutual interactions of hormone- and light-signalling in this response.

Firstly, our analysis of JA-deficient mutants showed, that OsAOS1, and not its homologue OsAOS2, mediates the wound induced JA-production in rice. In the course of these experiments we were additionally able to show, that MecWorm treatments are sufficient to induce JA-mediated responses in rice and that these responses are attenuated in mutant plants deficient in JA.

Secondly, we discovered, that also ABA accumulates in response to wounding. This effect was not observed in the JA-deficient mutants and thus is dependent on the presence of JA.

Thirdly, in the central part of this study, we analysed the wounding response of phy-deficient mutants and discovered, that while these mutants overproduce bioactive JA-Ile, the expression of JA-responsive genes was attenuated. This implies, that phytochromes modulate JA-signalling and that the JA-response is only executed properly in presence of active phy.

Lastly, we could observe, that the day and night rhythm exerts a certain influence on the production of JA and especially on the conversion of JA to JA-Ile in the wounding response.

Thus, during the response to wounding light and hormone signalling are linked on more than one level and can mutually influence each other. Moreover, this interaction appears to have negative effects on plant defence under certain light conditions like shading or darkness, an effect which possibly serves the regulation of resource allocation to growth and defence.

4.1 The JA-mediated wound-response is silenced in rice aoc and aos1 mutants and solely mediated via OsAOS1

In seedlings of the mutants *hebiba* and *cpm2*, both impaired in the function of OsAOC, it could be shown in earlier studies, that the production of JA and consequently JA-Ile is impaired in response to light and mechanical damage (Riemann *et al.*, 2003; Riemann *et al.*, 2013). We wanted to investigate the response of these mutants to wounding in a different developmental stage and chose a wounding technique which is as similar as possible to real insect damage but avoids the chemical influence by insect saliva. Therefore, we analysed the response to mechanical wounding in adult mutant plants of *hebiba* and *cpm2* and additionally examined another JA-deficient mutant, *kamakubi*, in which the *OsAOS1* gene is deleted.

We found that continuous mechanical wounding with MecWorm induced the production of JA as well as JA-Ile in the wild type very strongly as early as 30 minutes after wounding and that 1 hour and 6 hours of treatment elevated the JA and JA-Ile levels as well. Consequently, the increased amount of JA-Ile, a bioactive derivative of JA, caused an increase in the expression of the JA-responsive biosynthesis and signalling genes we tested. These results are in line with the findings of Bricchi *et al.*, who showed clearly that MecWorm is able to trigger stress hormone induction as well as gene expression responses on a similar level as natural herbivory (Bricchi *et al.*, 2010).

Interestingly, while the signalling genes *OsJAZ5* and *OsJAZ8* were induced very strongly by the MecWorm treatment in the wild type (see Figure 8, page 26), the biosynthesis genes *OsAOC* and *OsJAR1* were induced on a comparatively low level (see Figure 7, page 25).

The high induction rate of the examined JAZ-genes can be explained by their function in JA-signalling. The degradation of JAZ-repressors via the 26S proteasome is a key event in jasmonate signalling. These proteins are degraded very fast in response to stress. At the same time there has to be a fresh supply of new JAZ-repressors to prevent a constitutively active JA-response pathway leading to an overreaction of the plant (Thines *et al.*, 2007). Therefore a high expression of JAZ-genes during the stress response of plants is necessary to retain a balanced stress response.

For the low induction of the biosynthesis genes compared to the signalling genes in the wild type, the most likely explanation is, that OsAOC and OsJAR1 enzymes are already present in a sufficient abundance to sustain biosynthesis of JA and JA-Ile, respectively, in adult plants. In rice seedlings, it was observed that light irradiation elevated the expression of *OsAOC* and *OsJAR1* 6- and 4-fold, respectively (Svyatyna *et al.*, 2013). We conclude, that the inducibility of the respective JA-biosynthesis genes is highly dependent on the developmental status of the plant and the tissue examined. A sufficient level of JA-biosynthesis enzymes is of a certain advantage for the adult plant. In this way it can respond to external stress signals in a

fast manner by *de-novo* synthesis of JA (Koo & Howe, 2012). In contrast, in plants in the developmental stage a lot of different processes have to be orchestrated, which might be the reason why many enzymes are not produced by default but produced on demand.

Different from the wild type, in all the JA-deficient mutants tested in this study, JA and JA-Ile were only detected in trace amounts, independently of the different MecWorm treatment periods administered to these plants (see Figure 9, page 29 and Figure 10, page 31). These results are in line with the observations made in earlier studies (Riemann *et al.*, 2003; Riemann *et al.*, 2013), and show, that not only in the seedling stage but also in the vegetative leaves the JA-pathway is defective in these mutants.

Interestingly, the lack of JA-production in the *kamakubi* mutant (see Figure 10, page 31) implies, that OsAOS1 is the enzyme exclusively mediating the JA-response to mechanical stress in rice plants and that the other active AOS in rice, OsAOS2 (Haga & Iino, 2004; Kuroda *et al.*, 2005; Mei *et al.*, 2006), is not able to compensate the loss of OsAOS1 function in this physiological context. Mei *et al.* reported an inducibility of the *OsAOS2* transcript in response to infection with *M. grisea* and showed, that inducible *OsAOS2* overexpressor lines produced higher levels of JA upon pathogen infection than the wild type. Additionally, they observed, that the *OsAOS2* transcript could not be induced by wounding. Thus, although *OsAOS2* apparently plays an important role in the JA-biosynthesis in response to pathogen attack, the JA-production in response to wounding is solely dependent on *OsAOS1* in rice plants.

Additionally, for the selected JA-biosynthesis and -signalling genes, that were tested in this dissertation, no induction by wounding treatments could be detected in neither *hebiba*, *cmp2* (Figure 7, page 25 and Figure 8, page 26) nor in *kamakubi* (see Figure 11, page 33 and Figure 12, page 34). Therefore, the transcriptional stress response observed in the wounded wild type plants is exclusively dependent on the presence of JA, all four genes tested cannot be induced in response to mechanical wounding if the JA-biosynthesis pathway is impaired.

In *hebiba*, the *OsAOC* gene was not expressed at all, which was expected since the gene locus is included in the deletion on chromosome 3. In contrast, in *cpm2* expression of *OsAOC* was detectable, which indicates, that there might be a truncated form of this enzyme expressed in this mutant, as already proposed by Riemann *et al.* (Riemann *et al.*, 2013). The possibility for a residual activity of this truncated form is relatively low, as no JA could be detected in the tests they conducted. In the current study, we could make a similar observation: in the *cpm2* mutant the levels of JA and JA-Ile were as low as in *hebiba*, which does not express *OsAOC* at all (see Figure 9, page 29 and Figure 10, page 31). Thus, it is unlikely that there is a partially active OsAOC enzyme present in *cmp2*.

Interestingly, neither expression of *OsJAZ5* nor *OsJAZ8* could be detected in *cpm2*. It remains to be elucidated if this is an artefact, because these genes are expressed in such a marginal amount in *cpm2*, that they could not be detected due to technical limitations. The analysis of

OsJAZ5 and OsJAZ8 protein content in *cpm2* could give information if there really is no expression of the respective genes.

Summarizing, the results of this part of the study are in line with research done previously on the response of JA-deficient mutants. We could show that mechanical wounding is able to induce JA-dependent stress signalling in the wild type while in all JA-deficient mutants tested, no response to the MecWorm treatments could be observed, neither elevation of JA-levels nor induction of JA-responsive genes.

As a new finding, we discovered, that the *aos1* mutant *kamakubi* did show the same hormonal and transcriptional regulation patterns as the *aoc* mutants, suggesting that in rice exclusively OsAOS1 is responsible for the JA-production in response to wounding.

4.2 Rice plants accumulate ABA in response to mechanical wounding in a JA-dependent manner

To elucidate the influence of the changed JA-levels in the different mutant genotypes on the homeostasis of other plant hormones, we analysed the levels of different phytohormones in response to wounding with MecWorm.

In the course of this study, we made the intriguing discovery, that continuous wounding led to a continuous accumulation of ABA in leaves of the wild type over the wounding period (see Figure 13, page 35 and Figure 14, page 36). This effect was also observed in the tested *phy*-mutants, but not in the JA-deficient mutant plants.

As ABA is a phytohormone generally associated with functions in transpiration and water household of plants (Cutler *et al.*, 2010; Zeevaart & Creelman, 1988), the possibility exists, that the effect we observed was caused by endogenous factors in the plant in response to the day and night rhythm rather than the treatment duration. Thus, we wanted to examine whether the basal ABA-levels as well as the inducibility of ABA accumulation by wounding are influenced by exogenous factors like the changing light conditions or the day and night rhythm. Therefore, we analysed the production of ABA after short wounding treatments of 1 hour at different time points in the course of a whole 24 hour period. The results of these experiments showed, that the ABA levels of wild type rice plants were not influenced by light or darkness, control and wounded plants did not change their ABA levels significantly depending on the time of the day (see Supplemental Figure 2, page 68).

Therefore we conclude, that the observed effect of ABA accumulating in response to wounding with MecWorm in a time dependent manner is only marginally influenced by the light/dark rhythm or other external conditions the plants were exposed to, and is actually a direct response of the plant to the wounding event.

Consequently, the questions arise, what mechanism exactly underlies the induction of ABA and does the elevated level of ABA exert a physiological effect on the defence response of the plants?

The role of ABA in plant defence responses to biotic stresses is discussed controversial (Anderson *et al.*, 2004; Ton *et al.*, 2009). Especially in rice, various negative effects of ABA on pathogen defence have been reported (Jiang *et al.*, 2010; Nahar *et al.*, 2012). Nevertheless, the role of ABA is apparently highly dependent on the infesting organism and the status of the infection. As we examined the wound response of rice plants and used a machine mimicking herbivory, the effects we observed are more comparable to responses induced by feeding insects than by pathogen attack. In rice, no effects of herbivory on ABA have been reported until now, but in other organisms, observations have been made, that are in line with the results of our experiments.

In tomato and potato for example, an inducibility of ABA by wounding has been reported by different research groups (Birkenmeier & Ryan, 1998; Pena-Cortes *et al.*, 1991; Pena-Cortes *et al.*, 1995). Moreover, in maize plants, which are also monocotyledons, Erb *et al.* (Erb *et al.*, 2009) observed an accumulation of ABA after infestation with *S. littoralis* caterpillars. The elevation of ABA in response to the herbivore attack was moderate but nevertheless statistically significant. The authors discussed the desiccation of the leaves caused by the wounding as one possible reason for the induction of ABA by wounding. Another study also suggested that water loss of the damaged area might play a major role in the response to mechanical wounding (Reymond *et al.*, 2000). Therefore, one possible explanation for the induction of ABA by MecWorm treatment might simply be the desiccation of the treated tissue. In this case, the increase in ABA might lead to stomata closure and therefore prevent the leaf from losing more water as a first aid against one of the negative effects caused by the feeding herbivore. Preliminary results from the analysis of wound induced stomata closure in the wild type Nipponbare are supporting this hypothesis. After wounding with MecWorm, 23 % more stomata were closed in comparison to control plants (Bachelor thesis of Svenja Wehrle, 2013, see Supplemental Figure 3, page 69). All the more, this theory would also be in line with our observation of rising ABA levels over the wounding period, as longer wounding treatments damaged a larger area of the leaf and therefore potentially caused a higher water loss.

Disregarding the hypothesis that dehydration at the wounding site might lead to the observed increase in ABA, a physiological effect of ABA accumulation in response to wounding apart from stomatal closure should be considered and already has been observed in other plant species. While in tomato, a primary defence promoting function of the raised ABA content was challenged by Birkenmeier et al. (Birkenmeier & Ryan, 1998), another study reported that ABA-depressed tomato plants were more susceptible to caterpillar feeding (*S. exigua*) than control plants (Thaler & Bostock, 2004). The same observation, higher susceptibility to feeding herbivores correlating with a reduced ABA-level, was made in ABA-deficient *Arabidopsis* mutants (Bodenhausen & Reymond, 2007). Moreover, in *N.*

attenuata, it was shown that ABA signalling positively influences the accumulation of defence related metabolites associated with herbivore attack, while the depression of ABA led to an attenuation of the synthesis of the mentioned metabolites (Dinh *et al.*, 2013).

This evidence from other species strongly suggests a physiological role of ABA in the wound response of plants that is more complex than only the induction of stomata closure in response to desiccation caused by tissue damage. In support of this hypothesis, recently a study in herbivory challenged *Arabidopsis* plants revealed, that while JA is accumulated locally and systemically after wounding, ABA only increases locally on the wounding site. The authors suggested, that only the synergistic effect of JA and ABA induces costly defence responses in the local leaf, while the systemic leaves are primed for attack, ready to induce defences as soon as the ABA level in response to local tissue damage rises (Vos *et al.*, 2013).

More evidence, retrieved from our experiments with the JA- and *phy*-mutants, supports the idea of ABA as part of a complex defence response system, as we found light and JA to be modulators of ABA, which will be described in the next chapters.

4.2.1 JA-deficiency leads to attenuation of ABA-induction after wounding

The effect of ABA-levels rising in response to mechanical wounding, which we observed in wild type plants, was not visible in JA-deficient mutants. We analysed *hebiba* and *cpm2* mutants (see Figure 13, page 35), deficient in the AOC function, as well as *kamakubi* (see Figure 15, page 37), deficient in AOS1, and could not find any induction of ABA compared to control plants in response to the applied MecWorm treatments.

Therefore we conclude, that the presence of Jasmonates is required for the wound induced ABA accumulation in rice plants.

A connection of ABA and JA in wounding responses and an extensive interaction between JA and ABA in biotic stress responses was already proposed in other plant species (Bodenhausen & Reymond, 2007; Fan *et al.*, 2009; Lorenzo *et al.*, 2004; Pieterse *et al.*, 2012; Vos *et al.*, 2013).

Anderson *et al.* (Anderson *et al.*, 2004) first suggested a synergistic effect of JA and ABA in wounding response and at the same time an antagonistic relation of ABA and JA in pathogen defence. A similar observation was later made in microarray studies in *Arabidopsis*, ABA negatively affected pathogen inducible genes while promoting wound inducible genes (Bodenhausen & Reymond, 2007). A very good summary of these diverse effects of the interactions of ABA and JA in response to biotic stresses is depicted in Pieterse *et al.* (Pieterse *et al.*, 2012). In this review it is suggested, that ABA and JA are simultaneously activated by wounding and act synergistically on the induction of herbivore defence related

genes, at the same time suppressing the branch of the JA-pathway responsible for pathogen defence (see Figure 19).

The influence of ABA levels on JA-production has already been reported in various other studies. For example in potato, the presence of ABA is required for wound induced JA accumulation (Pena-Cortes *et al.*, 1995). In that study it was suggested, that ABA acts upstream of JA, as ABA-deficient mutants did not produce JA in response to wounding. Nevertheless, they could still induce their JA production and express defence responses when directly treated with linolenic acid, the substrate of the JA pathway, which lead to the hypothesis, that ABA signalling acts upstream of the release of fatty acid substrates for the octadecanoid pathway. However, recently it was shown, that the provision of free α-linolenic acid is sufficient to trigger JA-biosynthesis (Christeller & Galis, 2014). Consequently, the before mentioned hypothesis has to be considered with caution.

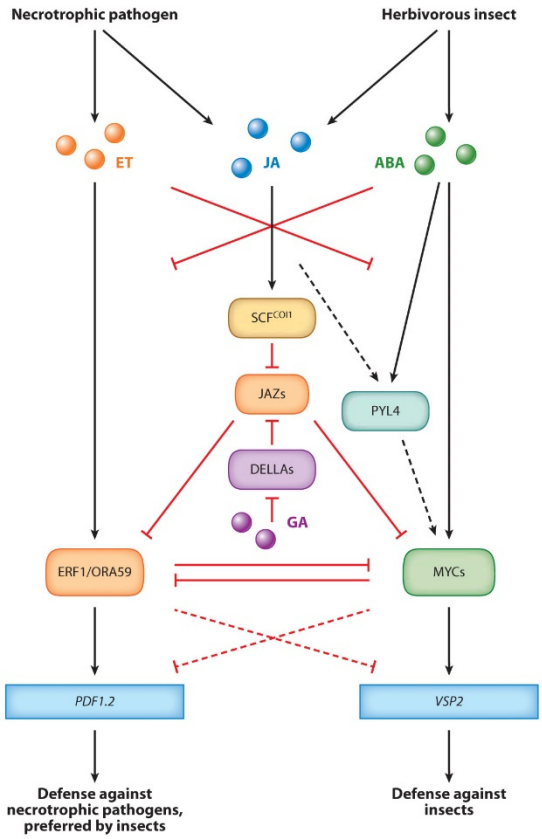


Figure 19: Model of the modulation of the JA-pathway in response to different biotic stresses
Herbivory induces JA and ABA accumulation simultaneously, which results in expression of the MYC-branch of the JA-response. Infection with necrotrophic pathogens induces JA and ET accumulation, leading to expression of the ERF-branch of the JA-pathway. MYC and ERF branches are mutually antagonistic.
Solid lines represent established interactions while dashed lines indicate proposed interactions.
(Figure taken from Pieterse *et al.*, 2012)

Another study, proposing a promotion of JA-biosynthesis by elevated ABA-levels, was conducted in *Arabidopsis* (Fan *et al.*, 2009), and in tobacco, the presence of ABA is apparently required for a correct JA signal transduction in defence responses to herbivory (Dinh *et al.*, 2013). More evidence for a modulating effect of ABA on the JA-signalling pathway in herbivore resistance can be found in the microarray studies conducted on ABA-deficient Arabidopsis mutants by Bodenhausen & Reymond (Bodenhausen & Reymond, 2007). They reported, that most of the genes regulated by ABA were COI1-dependent and known to be part of the JA-pathway, which indicates a considerable influence of ABA on the JA-pathway.

Thus, while many studies reported a dependency of JA-biosynthesis or signal transduction on the accumulation of ABA, we observed, that JA-biosynthesis is required for ABA accumulation in response to wounding. This is an intriguing observation, as to our knowledge, there are no studies reporting something similar. Additionally, the JA-dependency of ABA accumulation in response to wounding reinforces the idea, that tissue

desiccation is not the exclusive cause for the increased ABA levels, as JA would not necessarily be required in this response.

Therefore, taking together the available information from recent research, we propose a reciprocal interaction of both JA and ABA, fine-tuning the wound response of rice plants.

At this moment, it is not trivial to pinpoint the extent and the mechanistic background of this interaction. In recent studies, the interaction of JA and ABA has preliminary been discussed on the level of signalling and synergistic interactions. The transcription factor MYC2, for example, not only regulates JA-responsive gene expression but also is induced by ABA and potentially integrates JA-and ABA-signalling (Kazan & Manners, 2013; Vos *et al.*, 2013). In tobacco, the ABA receptor PYL4 was shown to affect JA-signalling (Lackman *et al.*, 2011). Moreover, even a moonlighting of hormone signalling repressors in different hormone signalling pathways was hypothesized (Robert-Seilaniantz *et al.*, 2011).

Nevertheless, these possible integration points are on the level of JA and ABA response and signalling and would imply a feedback of these responses on the hormone biosynthesis itself, which is known to work for JA, but to date was not observed for ABA. Therefore, the mechanistic background of the reciprocal modulation of hormone levels of JA and ABA remains elusive and needs further investigation.

4.2.2 Phytochrome B has an effect on the basal level of ABA

The induction factor of ABA in response to mechanical wounding was observed to be similar between the wild type, the *phyAphyC* and *phyBphyC* mutant plants. Nevertheless, we noticed an interesting effect in *phyBphyC* mutants: the levels of ABA in this genotype were generally elevated by a factor of 3 in comparison to the wild type and the *phyAphyC* mutant, in both, control and treated plants (Figure 15, page 36).

We conclude that the absence of phyB, but not phyA, is the cause of the elevated ABA levels and that generally phyB suppresses the basal level of ABA. Our findings are in line with a recent study in *Arabidopsis*, where a similar observation was reported. Leaves of *phyB* null mutants contained basically 39% higher levels of ABA in comparison to the wild type (Gonzalez *et al.*, 2012).

Additionally, in favour of our hypothesis, recent research on the link between ABA and the light signalling pathway especially emphasizes phytochrome B as a major player in this interaction. For example, an influence of phyB on drought tolerance in rice, directly connected with ABA, was proposed by Liu *et al.* (Liu *et al.*, 2012). They observed an increased tolerance to drought stress in rice *phyB* mutants and a reduced stomatal density together with a generally smaller total leaf area. Altogether they suggested a "[…] negative correlation between stomatal number and ABA level." (Liu *et al.*, 2012), hypothesizing, that the cause for the observed effects in the *phyB* mutant might be an elevated level of ABA.

Our study confirms this hypothesis, as we could show that mutant rice plants lacking phyB and phyC contain higher ABA levels. Moreover, the influence of phyB on ABA levels is not limited to the leaves only: Seo *et al.* (Seo *et al.*, 2006) showed that the ABA metabolism in *Arabidopsis* seeds is mediated by phyB. They reported that the reduction of ABA in response to R light pulses, which is crucial for the induction of seed germination, is attenuated in phyB mutants, leading to a reduced germination rate.

Thus, light signalling, mediated via phyB, appears to be tightly connected to the ABA hormone homeostasis in seeds as well as in mature plants and therefore the question arises, what physiological consequences result from this link. The physiological consequences in the seed have been thoroughly studied. In the presence of light, ABA accumulation is repressed by phytochrome signalling, and consequently the level of ABA decreases and the repression of seed germination is attenuated (Seo *et al.*, 2006; Seo *et al.*, 2009). To what extent the ABA-light interaction in mature plants is useful, remains to be elucidated, but as ABA acts in the regulation of transpiration by mediating the closing of stomata, a connection of light and ABA signalling in the regulation of photosynthesis is one possibility.

Another question, that comes up in this context is how light signals can mechanistically exert influence on the ABA levels. From different studies, it is known that light signalling and ABA signalling influence each other (Lau & Deng, 2010; Morker & Roberts, 2011; Tang *et al.*, 2013), but these reports are mostly descriptive. The molecular mechanism behind it remains largely unknown and needs further investigation.

Summarizing the results we obtained with respect to ABA, we could demonstrate that it is induced in response to mechanical wounding in a time dependent manner and this effect is dependent on the presence of JA. We suggest, that this rise in ABA is possibly, but not exclusively, induced by the desiccation of the damaged tissue and might lead to stomatal closure as a first aid in the wounding response, preventing water loss in the damaged leaf. Moreover, we hypothesize, that ABA and JA synergistically mediate the defence reaction of the plant to wounding. An additional role for ABA in the wounding response could be the induction of callose deposition into the cell wall, a defence strategy which is known from responses to pathogen attack that has recently been attributed to be mediated by ABA (de Torres-Zabala *et al.*, 2007; Oide *et al.*, 2013).

To what extent ABA is required in the JA-regulated defence response of rice plants remains to be elucidated, as well as the mechanistic reason for ABA accumulation, since ABA hormone levels are resulting from an equilibrium of biosynthesis and catabolism.

Additionally, we observed, that the basal level of ABA is modulated by phyB. In absence of phyB the production of ABA is elevated, possibly leading to an increased drought tolerance in *phyB* and *phyBphyC* mutants (Liu *et al.*, 2012).

4.3 The partial loss of the phytochrome function leads to an imbalance in JA-production and gene expression in response to wounding

In the previous chapters we described, that mechanical wounding with MecWorm generates a JA-mediated stress answer in wild type rice plants and that an interplay between phytohormones and light signalling potentially modulates this wound induced response.

Previous studies in rice seedlings have shown, that the response to light signals can be modulated by the stress hormone JA and that also the photodestruction of phyA is impaired in the absence of JA (Riemann *et al.*, 2003; Riemann *et al.*, 2009). These findings imply a reciprocal interaction of light sensing via phytochromes and the JA-response in rice plants. Recent studies in other organisms also suggest a connection of light and JA in the defence of plants against different stresses like herbivory or pathogen infection (Arimura *et al.*, 2008; Kazan & Manners, 2011; Moreno *et al.*, 2009; Radhika *et al.*, 2010, Zeier *et al.*, 2004), especially when plants exhibit the so called shade avoidance syndrome (SAS, see chapter 1.4, page 10). These studies propose an antagonistic relationship between defence responses and phy-mediated answer to shading, but were mostly conducted in *Arabidopsis* or other plants known to express the SAS. Rice plants are monocots and moreover culture plants and are therefore usually grown in dense canopies. No information is available on the existence of SAS in rice and the shade avoidance of grass plants only recently has emerged as a topic of interest for research (Kebrom & Brutnell, 2007).

We wanted to examine the effect of phy on the wounding response of rice plants and chose two rice *phy*-mutants, *phyAphyC* and *phyBphyC*, for experiments conducted with MecWorm.

The analysis of plant hormone levels and JA-responsive gene expression revealed two intriguing and at first view opposed effects in these mutants, which will be discussed in this chapter:

(i) In response to long wounding events, JA and especially JA-Ile were overproduced in *phy*-mutants

(ii) The expression of JA-responsive genes in response to wounding was reduced in *phy*-mutants

4.3.1 *Phy*-mutation leads to an overproduction of JA and JA-Ile in response to wounding

We measured the JA and JA-Ile levels in rice *phy*-mutants in response to wounding with MecWorm and observed, that after 6 hours of continuous wounding, *phyBphyC* produced 2-fold more JA and almost 3-fold more JA-Ile than the wild type, while *phyAphyC* produced similar levels of JA as the wild type, but significantly more JA-Ile (see Figure 10, page 31).

As the 6 hours wounding treatment ended in the late afternoon, shortly before the onset of dusk, it is possible, that the observed effect was not caused by the long wounding period but rather by alternatively regulated light signalling in the *phy*-mutants and the wild type in response to the diurnal rhythm. To rule out this possibility, we also conducted 1h MecWorm treatments in the wild type and *phyAphyC*, ending at the same time as the 6 hour experiments (for experimental setup see Table 2, page 18). The results of this experiment showed, that wild type plants accumulated significantly more JA and JA-Ile when wounded for 1 hour compared to a 6 hour wounding treatment at the same time of the day (see Supplemental Figure 4, page 70). Thus, in the wild type the extent of JA-accumulation was not dependent on the time of the day but on the wounding period.

On the contrary, in *phyAphyC* mutants, the wounding period did not influence the amount of accumulated JA or JA-Ile, the measured amounts were similar for 1 hour and 6 hours of MecWorm treatment (see Supplemental Figure 4, page 70). Therefore, we conclude, that the differential JA-accumulation of the *phy*-mutants compared to the wild type is an effect caused by the long wounding period of 6 hours. Additionally, this result implies a suppression of the JA accumulation in response to long wounding events in the wild type, which is absent in the *phy*-mutants and therefore appears to be under the mediation of phytochrome. We were not able to conduct 1 hour wounding experiments in *phyBphyC* mutants due to shortage of seed material. Therefore we cannot exclude that the high accumulation of JA and JA-Ile observed in *phyBphyC* was under the influence of external factors. However, as phyB is stable under continuous light (Furuya, 1993; Riemann *et al.*, 2009), it is rather unlikely that the effects observed in this mutant are caused by a time dependent light effect like the exposure to a 10 hour light period.

Taken together, we conclude, that after longer wounding events the accumulation of JA is repressed in the wild type, while repression of JA production is impaired in *phy*-mutants. A suppression of JA-biosynthesis by active phytochrome has already been reported by other studies, however, only little information is available on this effect. A very interesting study in chromophore deficient *hy1* and *hy2* mutants of *Arabidopsis* reported a constitutive overaccumulation of JA in absence of functional phy (Zhai *et al.*, 2007). Next to generally higher levels of JA and JA-Ile, these mutants also exhibited a constitutively activated JA-responsive gene expression. In the same study none of the mentioned effects was observed in *phyAphyB* mutants. However, as *Arabidopsis* contains 5 phys, it is possible, that the other,

still functional phys were sufficient to compensate for the loss of phyA and phyB. In line with these observations, more evidence from *Arabidopsis* implies, that deficiency in phyA leads to higher amounts of OPDA, a precursor of JA, in darkness and continuous FR light (Robson *et al.*, 2010). Another study, conducted in lima bean, also reported, that continuous wounding with MecWorm during darkness resulted in the accumulation of significantly higher amounts of JA compared to the light phase (Arimura *et al.*, 2008). The same applies for JA-accumulation in response to pathogen infection: in *Arabidopsis* more JA accumulated when plants were challenged with *Pseudomonas syringae* in the dark compared to light (Zeier *et al.*, 2004).

Thus, in a variety of organisms, challenged with either herbivores or pathogens, the JA-biosynthesis appears to be repressed in the light by phy, while in darkness, or under low R:FR ratios, or in absence of phy, more JA is produced. Our findings are in line with these studies as we observed a repressive effect of phys, especially phyB, on JA-accumulation in response to wounding.

Therefore it is intriguing to ask, what mechanism is underlying the light mediated repression of JA-accumulation. One explanation for the overaccumulation of JA in the dark could be, that certain JA modifying or catabolizing steps might require light. In lima bean, it was found that conversion of JA to JA-Ile did not work in darkness and moreover it was speculated, that different other metabolic steps like conjugation or hydroxylation of JA would need light for correct function (Arimura *et al.*, 2008; Radhika *et al.*, 2010). Attenuation of these metabolic reactions in the dark could lead to an accumulation of JA. We examined the production of JA and JA-Ile during dark and light in rice and were not able to observe enhanced hormone levels in the night compared to the day, neither in control samples, nor in wounded plants (see Supplemental Figure 1, page 67). Nevertheless, it cannot be excluded, that metabolic steps different from JA-Ile formation, for example serving catabolism of JA and JA-Ile, proceed in rice in the light and are attenuated in absence of phy function, which would result in higher accumulation of JA in absence of phy.

Another hypothesis, how light might influence the accumulation of JA, is connected to the processes that happens in the plant during photosynthesis. Photosynthesis not only produces energy but also consumes resources, gene expression capacity and generally a considerable amount of the cell metabolism. Thus, probably the lowered JA-levels in the light are due to shortage of metabolic capacity in the cells and the repression of JA is an issue caused by resource allocation, which is diminished as soon as photosynthesis is discontinued in the dark.

Summarizing, the inactivation of phy by mutation leads to increased JA- and JA-Ile levels in response to wounding. The physiological output as well as the molecular mechanism behind this effect are elusive and still require extensive investigation.

4.3.2 The loss of phytochrome function leads to a reduction of JA-induced gene expression in response to wounding

The results of the hormone analyses in the *phy*-mutants showed a higher accumulation of JA and JA-Ile after 6 hours of continuous wounding and we hypothesized, that more bioactive signalling molecule would lead to an increased JA-response. Therefore, we examined the expression of JA-responsive genes in *phyAphyC* and *phyBphyC* after wounding. Surprisingly, in contrast to our hypothesis, we found a reduced expression of the examined biosynthesis and signalling genes compared to the wild type (chapter 3.3, page 32).

In contrast to the impact of phy on JA- biosynthesis, the connection of phy and JA on the level of signalling responses has been extensively studied in different plant species. The results from these studies, however, are contradictory and point out the complexity and specificity of this interaction. For example, as already mentioned in the previous chapter, in *Arabidopsis hy1* and *hy2* mutants, deficient in phytochrome chromophore biosynthesis, a continuous upregulation of JA-response genes, among them *VSP1*, could be observed (Zhai *et al.*, 2007). Controversially, the same gene, *VSP1*, was downregulated in other phy-chromophore deficient *Arabidopsis* mutants in response to external JA-application (Costigan *et al.*, 2011). Additionally, also in *Arabidopsis*, the inactivation of phy by FR lead to attenuation of gene expression of the ERF-branch of JA-signalling, while other genes, among them also some JAZ genes, were upregulated (Moreno *et al.*, 2009). Based on these findings, Kazan & Manners suggested in their review, that light modulates the different branches of JA-induced defence differently, downregulating the ERF-branch and upregulating the MYC2-branch (Kazan & Manners, 2011, see also Figure 19, page 51). However, this hypothesis only roughly outlines the mechanisms behind the light modulated gene regulation in defence responses and cannot account for some of the mentioned findings.

Thus, the regulation of JA-responsive genes appears to be specific for different situations and diverse in between different plant species and tissues. In wounded rice plants, we also observed, that the loss of phy-function leads to a reduction of the JA-responsive expression of different biosynthesis and signalling genes. Of course we cannot exclude, that a variety of other genes, we did not examine, might be regulated differently. To rule out this possibility, an examination of the whole transcriptome, e.g. via microarray or RNA-seq analysis, would be required.

However, our findings are in accordance with other investigations on transcriptional responses of plants grown in either shade, darkness or under low R:FR ratio, which in most cases reported similar observations: deactivation of phy, by either mutation or through low R:FR ratios, leads to JA-insensitivity caused by attenuation of JA-responsive gene expression (Cerrudo *et al.*, 2012; Costigan *et al.*, 2011; de Wit *et al.*, 2013; Morker & Roberts, 2011). This conclusion is in concert with a variety of field studies, stating that plants during dark

periods or under low R:FR are more susceptible to herbivory and pathogen infection (Izaguirre *et al.*, 2006; Moreno *et al.*, 2009; Roberts & Paul, 2006).

4.3.3 The imbalance in JA-production and gene expression might be a result of altered JAZ-stability

At first view, our results from hormone analysis and gene expression analysis appear to be contradictory. While on the one hand more active signal in form of JA-Ile is produced in both *phy*-mutants in response to wounding, the response to this signal is reduced on the level of gene expression. Consequently the question arises, why an increase in signal compound is not leading to an increased signal response in the *phy*-mutants and what mechanism is underlying this effect.

An explanation for this enigma might be given by the mechanism which is mediating the JA-responsive gene expression. According to the present knowledge, a transcriptional signal response to free JA-Ile only occurs, if JAZ-proteins are ubiquitinated by the SCF^{COI1} complex and subsequently degraded by the 26S-proteasome, releasing MYC2 and other JA-responsive transcription factors from repression (Fonseca *et al.*, 2009).

Recent research has given rise to the assumption, that light can modulate the JAZ-degradation by influencing the stability of JAZ proteins. For example, in *Arabidopsis* it could be shown, that functional phyA is required for the degradation of AtJAZ1 in response to wounding (Robson *et al.*, 2010). The mutation of phyA lead to a stabilisation of AtJAZ1 and consequently the expression of JA-responsive genes was attenuated. More evidence for the influence of phytochromes on the stability of JAZ-proteins was communicated by Ballaré *et al.*, who observed that the stability of AtJAZ10 is increased by low R:FR ratios, mimicking canopy shading (Ballare, 2014).

The mechanistic background of this effect might be explained by posttranscriptional modifications. Recent studies have shown, that the stability of JAZ proteins is dependent on alternative splicing of the respective pre-mRNA and that certain, naturally occurring variants of JAZ, produced by alternative splicing, are stable and not degraded by the COI1-dependent pathway (Chung & Howe, 2009; Chung *et al.*, 2010). This finding is especially intriguing, as it has been shown recently, that light can modulate alternative splicing by induction of intron retention in a variety of different genes and a role of phytochromes in this process was proposed (Wu *et al.*, 2014).

Another possibility for the modulation of JAZ-stability by light, which also should be considered, is the crosstalk of DELLA and JAZ. DELLA proteins are repressors of the GA response, similar to JAZ in the JA-pathway, they are degraded upon activation of the GA-pathway (Hauvermale *et al.*, 2012; Hirano *et al.*, 2008). DELLAs have been shown to directly interact with JAZ, and subsequently preventing JAZ from interacting with MYC2 and repressing the JA-response (Hou *et al.*, 2010). The production of GA can be induced by light,

the increased levels of GA consequently leading to a degradation of DELLA (Feng *et al.*, 2008; Kazan & Manners, 2011). Thus, it has been proposed, that JAZ proteins are released from the interaction with DELLA and are therefore free again and able to suppress the JA-response. Evidence from a recent study supports this hypothesis, as it was found, that the sensitivity to JA is decreased in absence of DELLA (Moreno *et al.*, 2009).

Taking together these evidences, it is tempting to speculate, that light, mediated via phytochromes, regulates the stability of JAZ proteins, fine-tuning the JA-response to wounding in rice plants. In absence of phy, the balance of degradable and dominant JAZ repressors is possibly shifted to more dominant JAZ, leading to an attenuation of JA-response signalling, even in presence of high levels of bioactive JA-Ile. We therefore propose a model, combining the effects we observed (Figure 20). The accumulation of JA in response to mechanical wounding is repressed by active phyB, either by repression of JA-biosynthesis or by enhancement of JA-catabolism. In absence of phyB this repressive function is attenuated and consequently JA and JA-Ile levels increase in an excessive manner. Additionally, the stability of JAZ-proteins is modulated by active phytochromes.

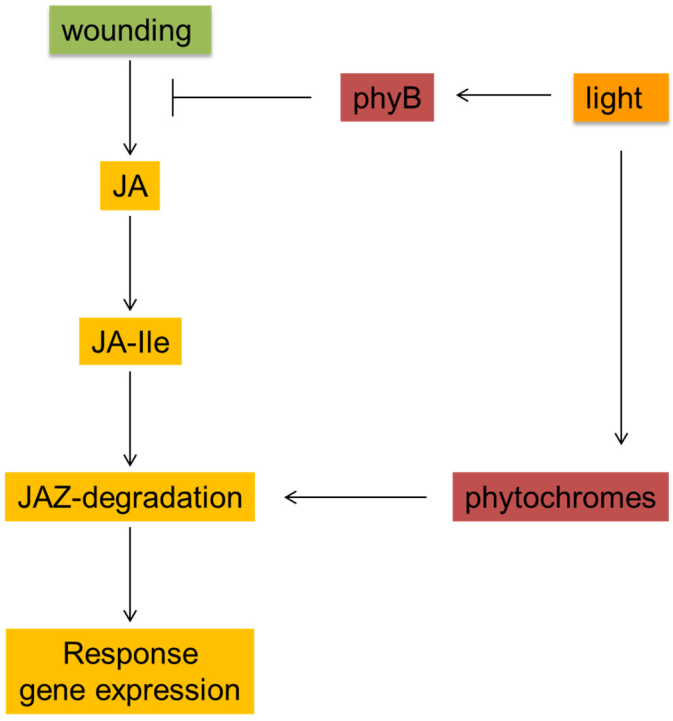


Figure 20: Working model for the modulation of wounding induced JA-biosynthesis and -signalling by phytochromes.
In response to wounding, the production of JA and JA-Ile is induced. Subsequently, in presence of JA-Ile, JAZ-proteins are degraded and JA-responsive gene expression commences. Active phyB suppresses the accumulation of JA in response to wounding, either by repression of JA-biosynthesis or by induction of JA-catabolism. The loss of phyB leads to increased levels of JA and JA-Ile. Additionally phytochrome function positively influences the degradation of JAZ, supporting the induction of JA-responsive gene expression. Loss of phy leads to a stabilisation of JAZ, probably by production of non-degradable JAZ splice variants, resulting in an attenuation of gene induction.

In absence of phy, JAZ proteins are not as readily degraded as under wild type conditions, which subsequently leads to an overaccumulation of JAZ, repressing the JA-response on the transcriptional level. At the moment it is not clear, which phytochrome exactly is responsible for the modulation of JAZ-stability and a synergistic effect of all phytochromes has to be considered.

Apart from the mechanistic background of the JA- and phy- interactions we proposed, of course also the physiological background of these apparently antagonistic effects is a challenging topic. Why would the same signal, light, exert a repressive function on one part of the pathway while simultaneously enhancing the other?

Probably the opposing effect of light on hormone production and response signalling serves the fine tuning of the JA-response. For plants it is crucial not to overreact to stress, as defence responses consume energy and resources. Therefore plants have to control and monitor their defences and consequently, the JA-response is tightly regulated by an array of feedback loops (Kazan & Manners, 2008).

Light sensing via phy could be one modulator of these feedback loops, ensuring that the appropriate response to different stresses is expressed. In a recent review it was proposed, that phy- and JA-signalling pathways act mutually antagonistic (Hsieh & Okamoto, 2014). As there appear to be a variety of intersections of JA-and phy-signalling and additionally the mutual influence is very variable and specific for plant, tissue and stress type, an approach like the one proposed by Hsieh *et al.* is not sufficient to describe the relations between phy- and JA-signalling. We therefore rather propose, that both, JA and phy, are reciprocally influencing each other and are equally important for building a regulative network, fine tuning the defence responses of plants.

4.4 The day/night rhythm has an impact on the JA-response to wounding

4.4.1 Modifications of JA and JA-Ile hormone levels by the day/night rhythm

To verify that the observations we made in 6 hour MecWorm treated plants were an effect of wounding period and not influenced by the dark/light changes during the short day conditions, we conducted short 1 hour wounding treatments at different times of a 24 hour period. During the analysis of the whole data set, we found, that the levels of JA and JA-Ile in untreated plants did not oscillate in the course of the day (see Supplemental Figure 1, page 67). In *Arabidopsis*, it was reported by Goodspeed *et al.*, that JA as well as SA levels are diurnally regulated, with JA-levels peaking in the middle of the subjective day (Goodspeed *et al.*, 2012). The physiological output of these oscillations is an entrainment of the plants to herbivory, making an anticipation of herbivore attack possible and thus enhancing the defence ability of the plant (Goodspeed *et al.*, 2013). In contrast to the experiments of this study, we did not examine the hormone levels of rice under so-called “free running”

conditions, exposing dark/light rhythm entrained plants to continuous light or darkness to examine endogenous oscillations without influences of external factors (Harmer, 2009). Nevertheless, we measured the hormone levels of dark/light rhythm entrained rice plants in the course of a whole day under physiological conditions. We could not observe any oscillations in the hormone levels in response to the dark-light changes and the JA-levels in the uninduced plants were generally very low. We therefore hypothesize, that in rice, JA is not regulated in response to the diurnal dark-light changes. To verify this hypothesis, of course it would be required to run experiments under a different setup, using "free-running" conditions, to examine the endogenous regulation. Nevertheless, our results indicate, that in rice in contrast to *Arabidopsis*, no diurnal oscillations of JA-levels take place. Hypothetically, this would imply, that rice plants do not anticipate attack, but respond to individual stresses with induced defences.

In contrast to the stable JA-levels of untreated rice plants, we could observe slight deviations in the hormone levels of 1 hour MecWorm wounded wild type and *phyAphyC* plants. While JA accumulated in higher amounts during the light phase and lower amounts in the dark (see Figure 16, page 40), JA-Ile levels were completely consistent during the course of the day (see Figure 17, page 42). The ratio calculated from JA-Ile and JA showed higher levels in the dark than in the light. This calculation method is not comparable to conversion rates of JA to JA-Ile, but nevertheless gives a hint on the hypothetical conversion rate. We therefore suggest, that the deviations in JA over the course of the day might be equalized by changing conversion rates. Thus, the JA-Ile levels are consistent throughout the day and the response to the 1 hour wounding treatment is always identical, regardless of the time of the day. Nevertheless it should be considered, that due to high deviations in the single measurements of the assay we only could observe tendencies without statistical verification. Thus, it would be very interesting to further investigate the conversion rate of JA to JA-Ile in wounded rice plants and to elucidate, if there are diurnal oscillations of JAR1 activity.

Moreover, in chapter 4.3.1 (page 55), we proposed a suppressive effect of active phy on JA-production, which results in increased JA-levels in response to wounding during dark phases (Arimura *et al.*, 2008; Zeier *et al.*, 2004). The results we retrieved from our short wounding experiments indicated the contrary, JA appeared to accumulate in higher amounts in response to wounding during the day compared to the night (see Figure 16, page 40). However, in rice wild type plants we observed the suppressive effect of phy on the JA-production only after long wounding periods of 6 hours, while in 1 hour MecWorm treated plants no difference between wild type and *phy*-mutants could be detected. Thus, we propose, that the suppressive effect of phy is only activated after a certain threshold of wounding period is reached. In this context the response to short wounding events of 1 hour might not be under the modulation of phy, which explains the similar JA-levels during day and night. To clarify this theory, a 6 hours wounding treatment during the dark phase should be conducted in the future.

Summarizing, over the course of a 24 hour day, no oscillations of JA or JA-Ile in untreated rice plants were noticed and the measured levels in these leaves were generally very low. While we could notice deviations in JA-levels of wounded plants, we could not verify these observations by statistical means. Thus, even though our results are suggestive of a slight diurnal oscillation of JA in response to wounding, more research is required to verify these data. Moreover, it should be examined, if the deviating hormone levels are a response to the changing light conditions during the day or actually caused by an endogenous circadian rhythm.

4.4.2 The conversion of JA to JA-Ile in wounded rice plants is not solely dependent on the JA-availability and potentially mediated by light

Throughout the experiments conducted in this study we could observe an interesting discrepancy in the proportion of JA to JA-Ile after wounding: the amounts of JA sometimes did not correlate to the JA-Ile levels measured at the same time point. To process these observations into comparable numbers, we calculated the ratio of JA-Ile to JA for every time point of the 24 hour series of 1 hour wounded plants (see Figure 18, page 43) and for the 6 hours MecWorm treated *phyBphyC* mutants (see Figure 10, page 31, JA-Ile/JA ratio: 32% opposed to 20% in the wild type, Student's *t*-test $p<0.05$). Even though these percentage ratios cannot be equated to a so called „conversion rate" of JA to JA-Ile, they give evidence on the turnover of JA to JA-Ile at the different time points.

Summarizing, these results indicate, that the production of JA-Ile is not exclusively dependent on the available amount of free JA, but that there are other regulation mechanisms, which might be influenced by the light conditions and/or the subjective time of day.

An influence of light conditions on the JA-Ile production implies that phy might be involved in this process, which can be supported by findings in other organisms. Next to the influence phy-signalling can exert on the JA-biosynthesis, also the bioactivation of JA appears to be modulated by light, sensed via phy. For example in *Arabidopsis* the abundance of the JAR1 enzyme, which catalyses the bioactivation of JA, appears to be positively regulated by FR light under the mediation of phyA (Robson *et al.*, 2010; Wang *et al.*, 2011). Other reports from experiments conducted in lima bean suggested, that certain JA modification steps like conjugation to amino acids as well as hydroxylation require light (Arimura *et al.*, 2008; Radhika *et al.*, 2010). Thus, the amount of active JAR1 or the conjugation reaction itself could be modulated by light, leading to an increased conjugation rate in the phytochrome mutants. Additionally, it should also be considered, that JA is not only converted to JA-Ile but a variety of other modifications of JA take place, which also might be subjected to activity deviations under different light conditions. Thus, even though JA might be available in the same amount in different situations, by modifications different from JA-Ile formation, available JA might be diverted from the conversion to JA-Ile.

Another very interesting hypothesis for the high JA-Ile levels in the *phyBphyC* mutant results from taking together all the information we gained by the hormone measurements. *PhyBphyC* not only overproduces JA in response to wounding but also contains generally elevated levels of ABA. We already discussed the possibly of an inductive effect of ABA on JA accumulation, which was reported by different studies (see chapter 4.2.2, page 52). Additionally, in wounded *N. attenuata* leaves, supplemented with exogenous ABA, an increase in JA-Ile accumulation could be observed in comparison to non-supplemented leaves, while the JA levels were not changed by the treatment (Dinh *et al.*, 2013). Thus, the increased levels of ABA in *phyBphyC* might contribute to the higher JA-Ile/JA ratio in this mutant. Nevertheless, the mechanism by which ABA can influence the conjugation of Isoleucin to JA remains elusive.

Additionally, we observed the interesting tendency that in 1 hour continuously wounded wild type plants, JA-Ile appeared to be produced more efficiently from JA during the dark phase of the 24 hour day (see Figure 18, page 43). In lima bean, Radhika *et al*. could show, that the conversion of JA to JA-Ile is light dependent (Radhika *et al.*, 2010). We did not observe such an effect in rice plants, but in contrast to the experiments conducted in this study, we did not expose the plants to continuous darkness or light. Thus, it might be, that the modulating effect of light on JA-Ile production is only noticeable after prolonged light or dark phases. Probably during the usual course of night and day, JA can be converted to JA-Ile during the night in day-like conversion rates, because required factors have accumulated during the preceding light phase and are only slowly wearing off. Nevertheless, our results hint at the possibility, that the formation of JA-Ile might be modulated differently in rice than in lima bean.

Summarizing, even if the mechanism underlying this effect remains to be elucidated and appears to be partly contrary to other studies, our results support the idea of JA-Ile formation being subjected to modulation by light.

4.5 Why are darkness and shading a potential danger for plant defence?

In this study we found evidence that light can influence the JA-mediated response to wounding on different levels in different ways, partially enhancing and partially downregulating single steps of the JA-pathway. The questions arise, why light exerts such a variable influence in the same pathway during the same response and why darkness apparently has a negative effect on plant defence.

The elevated accumulation of JA and JA-Ile in absence of active phy actually implies, that plants might express a better defence response during the night. However, our studies on the JA-responsive gene expression in the *phy*-mutants as well as evidence from a variety of reports indicate the contrary (see chapter 4.3.2, page 57). Thus, it is rather unlikely that JA-levels in response to wounding are elevated in the night to directly enhance plant defence

by increasing JA-responsive gene expression. However, one possible explanation for this effect might still be connected to a contribution to defence “enhancement”, in adaptation to the already lowered signal response. Maybe the plant tries to counteract the lowered signalling response on the level of gene expression by enhancing the levels of active hormone so at least a part of the defence response is sustained.

Still, as the JA-responsive gene expression is attenuated in absence of phy, the question remains, why darkness and shading can compromise JA-mediated defence responses. The most common hypothesis explaining this effect deals with the resource allocation problem between growth and defence (Herms & Mattson, 1992). Growth and gain of leaf material is important for photosynthesis, especially if the plant is growing in areas where not much photosynthetically active radiation (PAR) is available. As already described in the introduction, this holds especially true for plants growing in dense canopies, and shade intolerant plants are able to detect this competition via the R:FR ratio and subsequently express the SAS (see 1.4, page 10). Nevertheless, the hypothesis, that the expression of SAS leads to a deprivation of resources otherwise used for defence responses, causing a repression of defence in the process, could be challenged by studies in *Arabidopsis sav3* mutants (Moreno *et al.*, 2009). These mutants can sense the shade in canopies, but are not able to express the SAS phenotype due to an impairment specifically hindering the SAS induced auxin biosynthesis. Nevertheless, the defence against herbivory was compromised when these mutants were exposed to low R:FR ratios. This finding together with our observations in a crop plant, which presumably only marginally exhibits SAS, and the fact that not shading alone but also night can attenuate defence responses, hints at a conserved mechanism underlying this effect. During evolution features are mostly retained, if they positively influence fitness (Futuyma, 2005). Thus, it is very likely that, even if the fitness of the plant in respect of defence is lowered in the dark, the overall output of this whole interaction of signal is positive for the fitness of the plant in general. The lowered defence might be a “necessary evil” the plant has to accept in order to keep up the balance between defence and growth under certain unfavourable conditions like deprivation of light.

Thus, the downregulation of defence relevant JA-signalling at the level of transcription is presumably no side-effect of missing resources, but gene expression could be decreased deliberately to make a housekeeping of resources possible for the plant and to balance the costs of growth and defence.

4.6 Future challenges

In this study, we were able to gain intriguing new insights into the modulating functions of light and JA in the response to mechanical wounding. Still, many questions have to be answered in this field and our investigations gave rise to additional issues, which could be focus of research in the future.

4.6.1 Investigation of the ABA-JA interactions in wounding and the physiological output of ABA increase

We found, that ABA is increased by mechanical wounding, levels rising with increasing wounding period. Additionally, the induction of ABA by wounding turned out to be dependent on the presence of JA. Thus, we suggested that ABA and JA synergistically modulate the response to wounding in rice.

The next step in the investigation of the link between ABA and JA would be the examination of the wounding response in ABA-deficient mutant plants. The analysis of the JA and JA-Ile levels of such mutants could give evidence on the direction of the interaction of ABA and JA and clarify, if JA and ABA are equally contributing to the wounding response, or if one of the hormones is acting upstream of the other.

Moreover, the physiological effect of the ABA induction in response to wounding remains elusive. First evidences suggest stomatal closure upon wounding in rice, but these findings need to be verified. Thus, measurements of stomatal conductance (e.g. by a porometer) in wounded wild type and JA-deficient mutant plants could give insights into the impact of the ABA increase on the water household of the plant.

Moreover, the analysis of ABA inducible genes, like *OsLip9* (Nahar *et al.*, 2012), could clarify, if the measured amounts of ABA actually do induce a response on the level of gene expression. Additionally, to elucidate, if the rise in ABA is due to de novo production or rather a result of attenuated catabolism, the gene expression analysis of the gene families *OsNCED* and *OsABAox* could be a reasonable approach (Zhu *et al.*, 2009).

Additionally, it would be interesting to conduct these analyses in the *phyBphyC* mutant as well, as recent research already proposed a drought resistance for rice mutants lacking phyB (Liu *et al.*, 2012) and we could find a generally increased ABA level in these mutants.

4.6.2 Investigation of JAZ-protein stability in *phy*-mutants

The results of our studies in the *phy*-mutants were to some degree controversial, as we observed an increase of JA and JA-Ile while simultaneously the expression of JA-responsive genes was attenuated in these mutants. As an explanation for this enigma we suggested, that the degradation of JAZ-repressors, which are the crucial molecules mediating the JA-signalling response, might be disturbed.

In rice, it is not trivial to address the degradation of proteins, as transformation is not as fast and easy as in *Arabidopsis*. Thus, an approach like Robson *et al.* used in their study, visualizing the degradation of JAZ1 proteins in transformed seedlings by GUS-staining (Robson *et al.*, 2010), is rather complicated to conduct in rice plants.

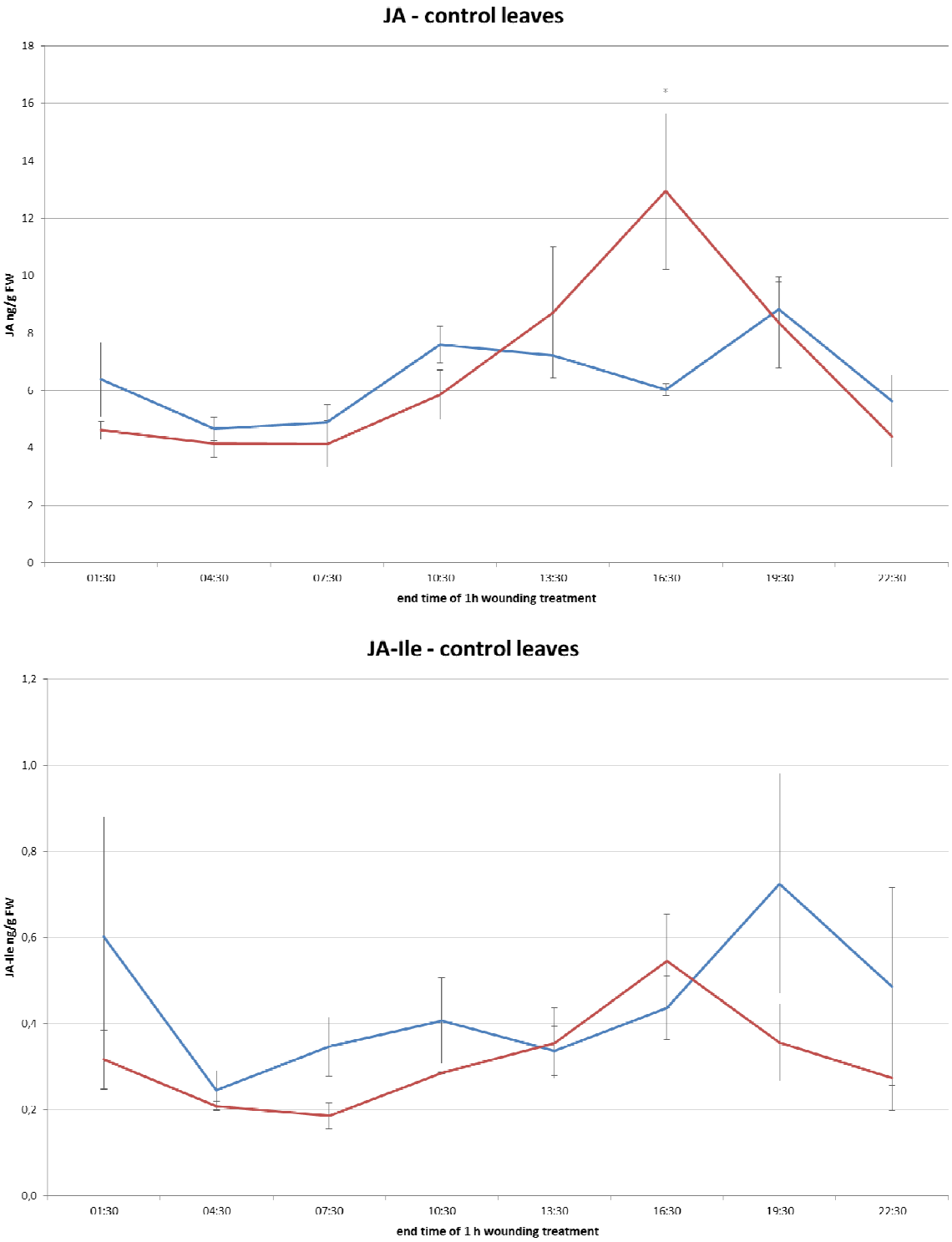
Nevertheless, monitoring of the JAZ turnover would be the procedure of choice to investigate our hypothesis further. So instead of directly examining the protein status, an analysis of the mRNA via RNA-seq might be a more feasible tool (Wu *et al.*, 2014). Certain splice variants of JAZ mRNA have been noticed to give rise to non-degradable JAZ proteins (Chung *et al.*, 2010). Thus, an extensive study of the mRNA pool in wounded wild type and *phy*-mutant plants could give evidence on the role of JAZ-stability in the light modulated wounding response.

4.6.3 Investigation of JA-Ile homeostasis in the diurnal context

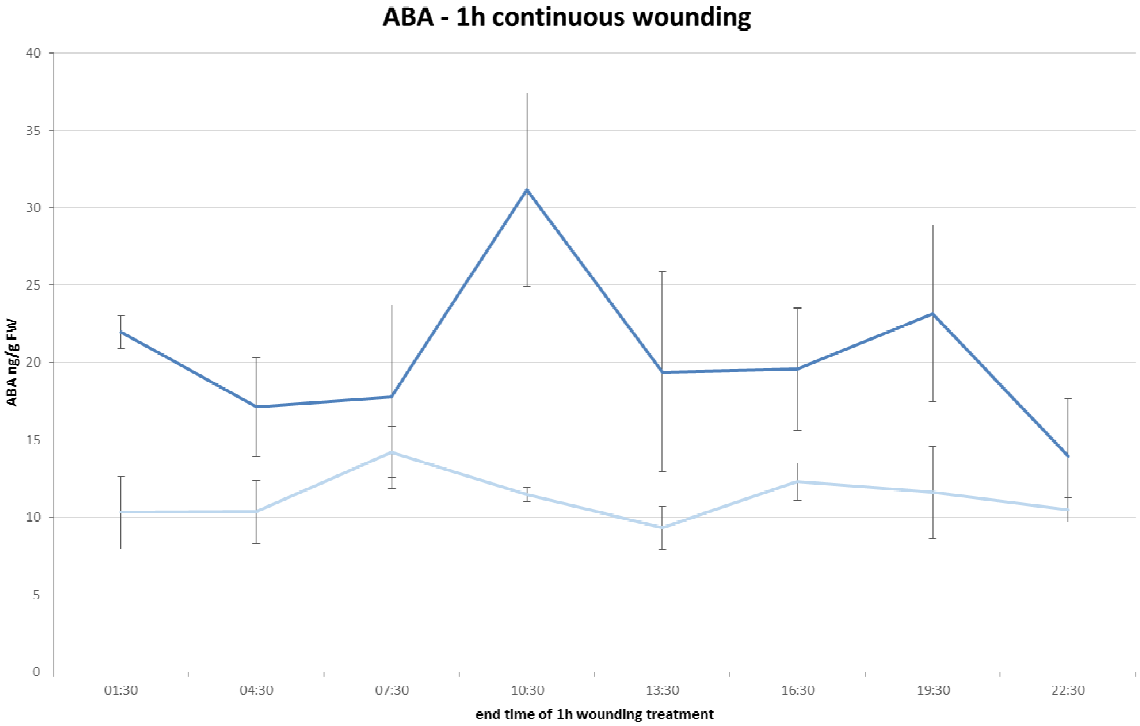
During our examination of hormone levels we found, that the JA-Ile content is not always correlated to the amount of available JA and that light appears to be a modulating factor in the JA-Ile production.

To further investigate the mechanism underlying this observation, it would be meaningful to monitor the JA-Ile turnover under different light situations or at different times of the day in wounded rice plants. Recently, a variety of metabolic products of JA and JA-Ile have been identified and the metabolic fate of JA and JA-Ile is at least partly elucidated (Koo & Howe, 2012). Therefore, the analysis of metabolic products like 12-OH-JA, 12-OH-JA-Ile and 12-COOH-JA-Ile could shed light on the metabolic pathways JA and JA-Ile are taking during the wounding response and if light conditions might change the equilibrium in one or the other direction. Especially the accumulation levels of JA-Ile catabolism products like 12-OH-JA-Ile and 12-COOH-JA-Ile could give new insights in the regulation of JA-responsive signalling, as the conversion into these products appears to be one route for deactivation of JA-Ile signalling capacity (Heitz *et al.*, 2012; Koo & Howe, 2012).

5 Appendix

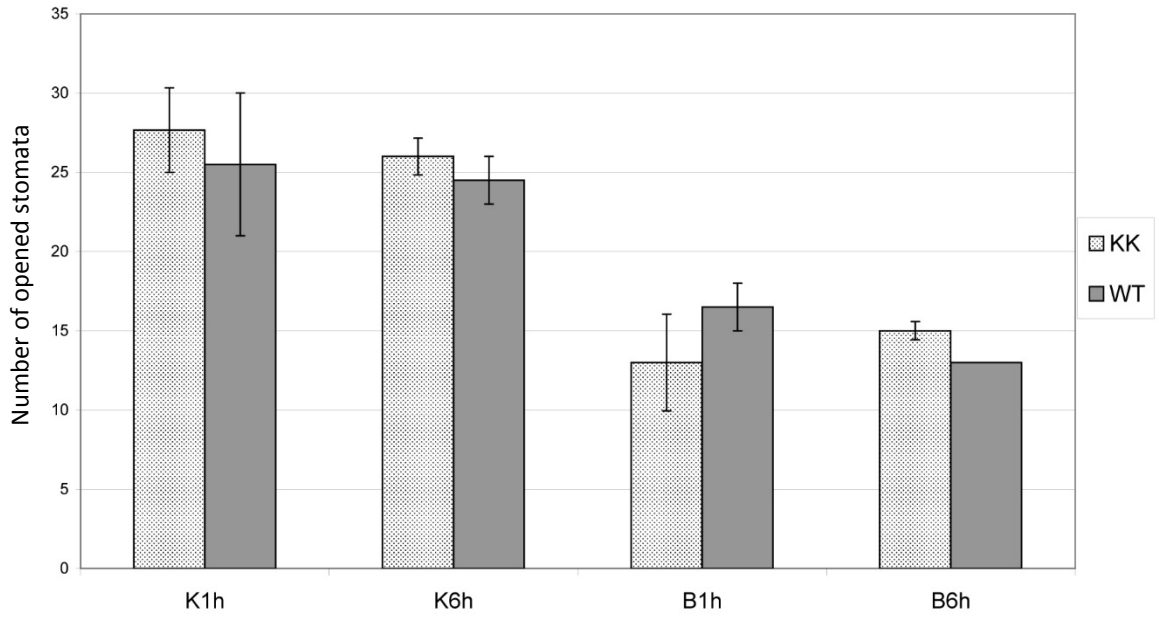


Supplemental Figure 1: JA and JA-Ile levels of rice plants. Comparison of diurnal JA (A) and JA-Ile (B) level changes in control samples of* phyAphyC *(red line) and the wild type (blue line).
Six weeks old rice plants were harvested at different time points during the day. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one asterisk (Student's t-test, $p < 0.5$).



Supplemental Figure 2: ABA levels of rice wild type plants (Nipponbare). Comparison of MecWorm wounded leaves (dark blue line) and control leaves (light blue line).

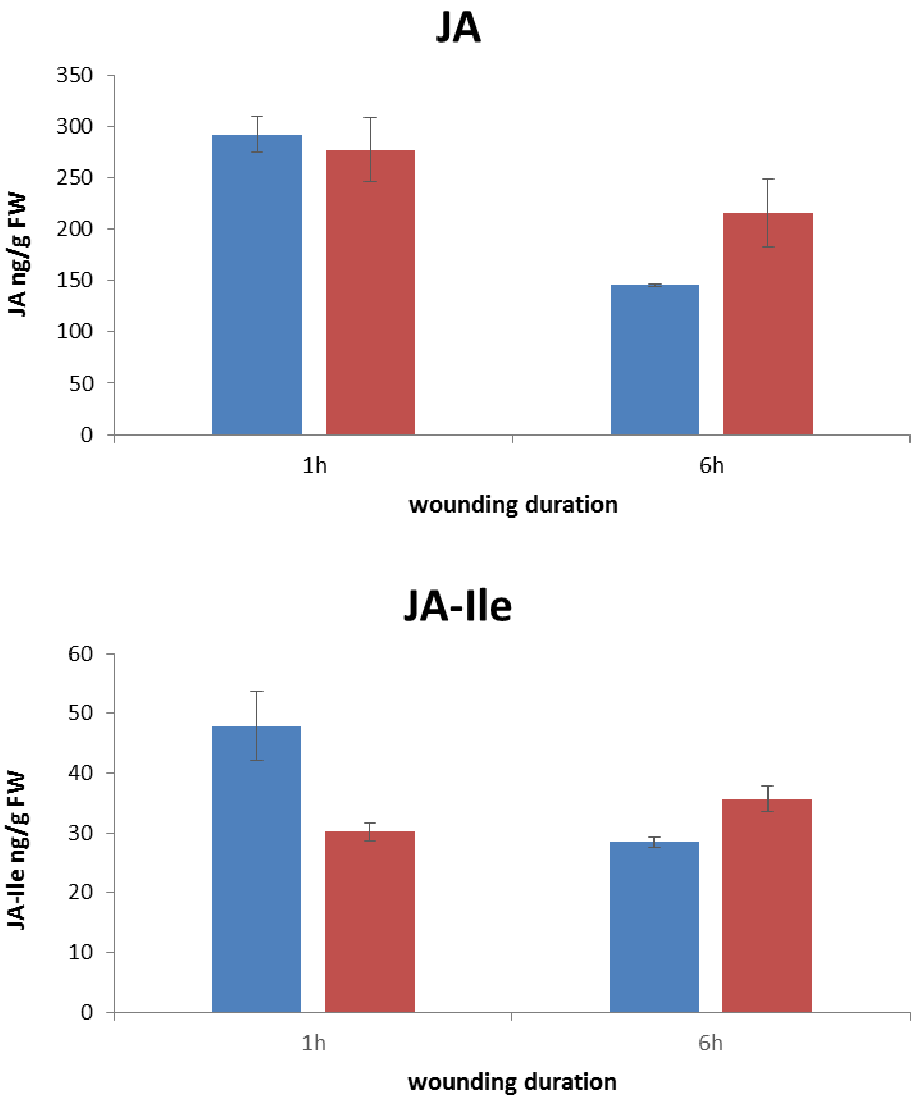
Six weeks old rice plants were wounded with MecWorm for 1 hour at different time points during the day, treatments ending at the indicated time points. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. Error bars indicate ±SE. Statistically significant differences to the wild type are indicated by one or two asterisks (Student's t-test, $p < 0.5$ or $p < 0.01$), respectively.



Supplemental Figure 3: Number of opened stomata in kamakubi (KK, light grey) and wild type (WT, dark grey) plants

Six weeks old rice plants were wounded with MecWorm for 1 hour or 6 hours. Leaf surface preparations of wounded (B) and control (K) leaves were made, using liquid glue, directly after the end of the treatment. 50 stomata were counted regarding their open/closed state. Results represent an average of at least two independent experiments. Error bars indicate ±SE.

(Figure taken from bachelor thesis of Svenja Wehrle, Botanical Institute, KIT, 2013.)



Supplemental Figure 4: JA (A) and JA-Ile (B) levels of rice plants at 4:30 p.m. Comparison of* phyAphyC *(red bar) to the corresponding wild type Nipponbare (blue bar).

Six weeks old rice plants were wounded with MecWorm for the indicated time intervals. Hormone levels are depicted relative to sample fresh weight and represented as an average of at least 3 independent experiments. Error bars indicate ±SE. Wild type plants accumulate significantly more JA and JA-Ile when treated for a shorter time period of 1 hour compared to 6 hours of treatment. (Student's *t*-test, $p < 0.05$ (JA) and $p < 0.01$ (JA-Ile)).

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