The Role of the Chloroplast Outer Envelope in Jasmonate Synthesis

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Summary

Jasmonates are vital plant hormones acting not only in stress response to biotic and abiotic influences, such as wounding, pathogen attack or cold acclimation, but also driving developmental processes in cooperation with other plant hormones. The biogenesis of jasmonates starts in the chloroplast, where several enzymatic steps produce the jasmonate precursor 12-oxophytodieonic acid (OPDA) from α-linoleic acid. OPDA is exported from the chloroplast and imported into peroxisomes where JA (jasmonate acid) is produced. Subsequently, JA is transported into the cytosol for further conversion into active jasmonates, which in turn induce the expression of multiple genes in the nucleus. Despite its obvious importance, the export of OPDA across the chloroplast membranes remains elusive.

In this study, I have characterized a novel protein residing in the chloroplast outer membrane, JASSY, which proved to be indispensable for the export of OPDA from the chloroplast. In the first part of my thesis, I show that JASSY is a chloroplast outer envelope membrane protein. Furthermore, I characterize the *Arabidopsis jassy* mutant. For the *jassy* mutant, no growth phenotype was detectable under the normal conditions, but the *jassy* mutant was much more sensitive to cold stress or pathogen attack compared with the WT. Moreover, we show that JASSY has channel-like properties and proposed that it thereby facilitates OPDA transport. Consequently, the lack of JASSY in *Arabidopsis* leads to a deficiency in the accumulation of jasmonic acids, which results in impaired expression of jasmonate-responsive gene upon exposure to various stresses.

Zusammenfassung

Jasmonsäurederivate sind Pflanzenhormone, die eine bedeutsame Rolle bei der Stressantwort auf biotische Einflüsse, sowie Verwundung, Pathogenbefall oder Kälteakklimatisierung spielen. Darüber hinaus sind sie essentiell für die pflanzliche Entwicklung, wobei sie mit anderen Pflanzenhormonen gemeinsam wirken. Die Synthese von Jasmonsäuren beginnt im Chloroplasten, wo verschiedene enzymatische Schritte die Vorstufe 12-Oxophytodiensäure (OPDA) aus α-Linolensäure herstellen. OPDA wird anschließend in den Peroxisomen und im Zytosol weiter zu den aktiven Jasmonsäuren umgewandelt, die daraufhin im Zellkern die Expression einer Vielzahl von Genen regulieren. Trotz seiner zweifelsfreien Wichtigkeit, bleibt der Exportprozess von OPDA aus dem Chloroplasten bislang im Dunklen.

In dieser Arbeit habe ich ein neues Protein in der Chloroplastenhüllmembran charakterisiert, JASSY, welches sich als unentbehrlich für den Export von OPDA erwiesen hat. Im ersten Teil meiner Arbeit zeigen ich, dass JASSY in der äußeren Chloroplastenmembran lokalisiert ist. Des Weiteren habe ich eine jassy Verlustmutante charakterisiert. Obwohl die Mutante unter normalen Wachstumsbedingungen keinen Phänotyp zeigt, stellte sich heraus, dass *jassy* deutlich sensitiver auf Kältestress oder Pathogenbefall reagiert. Darüber hinaus konnten wir zeigen, dass JASSY kanalähnliche Eigenschaften besitzt und schließen daraus, dass das Protein am Export von OPDA beteiligt ist. Im Einklang damit wird auch gezeigt, dass in der Mutante keine Jasmonsäure akkumuliert, was zu einer Störung der jeweiligen Genexpression als Reaktion auf verschiedenste Stressoren führt.

Abbreviations

ABA	Abscisic acid
At	Arabidopsis thaliana
BR	brassinosteroids
C-terminus	carboxyl-terminus
cTP	chloroplast transit peptide
DEPC	Diethylpyrocarbonate
dNTPs	Deoxyribonucleic Acid
DTT	dithiothreitol
EDTA	Ethylene Diamine Tetra-acetic Acid
E.coli	Escherichia coli
FNR	ferredoxin-NADP[H] oxidoreductase
gDNA	genomic DNA
GFP	green fluorescence protein
HPLC	High-performance Liquid Chromatography
IE	inner envelope/inner envelope membrane
kDa	kilo Dalton
Ile	isoleucine
IPTG	isopropyl-ß-D-thiogalactopyranoside
JA	Jasmonic acid
MeJA	Methyl jasmonate
Met	methionine
mRNA	messenger-RNA

Ni-NTA	nickel-nitrilotriacetic acid
N-terminus	amino-terminus
OE	outer envelope/outer envelope membrane
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
Ps	*Pisum sativum*
PVDF	polyvinylidene difluoride membrane
RT	room temperature
RT-PCR	Reverse Transcription-PCR
RT-qPCR	Real- time quantitative PCR
SA	Salicylic acid
SDS	sodium dodecyl sulphate
Sec	secretory
StAR	Steroidogenic acute regulatory
START	StAR-related lipid transfer
TEMED	Tetraacetylethylenediamine
Tic	translocon at the inner envelope of chloroplasts
Toc	translocon at the outer envelope of chloroplasts
TP	transit peptide
Tm	Temperature melting
TM	transmembrane (domain)
WT	wild type

1. Introduction

1.1 Introduction of Jasmonates

Plants are continuously challenged by various biotic (e.g. cold, high light, drought and salt) and abiotic factors (e.g. pathogens and insect wounding). The survival of the plants under different adverse conditions relies on their ability to perceive external signals and respond to these signals in time (Zhu, 2016). Plant hormones are known as phytohormones which are produced within plants and expressed in extremely low concentrations under normal conditions, but their synthesis pathways are activated rapidly and substantially upsurge after the plants receive the threatening signal from the environment (Davies, 2010). To date, current studies of signaling pathways by which plants responsed to the different biotic and abiotic stresses are still in processing.

Plant hormones (like abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA)) play a key role in almost every processe of plant growth and development, from seedllings to mature plants (Waadt et al., 2015). Also, they play an important role in plants stress tolerance and pathogen defense (Davies, 2010). Depending on chemical structures, plant hormones can be separated into different families. Within a certain family, different hormones have similar structures but their physiological effects may vary (Wang and Irving, 2011). To start with, plants' hormones can be classified into five major families: ABA, auxin, cytokinins, ethylene and gibberellins. Eventually, this list expands to include brassinosteroids (BR), jasmonates (JAs) and salicylic acid (SA) (Wang and Irving, 2011). Among these members, JA and its metabolites are collectively called jasmonates (JAs) which response to environmental stimuli via transcriptional programming and orchestrate the growth and development of plants (Wasternack, 2007).

JAs are known as a family of cyclopentanone oxylipins derived from the fatty acid linolenic acid via lipid peroxidation (Wasternack and Hause, 2013). JA was originally isolated and purified from *Jasminum grandiflorum* by Hesse and Muller in 1899 (Patent, 1899). In 1980, JA was first documented to play a role in leaf senescence (Ueda and Kato, 1980) and seedling germination (Dathe et al., 1981). Later on, JA and its derivatives have been proven to be involved in fruit ripening (Ziosi et al., 2008),

production of viable pollen (Browse, 2009) and root growth (Xie et al., 1998; Lorenzo, 2004), tendril coiling (Malabarba et al., 2018). Besides playing a role in plant growth and development, JA also plays an important role in plant responses to many biotic and abiotic stresses. Upon injury, JA is induced to regulate the expression of certain genes and proteins, which in turn slow down the metabolic processes and plant growth (Acosta and Farmer, 2010). JA modulates gene expression at the level of transcription, RNA processing, and translation (Acosta and Farmer, 2010).

1.2 Biosynthesis of JA

The pathway of JA biosynthesis has been identified as a route of oxidization of fatty acid that takes place in both, chloroplasts and peroxisomes (Fig. 1) (Wasternack, 2007). The JA biosynthesis pathway starts from 18C fatty acid α-linolenic acid 18:3 (α-LA), which is released by phospholipase 1 from galactolipids in the chloroplast thylakoid membranes. Then, α-LA is oxidized by a lipoxygenase (LOX) and HPOT (13S-hydroperoxy linolenic acid) is produced (Schaller and Stintzi, 2009). In Arabidopsis, there is a total of six LOXs, and LOX2 is thought to drive the bulk of the JA formation during the first two hrs after initiation (Bell et al., 1995; Feussner et al., 2002). Subsequently, 13-allene oxide synthase (AOS) catalyzes dehydration of HPOT and forms allene oxide which is cyclized by 13-allene oxide cyclase (AOC) and produces OPDA (12-oxo-phytodienoic acid) (Fig. 1) (Hamberg and Fahlstadius, 1990; Song and Brash, 1991), all the reactions from α-linolenic acid to OPDA proceed within the chloroplast (Fig. 1). The second half of JA biosynthesis takes place in peroxisomes as OPDA is transported out of the chloroplast and imported into the peroxisome (Wasternack and Hause, 2013). However, how OPDA is transported through chloroplast envelope membranes is still unknown. Also, how OPDA is transported into the peroxisome is still under debate. So far, there is just one peroxisome localized protein known so far, COMATOSE, which might play a role in OPDA transport. It has been shown that COMATOSE (CTS) belongs to the ATP binding cassette (ABC) transporter class and links the JA transporter with the peroxisome (Theodoulou, 2005). However, the CTS mutant (in Arabidopsis) still can

produce some JA (Nyathi et al., 2010). According to this, CTS might not be the only OPDA transporter in peroxisome.

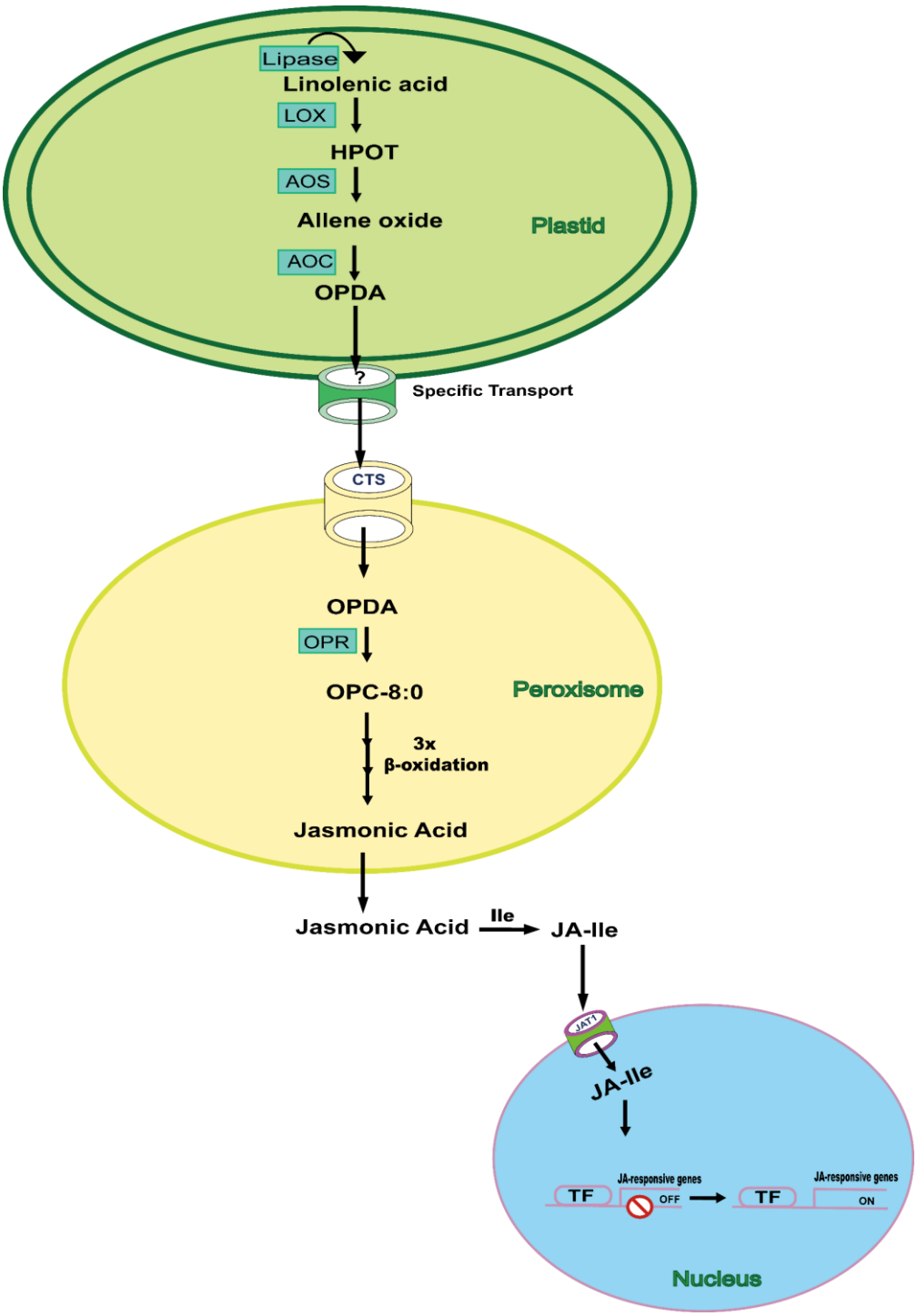


Figure 1. The JA biosynthesis pathway: The biosynthesis of JA takes place in both chloroplasts (green) and peroxisomes (yellow). In brief, the JA biosynthesis starts from the cleavage of the membrane lipids via lipase to linolenic acid. Linolenic acid is converted to OPDA by three different enzymatic (LOX, AOS, AOC) steps. OPDA can be transported out of the chloroplast with unknown machinery and transported into the peroxisome. In the peroxisome, OPDA is further converted to JA through OPR and subsequently ß-oxidation steps. Subsequently, JA released to the cytosol and conjugated with Ile via JAR1. The transporter JAT1 allows JA-Ile import to the nucleus to active the expression of specific genes. Abbreviations: LOX, 13-lipoxygenase; 13-HPOT, 13S-

hydroperoxyoctadecatrienoic; AOS, Allene oxide synthase; AOC, allene oxide cyclase; OPDA, 12-oxo-phytodienoic acid; OPR3, 12-Oxophytodienoate reductase 3; OPC: 3-OXO-2(2' 2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid reductase.

After OPDA is transported into the peroxisome, it is reduced by the peroxisomal OPDA reductase (OPR3) (Schaller et al., 2000). These cyclic intermediates are then processed by the peroxisomal fatty acid β-oxidation machinery via three steps of oxidation and the last produced, JA, is formed (Fig. 1) (Wasternack and Hause, 2013).

1.3 Metabolism of JA

JA is in turn exported to the cytosol from the peroxisome. In the cytosol, there are many conjugation forms of JA, like JA-Ile, MeJA, JA-ACC, JA-Glc and 12-HSO_4-JA produced. However, it was demonstrated that only MeJA (Farmer and Ryan, 1990) and JA-Ile (Li et al., 2017) are the active forms.

When plants are under the stress, there is a rapid increase of endogenous JA which can be conjugated to isoleucine via JAR1 (JASMONATE RESISTANT 1) (Staswick et al., 1998), and thus produces the major biologically active form JA-Ile (Staswick, 2004; Li et al., 2017) (Fig. 1). Subsequently, JA-Ile binds to the F-box protein COI1 (CORONATINE INSENSITIVE 1) changing the conformation of COI1 (Xie et al., 1998). The structural changes allow the binding between COI1 and JAZ (JASMONATE ZIM DOMAIN), wherein JA-Ile acts as a molecular glue to stabilize the association of JAZ and COI1 (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). COI1 in turn is involved in the ubiquitin proteasome degradation machinery and forms a Skp1/Cullin/F-box $^{(SCFCOI1)}$ complex, which has an E3 ubiquitin ligase activity. The formation of this complex results in ubiquitination and degradation of JAZ, thus removing the suppression effects of JAZ and allowing the transcription factors to activate the expression of JA response genes (Fig. 2B) (Thines et al., 2007; Chini et al., 2009; Yan et al., 2009; Wasternack and Song, 2017). However, under normal conditions, the formation of JA-Ile is impeded due to the low levels of endogenous JA. The JAZ protein binds to various transcription factors, and inhibits the activity of these transcription factors thereby preventing the expression of JA-responsive genes (Fig. 2A) (Chung et al., 2008).

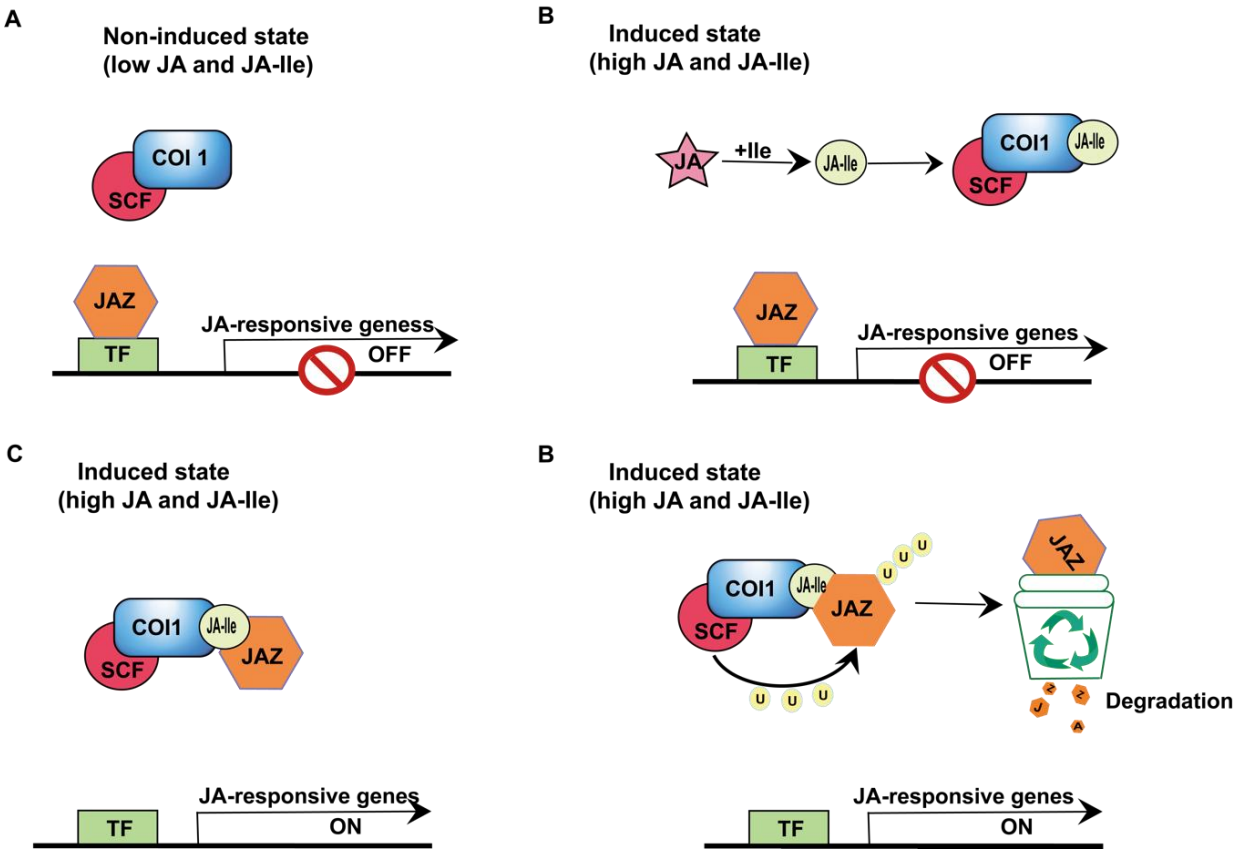


Figure 2. A Model of JA-Ile signalling in Arabidopsis. (A) Under the normal conditions, JAZ proteins bind to transcription factors (TFs) and represses the expression of TFs. **(B)** Under the stress conditions, JA-Ile is formed and binds to SCF^{COI1} **(C)** JAZ is released from the TFs and subsequently binds to the JA-Ile and SCF^{COI1} complex. **(D)** SCF^{COI1} targets JAZ protein for ubiquitination and subsequent degradation. As a direct consequence of JAZ removal, JAZ-associated TFs are de-repressed, initiating the transcription of JA-dependent genes.

In total there are two different branches of JA signalling downstream of JAZ repressors: the MYC branch (Dombrecht et al., 2007) and the ERF branch (Lorenzo, 2003). The MYC branch is responsible for the wounding response (Lorenzo, 2004; Verhage, 2011; Schmiesing et al., 2016) and the ERF branch is activated upon pathogen threatening (Pieterse et al., 2002). MYC2, a basic helix-loop-helix transcription factor, was the first transcription factor identified and found to regulate the expression of multiple genes which are involved in reacting to various stresses, such as insects/herbivores attack (Pieterse et al., 2002; Lorenzo, 2004). It has been reported that MYC2 is a major transcription factor which is regulated by the JA pathway (Lorenzo, 2004). MYC2 binds to the G-box sequence (5'-CACGTC-3'), and this binding can regulate the expression of JAZ2 (Fig. 3A) (Pieterse et al., 2002;

Figueroa and Browse, 2012; Ezer et al., 2017). Under the unchallenged environment, MYC2 binds to the G-box sequence and JAZ. JAZ represses the expression of JA-responsive genes. When plants were under stress, the degradation of JAZ frees MYC2 and triggers the expression of JA-responsive genes (Fig. 3B) (Figueroa and Browse, 2012).

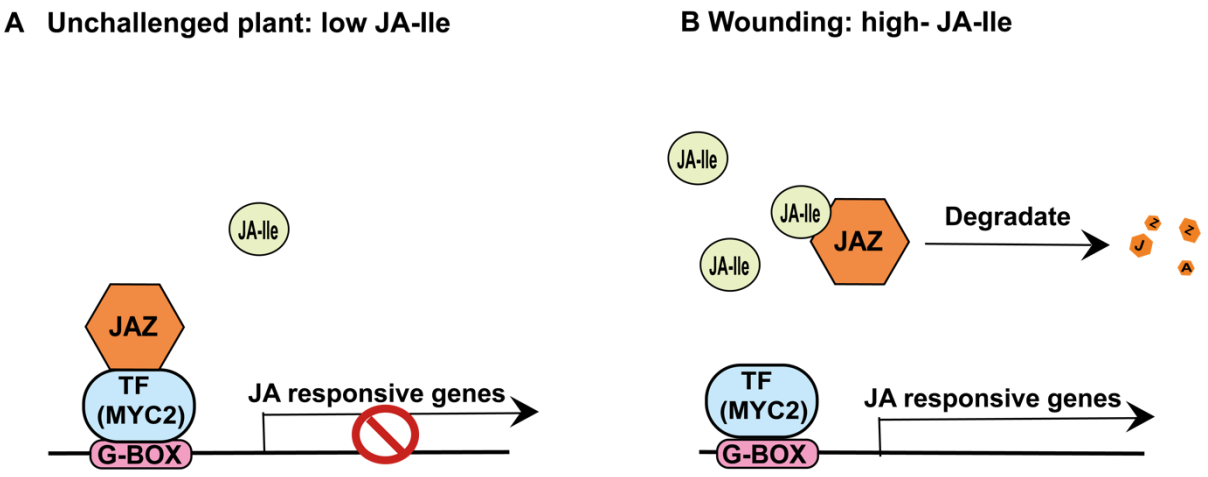


Figure 3. MYC branch in JA signaling pathway. (A) Under normal conditions, due to the low concertation of JA-Ile, JAZ inhibits the transcriptional factor MYC2, and prevents the expression of JA-responsive genes. **(B)** When wounding, formation of JA-Ile promotes degradation of JAZ, thus releasing MYC2 to trigger expression of JA-responsive genes via binding to G-box motifs.

The ERF branch contains the ETHYLENE RESPONSE FACTOR1 (ERF1) and OCTADECANOID RESPONSIVE ARABIDOPSIS AP2 59 (ORA59) which regulate the expression of JA-responsive marker gene PLANT DEFENSIN1.2 (PDF1.2) via binding to the specific sequence (5'-GCCGCC-3') (Pre et al., 2008). Interestingly, the ERF branch and MYC branch were reported to be antagonistic to each other. For example, MYC2 negatively regulates some genes in the ERF branch like PDF1.2. However, the antagonistic mechanism is still unclear (Fig. 4) (Pieterse et al., 2002; Zarei et al., 2011).

Under the normal environment, due to the absence of JA or low concertation of JA-Ile in the plant, JAZ inhibits the activation of the transcriptional activator ERF1, preventing the transcription of ORA59. ORA59 is another important transcription

factor which regulates the expression of JA-responsive genes. But under pathogen attack, JA-Ile promotes degradation of JAZ, thus releasing ERF1 to trigger expression ORA59. ORA59 in turn interacts with specific sequence (5'-GCCGCC-3') to induce the expression of JA-responsive genes (Leon-Reyes et al., 2009) (Fig. 4).

Besides being involved in response to biotic stresses, JAZ has been identified in several studies as a repressor of the transcription factors *ICE1* and *ICE2* (INDUCER OF CBF EXPRESSION), which activate the C-repeat binding factor (CBF) pathway. Thereby, several genes responsible for cold and freezing tolerance are activated (Sanders, 2000; Chinnusamy et al., 2007; Hu et al., 2017).

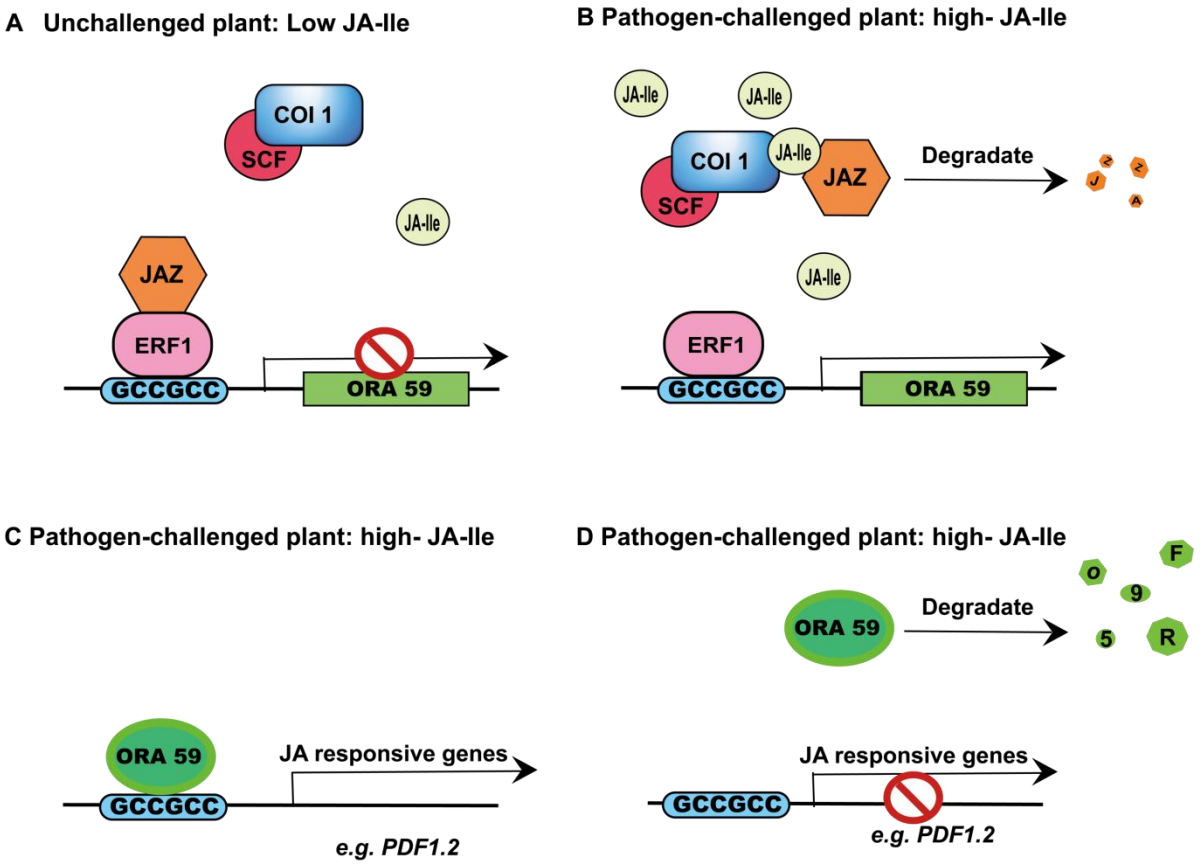


Figure 4. ERF branch of JA signaling pathway. In the absence of JA, JAZ inhibits the transcriptional activator ERF1, thereby preventing the transcription of ORA59 **(A)**. Under the pathogen challenge, JA-Ile promotes degradation of JAZ, thus releasing ERF1 to trigger expression of ORA59 **(B)** Subsequently, ORA59 is induced that activates the ERF branch of the JA pathway via binding to specific sequence (5'- GCCGCC-3') and induces the expression of JA-responsive genes **(C). (D)** The degradation of ORA59 slowdown or stop the expression of JA-responsive genes.

A novel protein, we termed as JASSY(AT1g70480), was first reported to be a novel protein in the chloroplast outer membrane and its function prediction suggest it to be involved in the JA biosynthesis pathway. Jassy contains a START (StAR (Steroidogenic Acute Regulatory) -related lipid transfer) lipid transfer domain. It has a conserved motif which belongs to the Bet v1-like superfamily.

1.4 The Bet v1-like superfamily

The major birch pollen allergen, Bet v1, is a member of the ubiquitous PR-10 family of the plant pathogenesis-related protein (Radauer et al., 2008). The Bet v1 superfamily consists of eleven sub-families, including the START domain family, phosphatidylinositol transfer proteins family, ring hydrolases α-chain protein family, polyketide cyclases, AHSA1 domain protein family, CoxG family, CalC-related proteins family, homotrimeric ring hydroxylases family, PA1206-related proteins family and the Smu440-related proteins family (Radauer et al., 2008). The members in this superfamily share a similar three-dimensional structure, but have low sequence identity. They fold into a prototypic Bet v1 structure and the common secondary structure is characterized by a β-α2-β6-α fold, forming an U-shaped incomplete β-barrel wrapped around a long C-terminal α-helix, which is forming a large hydrophobic binding cavity (Fig. 5A). The structure of the START domain differs from the Bet v1 structure by an N-terminal extension which contains an α-helix on the outer facing to the β-sheet part and two additional β-strands adding to the β-sheet on the side of N-terminal stand (Fig. 5B). PF01852 is the START family signature on Pfam (https://pfam.xfam.org). More than 2500 sequences from 400 species ranging from bacteria to eukaryotes that contain the START domain can be found on this website. Moreover, 40 structures are available, most of them derived from human proteins. The prototypic member of this family, STAR, plays a role in stimulating and transporting of cholesterol from the outer to the inner mitochondrial membrane, whereas it is required as a substrate for steroidogenic enzymes (Miller, 2007). Most of the protein which contain a START domain are mammalian cytosolic acetyl-CoA

hydrolases (Suematsu et al., 2001), and the rest members are HD-ZIP family of homeodomain transcription factors (Ariel et al., 2007).

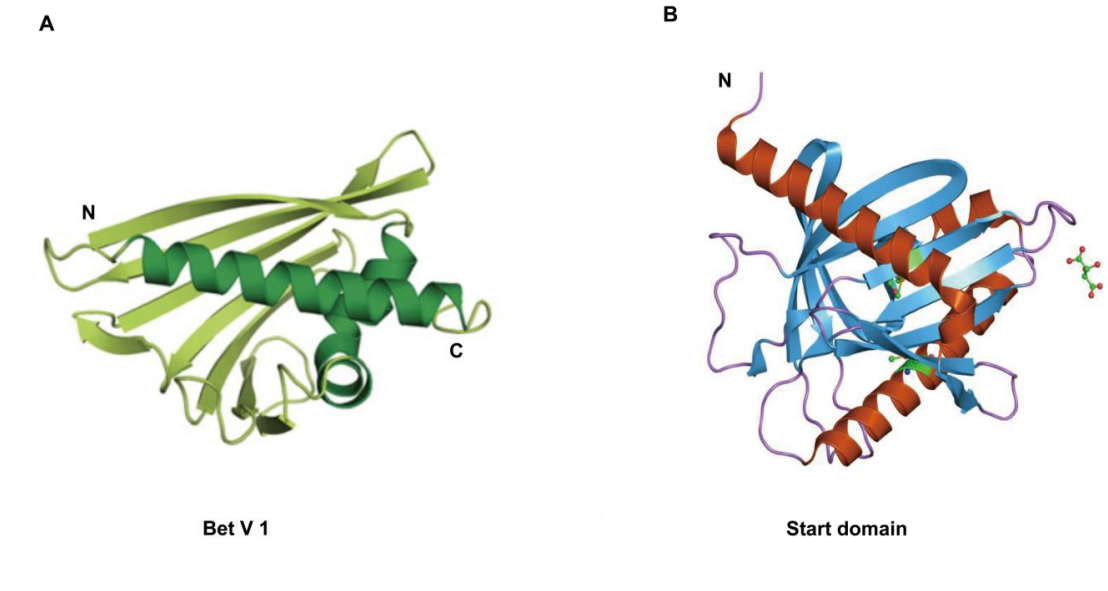


Figure 5. Structures of representative members of Bet v1-like superfamily. (A) The birch (Betula verucosa) pollen allergen Bet v1 (Kofler et al., 2012). **(B)** The human (Homo sapiens) START-related lipid transport domain (Radauer et al., 2008).

1.5 START domain containing proteins

The StAR (Steroidogenic acute regulatory) proteins play a role in binding and transferring lipids between the intracellular membranes in mammals (Miller, 2007). The START (StAR-related lipid transfer) domain is defined as a motif of around 200 amino acids implicated in lipid or sterol binding (Schrick et al., 2004). This kind of binding is important for the stability of the membrane (Eyster, 2007). START domain containing proteins are present in a wide range of species like animals, plants and a few bacteria but absent in yeast. It is suggested that a conserved mechanism of the START containing proteins is ligating the lipids and sterols (Ponting and Aravind, 1999). In humans, most of the START domain proteins are found as multi-domain proteins that play different roles in various processes. For example, mutations of the START domain caused congenital adrenal hyperplasia. Similarly, a protein homologous to StAR protein was found to be over-expressed in breast cancer in humans (Ponting and Aravind, 1999). Moreover, the START1 protein plays a key role in the synthesis of ecdysteroid (Roth et al., 2004). In 2002, X-ray assays revealed the

α-helix fold secondary structure of the START domain (Roderick et al., 2002; Romanowski et al., 2002). Based on the solved structure, it was demonstrated that one protein of the family of START domain proteins, named phosphatidylcholine transfer proteins, is conserved from animals to plants (Schrick et al., 2004).

In plants, it has been reported that the purified START domain containing proteins were predominantly associated with homeodomain transcription factors (Schrick et al., 2004). In Arabidopsis, the START domain containing proteins can be separated into many different families. (1) HD-START (homeodomain, a DNA-binding domain) family, to which majority of Arabidopsis START domain containing proteins belong and act as plant-specific HD transcription factor. (2) Another Arabidopsis START domain containing protein family consists of proteins with a PH (Pleckstrin Homology) domain together and DUF domain. The DUF (Domain of Unknown Function) domain is specific for the plants but with unknown function. (3) A small part of the proteins just contains START-DUF domain alone and (4) few of them just have the START domain only (Schrick et al., 2004).

The function of the START proteins with HD and PH domains has been reported to regulate plant development, and the corresponding knock-out mutants involved in the sterol biosynthesis have a similar phenotype. For example, the Arabidopsis EDR2 gene encodes a START protein with PH and DUF domain that acts as a negative regulator of pathogen induced resistance (Tang et al., 2005). The EDR2 is involved in binding of lipids, probably via its active START domain (Vorwerke et al., 2007).

1.6 Aim of this work

The synthesis of the JA precursor, OPDA, in chloroplast is fairly well understood. However, the of OPDA transport out of the chloroplast remains elusive. Recently, a protein of yet unknown function was identified in a proteomics study of outer chloroplast envelopes (Simm et al., 2013). Interestingly, the corresponding gene was found to be co-expressed with a number of genes involved in JA response. This protein, which we termed as JASSY (AT1g70480), contains a START lipid transfer domain, thus suggesting a function in the binding and/or transport of hydrophobic molecules. Based on these information, we suspected that the JASSY protein might be

involved in the JA mediated stress responses. Considering the function of the JASSY domain and the biosynthesis of JA, it sounds reasonable that the JASSY might participate in transport of JA biosynthesis intermediates out of chloroplast membrane. Our work is to study the function of the JASSY protein and decipher its role in the JA biosynthesis/signaling.

2.Material and Methods

2.1 Material

2.1.1 Chemicals

All chemicals used in the following experiments were purchased from Sigma (Steinheim), Roth (Karlsruhe), Duchefa Biohemia (Haarlem, Netherlands), J.T. Baker Chemicals (Deventer, Netherlands), Fluka (Buchs, Scheiz), Merck (Darmstadt), Serva (Heidelberg), Roche (Penzberg, Germany). The Whatman paper used for immunoblot was received from Macherey-Nagel (Duren) and the PVDF membrane was from Millipore Corporation (Billerica, USA). [^{35}S-Met/Cys] amino acid mix was ordered from Perkin Elmer (Dreieich, Germany).

2.1.2 Enzymes and Kits

Phusion DNA polymerase, Taq DNA Polymerase, restriction enzymes (SalI and NotI), SP6 RNA polymerase and M-MLV Reverse Transcriptase were ordered either from New England Biolabs (Frankfurt am Main, Germany) or Thermo Fisher Scientific. RNase-free DNase I was ordered from QUIAGEN, Germany. BP and LR Clonase TMII kit and Proteinase K for Gateway cloning were purchased from Invitrogen. Macerozyme R10 and Cellulase Onzuka R10 for protoplast isolation were obtained from Serva. The QIAprep Spin Miniprep Kit, Nucleobond Xtra Midi Kit and the NuceloSpin Plasmid Easy Pure Kit for isolating plasmid DNA from *E.coli* were purchased from QUIAGEN. In order to extract PCR products from agarose gels, the NucleoSpin plasmid Kit and PCR Clean-up Kit were used and were ordered from Macherey-Nagel in Duren. RNase Plant Mini Kit was used for RNA isolation from Qiagen (Hilden). Fast Start DNA Master SYBR-Green Plus Kit was used for quantitative RT-PCR which were purchased from Roche. For *in vitro* translation the reticulocyte lysate translation kit was bought from Promega.

2.1.3 Molecular weight and size markers

Pep Gold protein marker I was used for SDS-PAGE (VWR, Ismaning, Germany). Lamda phage DNA marker was used for DNA fragment size analysis on agarose gels.

2.1.4 Oligonucleotides

DNA oligonucleotides were ordered from Metabion (Martinsried, Germany). The sequence is on the list Table 1.

Table 1: List of oligonucleotides

name of oligonucleotide	5'-3' oligonucleotide sequence	purpose
At1g70480 Not I R	ATATGCGGCCGCGAACAAGCAGCTTTGTGG	pET51b(+)
At1g70480 SalI F	ATATACACTTGCTTGAATTTCATGTCGAC	pET51b(+)
At1g70480 LP	TCTGCATCGTTTTCATCACAG	genotyping
At1g70480 RP	TGTTGGTTTACCTCCTGATGC	genotyping
65-At1g70480 Smal1	TATACCCGGGATGGGCAATCCGGGATCAGA	pSP65
65-At1g70480 NotI	ATATTCTAGATTAACACAAATCATTGCGTCG	pSP65
At1g70480 GW F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAA GGAGATAGAACCATGGGCAATCCGGGATC	pAUL11
At1g70480 GW R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCT CCGGATCCACACAAATCATTGCGTCGTCT	pAUL11
At1g70480 GW stop F	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCT CCGGAACTTCAATCTGTGTACAAAACGAA	pK7FWG2.0
At1g70480 GW stop F	GGGGACCACTTTGTACAAGAAAGCTGGGTCTC CACCTCCGGATTCACCAAATCATTGCGTCGTCT	pK7FWG2.0
pDONR207 F	TGCCATCCAGCTGCAGCTCTGGCCCGT	scequcing

pDONR207 R	AATCATTATTTGGGGCCCGAGATCCATGC	scequcing
LOX2 RT F	ACGCTCGTGCACGCCAAAGT	RT-PCR
LOX2 RT R	TCCTCAGCCAACCCCCTTTTG	RT-PCR
AOS RT F	AAGCCACGACGCGGCGTTTA	RT-PCR
AOS RT R	GGAGTCTCCGTCTCCGGTCCA	RT-PCR
b-actin JA F	AGTGGTCGTACAACCGGTATTGT	RT-PCR
b-actin JA R	GATGGCATGAGGAAGAGAGAAAC	RT-PCR
AtMYC2 RT F	TTTGCAACGGGTAACGCGGTTT	RT-PCR
AtMYC2 RT R	CACGCAATAGTATGCATCCCAAA	RT-PCR
JAZ10 RT F	AGCAACGACGAAGAAGGCTTCAA	RT-PCR
JAZ10 RT R	ACGACTCCAAAACCGTGTGCAA	RT-PCR
PDF1.2 RT F	ATGGCTAAGTTTGCTTCCA	RT-PCR
PDF 1.2 RT R	TTAACATGGGACGTAACAGATAC	RT-PCR
ICE1 RT F	GGGAACAGAGCGAAAGTTCTG	RT-PCR
ICE1 RT R	TCCATATCTCCATCATCACTAAA	RT-PCR

2.1.5 Vectors

In order to overproduce proteins, pET51b (+) was used. pSP65 and pF3A vectors were used for *in vitro* transcription and translation. For transient or stable expression of proteins in plant cells, the binary Gateway vectors pK7FWG2.0, pAUL2 and pAUL11 via pDONR207 were utilized. All plasmids used for this thesis are listed in Table 1.

2.1.6 Bacterial strains

For propagating plasmid DNA in *E. coli*, competent TOP10 and DH5α (Invitrogen, Karlsruhe, Germany) cells were used. The *E. coli* BL21(DE3) pLysS strains (Novagen or Merck, Darmstadt, Germany) were used for protein overexpression. The *Agrobacterium tumefaciens GV3101*:: pMP90RK strain (Koncz and Schell, 1986) was used for *Arabidopsis* stable transformation. For transient expression of proteins,

Tobacco (*Nicotiana benthamiana*) leaves were infiltrated with AGL1 (*A. tumefaciens*) cells (Lazo, 1991). For pathogen infection *Botrytis cinerea* was used.

2.1.7 Antisera

Jassy antisera were generated by Pineda (Berlin, Germany). OEP37, TOC75, FPBase, LHCII were obtained from Dr. Bettina Bölter.

2.1.8 Bacteria and plants growth media and plates

For *E. coli*:
LB medium: 1% NaCl, 0.5% yeast extract and 1% trypton (Roche, Karlsruhe, Germany). For agar plates, 2% Agar was added.
For *A. tumefacients*:
YEB medium: 2 mM $MgCl_2$, 0.5% sucrose, 0.5% peptone, 0.1% yeast extract, 0.5% beef extract (Roche, Karlsruhe, Germany).
For plants:
MS (Murashige and Skoog) medium plates: 1/2xMS, 1.5% sucrose, 0.05 MES, 0.3% Gelrite, pH 5.7.

2.1.9 Column material

Ni-Sepharose and Strep-Tactin Sepharose for protein purification were obtained from GE Healthcare.

2.1.10 Software

To align DNA sequences, BioEdit (Ibis Bioscience, Carlsbad, USA) was used. Target P (http://www.cbs.stu.dk) server was used to predict the targeting peptides. To measure the secondary structure of the proteins by CD spectroscopy, Spectra Manager was used. DICHROWEB was used to analyze the CD results. All figures were illustrated by using Microsoft Powerpoint. Immunoblot images were processed by Image J. Sequences were obtained from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Trees were generated by using the

CLC Main Workbench software (CLC bio, Aarhus, Denmark). Alignments were generated by using the algorithm provided by CLC Main Workbench (developed by QIAGEN Aarhus). Graphs and statistical analyses were generated by using GraphPad Prism version 6.0, GraphPad Software, La Jolla, California, USA (www.graphpad.com).

2.1.11 Plant material and growth conditions

The T-DNA insertion lines of At1g70480 (SAIL_860179) was purchased from the European Arabidopsis Stock Centre (NASC, USA). Arabidopsis ecotype Columbia was established as WT and obtained from Lehle Seeds (Round Rock, USA). Plants were grown on soil under 22℃, 100 µmol/m^2 with light and dark rhythm of 16/8 h.
For selection of transformed plants which contained the pK7FWG2-construct, plants were grown on MS medium with hygromycin (100 µg/ml) under 22℃, 100 µmol/m^2 with a light and dark rhythm of 16/8 h. Transformed plants with pAUL2 and pAUL11-constructs were grown on soil for one week, then 10 µg/µl BASTA solution was sprayed on top of the leaves.
Nicotiana benthamiana seeds were used for the tobacco plants. Pea plants (P. sativum L., cv. 'Arvica', Prague, Czech Republic) were grown under a 16 h light (220 µmol m-2 s-1) and 8 h dark regime at 21° C. *Nicotiana benthamiana* was grown in soil under greenhouse conditions.

2.1.12 Wounding, cold and Pathogen treatment

Wounding of Arabidopsis leaves was performed as follows: leaves of four-week-old plants were wounded by cutting with sharp razor blades. Each leaf was wounded on each occasion for three times. No leaf was wounded more than once. Wounded plants were covered for 1.5 hrs. Harvested leaves were immediately stored in liquid nitrogen.
For cold treatment, plants were first grown under long day conditions for 14 days and then transferred to 4°C, 16 h light / 8 h dark, 22 °C, 120 µE m-2 s-1, for the indicated time periods.

For pathogen assay, Four-week-old plants grown under short day conditions (8 h light / 16 h dark, 22 °C, 120 µE m^{-2} s^{-1}) were inoculated by applying one drop of 20 µl *B. cinerea* culture to each leaf. Extent of the disease was analyzed by measuring lesion size (ImageJ software).

2.2 Methods

2.2.1 Cloning

For PCR amplification, appropriate oligonucleotides were used for the PCR reactions. The master mix can be found in Table 3. PCR products were purified from agarose gels using the Nucleospin Extract II Kit (Macherey and Nagel, Düren, Germany).

In order to produce compatible sticky ends, the appropriate vector and amplified fragments were digested by restriction endonucleases for 2 hrs at 37℃. Subsequently, the products from digestion were purified by the clean up kit. Ligation between insert and vector was performed by using T4 DNA ligase and incubated at 16 ℃ overnight. The products were transformed into TOP10 cells and spread on LB plates containing the appropriate antibiotics. Colony PCR was performed for selecting the positive colony. A single positive colony was inoculated in 5 mL LB medium and grown overnight at 37℃. The plasmid was purified by using the QIAprep Spin Miniprep Kit.

Table 3: Reaction Mix for the Phusion PCR

Component	Concentration
Template (cDNA)	250 ng
5 x High Fidelity Buffer	1x
10 mM dNTP	200 µM
20 µM Forward Primer	0.5 µM
20 µM Reverse Primer	0.5 µM
Phusion Polymerase	1.0 units/ 50 µl PCR
50 mM $MgCl_2$	200 µM
DMSO	3%
ddH_2O	Up to 50 µl

For the GATEWAY cloning, inserts were cloned into the vector pDONR207, following the strategies of the Invitrogen Gateway Cloning Technology online (http://www.thermofisher.com/de/de/home/life-science/cloning). 10 µl out of the 25 µl PCR reaction were used for agarose gel electrophoresis. The remaining PCR products were purified according to the instructions from the PCR purification kit.

BP reaction was performed by adding 75 ng of PCR product, 75 ng pDONR 207 vector and 1 µl BP Clonase II. Reactions were incubated at 25℃ overnight. The reactions were stopped by adding 0.5 µl Proteinase K at 37℃ for ten min. BP reactions products were transformed into Top10 *E.coli* cells and selected on LB medium with gentamicin (20 µl/ml). The plates were incubated at 37℃ overnight. A positive colony was selected and the plasmid was isolated and analyzed by sequencing. For LR reaction, the pDONR207 plasmids with inserts were incubated with 75 ng of the destination vector and 1 µl of LR Clonase II overnight at 25℃. By adding Proteinase K for ten min the LR reaction was stopped. The products of the LR reaction were transformed into Top10 *E.coli* cells and selected on LB plates with the appropriate antibiotics at 37℃ overnight. A positive colony was chosen by the same manner as above. The following constructs were generated in this work.

Table 4: **List of constructs and their selection marker in *E.coli* and *A.thaliana***:

Construct	**Vector**	**Resistance in *E.coli***	**Resistance in *Arabidopsis***
pDONR207::JASSY	pDONR207	Gentamycin	n.a
pAUL11::JASSY	pAUL11	Kanamycin	Basta
JASSY::GFP	pK7FWG2	Spectinomycin	n.a

2.2.2 Sequencing

200 ng plasmid DNA was sent to the sequencing service of the Faculty of Biology (Ludwig-Maximilians-University Munich, Germany) using appropriate oligonucleotides. The inserts were fully sequenced and analyzed by NCBI (http://blast.ncbi.nlm.nih.gov) blast server.

2.2.3 Genomic DNA isolation from *Arabidopsis* for genotyping PCR

By using the tissue lyser, two to three leaves were homogenized in 450 µl extraction buffer which contained 0.5% SDS, 25 mM EDTA, 200 mM Tris and 250 mM NaCl. The samples were incubated at 37℃ for 5 min then centrifuged at 4℃, 10000 x g for 15 min. The supernatant was transferred into a new tube and equal volumes of isopropanol were added the samples were kept in -20℃ for 20 min. The samples were centrifuged at 4℃, at 14000 x g for 15 min. The pellet was washed twice with 70% ethanol then dried at 37℃. gDNA was dissolved in 70 µl water.

2.2.4 RNA isolation from Arabidopsis leaves for cloning and real-time quantitative PCR

In order to extract RNA from three to five weeks old *Arabidopsis* leaves, RNeasy Plant Mini Kit (Qiagen) was used according to the manufacture's introduction. Subsequently, the isolated RNA was digested with DNase (DNasel, Roche, Mannheim, Germany) for 30 min and eluted with RNAse free water. The quality of RNA was controlled by loading 2 µl on to 1% agrose Gel. And the concertation was demining the 260/280 nm ratio.

2.2.5 cDNA Synthesis

1 µg RNA was used to for cDNA synthesis with M-MLV reverse transcriptase according to the manufacturer's instructions (Promega)

2.2.6 qRT-PCR (Quantitative Real-Time PCR)

RNA was extracted from untreated and treated plants as indicated using the RNeasy Plant Kit (QIAGEN, Hilden, Germany). After quantification of the RNA and digestion of DNA with TURBO DNA-free kit (Life Technologies), first strand cDNA was synthesized using M-MLV reverse transcriptase (Promega) from 1 µg RNA. cDNA

was diluted 10-fold with ddH2O and 0.1 µg/µl tRNA from *E. coli* was added. Quantitative PCR was performed in 96 well plates in a CFX96 real-time PCR detection system (Bio-Rad) with SYBR Green Real-Time PCR Master Mix (Roche). PCR process was set up by the following program. 95℃, 2min; (95℃, 20sec; 60℃, 30sec; 72℃, 20sec) * 39 cycles; 95℃, ten min. Expression levels were normalized to the expression of ACTIN2 (AT3g18780). Gene-specific oligonucleotides are listed in Table 1.

2.3 Biochemical methods

2.3.1 Protein overexpression in *RosettaII* cells

RosettaII (DE3) cells harboring the Jassy pET51(b)+ plasmids were grown at 37℃ until an OD600 of 0.6-0.8 was reached to. Protein expression was induced by adding 1 mM IPTG (isopropyl ß-D-1-thiogalactopyranoside). Bacteria were incubated at 110 x g either at 18 ℃ overnight or 37℃ for four hrs. Cells were harvested by centrifugation at 4000 x g for 30 min at 4 ℃. In order to assess whether the overexpressed protein is soluble or accumulated as inclusion bodies, the cells were suspended in resuspension buffer (150 mM NaCl, 50 mM Tris pH7.5) and cells were disrupted using a french press. Broken cells were centrifuged at 10000 x g for 30 min at 4℃. Proteins were analyzed by SDS-PAGE.

2.3.2 Purification of soluble overexpressed Jassy protein from soluble proteins

To obtain recombinant, soluble JASSY protein expression was performed in twelve liters of *RosettaII* cells at 28℃ overnight. The supernatant after French Press was incubated with Ni-Sepharose (Ni-NTA) at 4℃ overnight. Prior to the incubation, the beads were washed four times with 5 ml resuspension buffer. The unbound proteins were washed off the beads by using Washing buffer (150 mM NaCl, 50 mM Tris, 50 mM Imidazole, pH 7.5) to wash five times. The bound proteins were eluted in 1 ml Elution buffer (150 mM NaCl, 50 mM Tris, 500 mM Imidazole, pH 7.5) for four times. The eluted proteins were analyzed on 12% SDS gel.

In order to obtain a higher degree of purity, the eluted Jassy protein was incubated with Strep-Tactin Sepharose at 4℃ overnight. The Strep-beads were washed three times by using 5 ml resuspension buffer. Also, the unbound proteins were washed out by using washing buffer (150 mM NaCl, 50 mM Tris pH7.5) and the recombinant proteins were eluted in 500 µl elution buffer (2.5 mM D-Desthiobiotin, 50 mM Tris, 150 mM NaCl, pH 7.5). All of the elution fractions were analysed on 12% SDS gels.

2.3.3 Purification of Jassy out of inclusion bodies

The insoluble JASSY protein was purified from inclusion bodies as follows. Bacteria from twelve Liters overexpression of *RosettaII* cells was induced by 1mM IPTG for three hrs at RT. The cell pellet was resuspended in Resuspension buffer (50 mM Tris,150 NaCl, 15 mM Imidazole, 10 mM β-Mercaptoethanol, pH 7.5), and cells were broken by the French Press. After centrifugation at 20000 x g for 30 min at 4℃, the pellet was resuspended in the 30 mL Detergent buffer (20 mM Tris, 150 mM NaCl, 1% Deoxycholic Acid, 1% NP40, 10 mM BME, pH 7.5). The suspension was vortexed for 5 min and centrifuged at 10000 x g, RT for 5 min. This step was repeated three times. Subsequently, the pellet was resuspended in 30 ml Triton Buffer (20 mM Tris, 5% Triton X-100, 5 mM BME, pH 7.5). The suspension was vortexed for 5 min and centrifuged at 10000 x g for 5 min at RT. This step was repeated twice. The pellet was resuspended in 30 ml Tris buffer (50 mM Tris, 10 mM DTT, pH 7.5). The suspension was vortexed for 5 min and centrifuged at 10000 x g for ten min at RT. And this step was repeated twice. Samples were analyzed on 12% SDS gels.

2.3.4 Measuring the concentration of proteins

Protein concentration was measured by using the Bradford reagent (0.1% Coomassie brilliant blue G-250, 10% phosphoric acid, 5% ethanol). 10 µl samples were mixed with 1x Bradford reagent. The absorption was measured at 595 nm.

2.3.5 Isolation and fractionation of *Arabidopsis thaliana* chloroplast

Intact chloroplasts were isolated from 200 g fresh weight leaf material from three weeks old plants grown on soil essentially as described in Seigneurin-Berny *et al*., 2008. Subsequently, intact chloroplasts were resuspended in 15ml of "Chloroplast Burst" buffer (5 mM $MgCl_2$, 10 mM Hepes KOH (pH 7.6)) and lysed the chloroplast by 50 strokes in a small Dounce-homogenizer (Wheaton, Millville, NJ, USA). Further separation for the envelopes was done according to Li *et al*., 1991.

2.3.6 Chloroplast isolation from *Pisum sativum*

About 200 g of 9-14 days old peas were ground in a kitchen blender with 330 ml isolation buffer (330 mM sorbitol, 3 mM $MgCl_2$, 20 mM MOPS, 0.1% BSA, 13 mM Tris-HCl, pH 7.6) at 4℃. The suspension was filtered through 4 layers of mull and 1 layer of gauze and centrifuged for 1 min, 1900 x g at 4 ℃. Intact chloroplasts were isolated by a percoll gradient which contained twelve ml 40% percoll solution (330 mM sorbitol, 50 mM HEPES pH 7.6, 40% percoll) and 8 mL 80% percoll solution (330 mM sorbitol, 50 mM HEPES, pH 7.6, 80% percoll), which was centrifuged for 5 min at 7000 g in a swing-out rotor. After centrifugation two bands were observed, the bottom band represented the intact chloroplasts. The intact chloroplasts were isolated and washed twice with washing buffer (330 mM sorbitol, 250 mM HEPES pH 7.6, 3 mM $MgCl_2$). To order to measure the concentration of chlorophyll, 1 µl chloroplasts was added to 1ml 80% acetone and calculated from the following formula:

$$\text{mg chlorophyll / ml} = 8.02 * (E663 - E750) + 20.2 * (E645 - E750)$$

2.3.7 Isolation of outer and inner envelope membranes from *Pisum sativum*

Intact chloroplasts were washed twice by Washing Buffer (330 mM sorbitol, Tris-base (pH 7.6)), homogenized and further treated according to the modification

(Waegemann *et al*., 1992) of previously described method (Keegstra and Youssif,1996).

2.3.7 *In Vitro* transcription

5 ug of pSP65 (*JASSY*) was digested with Buffer R and HindIII in 50 µl volume and incubated at 37℃ for 60-90 min. *In vitro* transcription, with linearized plasmids was carried out in a 50 µl reaction mix which contained 2% BSA, 0.5 mM ACU, 50 units of RNase Inhibitor, 30 units of T7 RNA polymerase in 5 µl 5x transcription buffer. After incubation at 37℃ for 15 min then 1.2 mM GTP was added. The samples were incubated for another 120 min at 37℃.

2.3.8 *In Vitro* translation

10 µg in vitro transcription products were used for 50 µl in vitro translation reaction. The 50 µl reaction contains 35 µl rabbit reticulocyte lysate (Promega), 1 mM amino acid mixture minus methionine, 30 µCi ^{35}S Methionine and Cystein (Perkin Elmer, Walluf, Deutschland), 40 u/µl RNase ribonuclease inhibitor. Using nuclease-free water up to the final volume of 50 µl. The samples were kept at 30℃ for 45 min.

2.3.9 Chloroplast *in vitro* Import from *Pisum sativum*

10 µg chlorophyll was used in a 100 µl final import mix buffer (250 mM methionine, 250 mM cysteine, 5% BSA, 100 mM ATP, 1 M calcium gluconate, 1 M $NaHCO_3$, 10x HMS (50 mM HEPES, 3 ml $MgSO_4$, 0.3 M Sorbitol), 5µl translation product, added water up to 100 µl). The reaction was incubated at 25℃ for 15 min. Samples were transfered on top of a 300 µl percoll cushion (330 mM Sorbit,1 M HEPES/KOH, 40% Percoll) and centrifuged at 7000 x g or 5 min at 4℃. The pellet was washed two times with washing buffer (330 mM Sorbit, 1 M HEPES, 1 M $MgCl_2$). Chloroplasts were collected by centrifugation at 3400 x g or 1 min at 4℃. The pellet was resuspended in 100 µl wash buffer II (330 mM Sorbit, 50 mM HEPES, 0.5 mM $CaCl_2$) which contained 1 mg/mL Thermolysin and incubated on ice for 20 min. Subsequently, 0.5

M EDTA was added to the suspensions to stop the reaction. Chloroplasts were collected by centrifugation at 3200 x g for 1 min at 4 ℃. Chloroplast were washed once in washing buffer III (330 mM Sorbit, 50 mM HEPES, 5 mM EDTA), centrifuged at 3200 x g or 1 min,4 ℃. The pellet was resuspended in 20 µl protein loading buffer and boiled for three min at 95℃ before analysis by SDS-PAGE.

2.3.10 Stable transformation of *Arabidopsis*

For *A. thaliana* stable transformation, 2 µg of the appropriate plasmid was transformed into competent Agrobacteria (strain GV3101). The cells were kept on ice for 5 min, 5 min in liquid nitrogen and another five min at 37℃. 800 µl LB medium were added and incubated at 800 x g for four hrs. at 28℃. The suspension was centrifuged at 3700 x g for three min at RT. The pellet was resuspended in 100 µl LB medium and plated on LB plates with appropriate antibiotics. Plates were incubated at 28℃ for two-three days. Cells were transferred into 10 ml LB medium and grown for two days at 28℃ for two-three days, shaking at 110 x g. Then the culture was transferred into 500 ml LB medium and grown overnight at 28℃ and moderate shaking. Bacteria were pelleted for ten min, 5500 x g at 4℃. After resuspending the pellet in 400 ml infiltration medium (5% (w/v) sucrose, 0.5x MS salts, 0.05% (v/v) Silwet L-77), the OD600 was adapted to 0.8-1.

Five week-old mutant and WT plants were dipped into the bacterial suspension for ten seconds and covered overnight with plastic lids. Dipping step was repeated after seven days. Transformed plants were selected by BASTA (50 µg/ml) treatment.

2.3.11 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

In order to separate proteins, discontinuous SDS-PAGE was used according to Laemmli, 1970. 5% stacking gel (1% TEMED, 0.1% APS, 5% polyacrylamide, 0.1% SDS, 125 mM Tris/HCL pH 6.8) and 12 or 15% separating gel (390 mM Tris/HCl pH 8.0, 0.4% TEMED, 0.1% APS, 12% or 15% polyacrylamide (acrylamide:bisacrylamide=29:1) ,0.1% SDS). SDS loading buffer (0.004%

bromphenol blue, 5% ß -mercaptoethanol, 10% glycerol, 2% SDS, 62.5 mM Tris pH 6.8) was added into the protein samples. The gels were run in SDS running buffer (0.1% SDS, 192 mM glycine, 25 mM Tris). Afterwards gels were either used for western blotting or for coomassie (0.2% coomassie brilliant blue R-250, 9% acetic acid, 45% methanol) staining.

2.3.12 Western blotting

In order to transfer the proteins from the SDS gel to PVDF membrane, six Whatman papers were soaked in Towbin buffer (20% methanol, 0.1% SDS, 192 mM glycine, 25 mM Tris/ HCl). The PVDF membrane was activated with 100% methanol for 90 sec and rinsed with Towbin buffer. Three soaked Whatman papers were set up onto the anode of the semi-dry electro blot. The SDS gel was placed on top of the PVDF membrane and three more whatmann papers were added. The blotting was performed at 0.8 mA/cm^2 for 1 hour. To mark the marker bands, Ponceau-S was used (5% acetic acid, 0.3% Ponceau S) to stain with the membrane for 5 min and removed by using water.

2.3.13 Immunodetection

After Ponceau-S staining, the membrane was blocked for 1 h or overnight in either 5% milk buffer (5 g skimmed milk powder in 100 ml TBST). The membrane was incubated with the first antibody for three hrs or overnight at 4 ℃. To remove unbound antibodies, the membrane was washed in TBS-T three times, for ten min. The membrane was incubated with an appropriate secondary antibody conjugated to horseradish peroxidase for 1 h and also washed three times with TBS-T buffer for ten min.

In order to detect the signal, ECL solution 1 (0.44% coumaric acid, 1% luminol, 0.1 M Tris/ HCl pH 6.8) and solution 2 (0.018% H_2O_2, 0.1 M Tris/HCl pH 6.8) were applied in equal amounts on top of the membrane. After exposure for ten min, the

chemiluminescence signal was observed by an ECL Reader. The software is Image Quant LAS 4000.

2.3.15 Transient transformation of *Nicotiana benthamiana*

Four-week-old *Nicotiana benthamiana* plants were used for transient transformation. *Agrobacterium tumefaciens* (AGL1) cells, which carried the plasmid of interest were grown in 10 ml LB medium until an OD600 of 0.5-0.6 was reached. Bacteria were collected by centrifugation at 4500 x g for 15 min at 25℃. The pellet was resuspended in an appropriate amount of infiltration buffer (10 mM MES pH6, 10 mM $MgCl_2$, 150 μM Acetosyringone) in order to reach an OD600 of 1. The bacterial solution was kept in dark and rotated for two hrs. The solution was injected into *Nicotiana benthamiana* leaves using a 1 ml syringe. Expression of GFP-fusion proteins was observed with a TCS-SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany)

2.3.16 Protoplast isolation from *Nicotiana benthamiana*

Transient transformed *Nicotiana benthamiana* was used for protoplast isolation. The leaves were cut into 0.1 cm wide strips and placed into a petridish with 10 ml freshly prepared enzyme solution 81% cellulase R10, 0.3% macerozyme R10, 0.1% BSA in F-PIN buffer (20mM MES, pH 5.8, 80g/l glucose) and shaken at 40 x g in darkness for 90 min. The suspension was filtered through 100 μM Nylon-membrane and transferred into a centrifuge tube. The filtered suspension was overlayed with 2 ml F-PCN medium and centrifuged for ten min at 70 x g. The intact protoplasts were collected from the interface between F-PIN and F-PCN. The intact protoplasts were washed two times withW5 buffer and GFP fluorescence signal was observed with TCS-SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany)

2.3.17 Microscale Thermophoresis

MST was performed with purified recombinant JASSY protein and OPDA or JA. The concentration of JASSY was kept constant at 25 nM, while the concentration of OPDA and JA was varied between 30 nM and 1 mM. The assay was performed in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.05% Tween-20. After 30 min of incubation the samples were centrifuged at 1400 x g for 5 min and loaded into MST. Label Free standard glass capillaries (NanoTemper, München). The MST analysis was performed using the Monolith NT.115 Red/Green device (NanoTemper) with 40% LED Power and 40% MST Power. The overall affinity (KD) was determined using the NanoTemper Analysis Software.

2.3.18 Carboxyfluorescein Assay

20 mg of phosphatidylcholine lipids (Larodan Fine Chemicals AB, Solna, Sweden) were washed with chloroform/methanol (1:1) and dried under N2. The lipids were resuspended in 1x PBS, pH 7.4 to a final concentration of 20 mg/ml and 20 mM of carboxyfluorescein was added to 20 mg/ml PC lipids. Subsequently, lipids were subjected to a five times freeze/thaw cycle and the mixed-lamellar liposomes were extruded through a membrane with 200 nm pore size to generate unilamellar vesicles. To remove non-encapsulated dye the liposomes were dialysed against 1x PBS buffer pH 7.4 overnight at 4°C. For fluorescence measurements, 5 µl of liposomes were mixed with 995 µl 1x PBS pH 7.4 to generate a suitable fluorescent signal. After addition of purified proteins fluorescence was recorded every millisecond for 300 or 600 s with a LS55 fluorescence spectrometer (PerkinElmer, Waltham, USA) with an excitation wavelength of 494 nm and an emission wavelength of 515 nm.

2.3.19 Electrophysiology Assay

To prepare the samples for electrophysiological characterization, La-phophstidylcholine and L-a-phosphatidylethanolamine (both purchased from Avanti Polar Lipids, AL, USA) were mixed in molar ratio 80:20, dried first under nitrogen then desiccated under vacuum. The dried lipids were fully resuspended in liposomes

buffer (150 mM NaCL, 50 mM Tris/HCl, pH 7.5) to 5 mg/ml and subjected to seven freeze/thaw cycles before being extruded through a 200 nm filter. The liposomes and purified recombinant JASSY, or the identical treated mock expression using an empty plasmid, were solubilized with 80 mM of the dialyzable detergent MEGA-9 (Glycon, Germany) separately for 15 min at room temperature. Both parts were pooled and incubated for another 30 min at room temperature with a final lipid and protein concentration of 1.5 mg/ml and 0.2 mg/ml, respectively. The mixture was dialyzed in a 3.5 kDa cutoff dialysis tube against 5 l of liposome buffer to remove detergent, first for 2 h at room temperature and then overnight at 4°C. Incorporation success was monitored by density gradient flotation assay and sodium carbonate extraction as described before (please insert Tarasenko et al. 2017 Journal of Cell Biology).

The electrophysiological experiments were performed were performed using the planar lipid bilayer technique as described elsewhere (please insert Montilla-Martinez et al. 2015 Cell Reports). Briefly, liposomes with incorporated JASSY were added below the lipid bilayer to allow osmotically-driven fusion in the cis chamber (250 mM KCl, 10 mM MOPS/Tris, pH 7) while the trans chamber contained a low salt buffer (20 mM KCl, 10 mM MOPS/Tris, pH 7). After insertion of a channel into the bilayer, buffers in both chambers (cis and trans) were perfused to symmetrical standard buffer conditions using 20x chamber volumes. For determination of the reversal potential, asymmetric buffer conditions as used for liposome fusion, were applied. Electrical recordings were performed using Ag/AgCl-electrodes in a 2 mM KCl agar-bridge, connected to a Geneclamp 500B amplifier via a CV-5-1GU headstage, with the cis-electrode connected to the ground. The signal was digitized by a Digidata 1440a A/D converter and recorded with the AxoScope 10.3 and Clampex 10.3 software (Molecular Devices). Automated data analysis was performed as described before (please insert Denkert et al. 2017 eLife). To determine substrate effects, OPDA in EtOH was added to both sides of bilayer-incorporated JASSY channels. The buffer in both chambers was circulated by stirring for two min after addition, then left alone for another two min before recording.

2.3.20 Jasmonate Measurements

Jasmonate and OPDA were extracted as described in Glauser and Wolfender. In brief, 200 mg FW frozen material was ground in a 2 ml reaction tube using a ball mill (TissueLyser II; Retsch) at 30 Hz for 1 min. For extraction 1.5 ml of pre-cooled isopropanol are added, including 0.5% (v/v) formic acid and 5 ng ml^{-1} chloramphenicol as internal standard. After extensive mixing until the tissue was fully re-suspended, the samples were mixed using the ball mill at 30 Hz for 4 min. Afterwards the samples were centrifuged at 16400 x g (Centrifuge 5417R; Eppendorf). The supernatant was transferred to a new 2 ml reaction tube and dried using a vacuum concentrator (Concentrator 5301, Eppendorf). Dry pellets were resuspended in 85% methanol using sonication (Sonifier B-12, Branson Sonic Power Company). The solution was centrifuged at 16400x g for 90 s and 1 ml of the supernatant was loaded to a C18 solid phase extraction (SPE) column (Sep-Pak Vac 1cc 50 mg; Waters). SPE columns were equilibrated before, using 1 ml 100% and 1 ml 85% (v/v) methanol. After SPE separation the total flow trough was collected followed by a 1 ml 85% (v/v) methanol washing step. The flow through and the methanol wash were combined and dried overnight using the vacuum concentration. Dry samples were filled with argon and stored at -80°C until further measurement. For LC-MS analysis the Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) in combination with an Impact II QToF (Bruker Daltonik) was used. Therefore, the dry sample were resolved in 100 µl 85% (v/v) methanol. For separation 20 µl were injected on a C18 reversed phase column (Ultra AQ C18, 3 µm100 x 2.1 mm; Restek) with 400 µl min^{-1} flow at 30°C. The solvents used are (A) water and (B) acetonitrile, both including 0.1% (v/v) formic acid. The 30 min gradient started at 5% B for two min, followed by a ramp to 95% B within 20 min. After a three min washing step at 95% B the gradient turned back to 5% B within 1 min and kept constant for 4 min equilibration. For MS detection, an electrospray ionization (ESI) source was used in positive mode at 4 kV capillary and 0.5 kV endplate voltage. Nitrogen was the dry gas with 8 l min^{-1}, 8 bar and 200 °C. The Impact II mass spectra were recorded in MS mode from 50-1300 *m/z* with 40,000 resolution, 1 Hz scan speed and 0.3 ppm mass accuracy. Compounds were annotated in a targeted approach using specific mass (*m/z*) at retention time and the isotopic

pattern. All data were acquired by otofControl 4.0 and HyStar 3.2. The evaluation was performed by DataAnalysis 5.1, ProfileAnalysis 2.3 and MetaboScape 1.0. Bruker provided all software tool. Additional data evaluation was done with Excel. BioSolve or Sigma-Aldrich supplied all solvents and standards in LC-MS-grade.

3. Results

3.1 JASSY is a novel protein with a conserved START domain

A wide scale proteomic investigation of chloroplast envelope membranes lead to the discovery of JASSY. JASSY was first identified as a chloroplast outer envelope protein with unknown function in peas (Simm et al., 2013). JASSY has a conserved motif which belongs to the Bet v1-like superfamily. The Bet v1-like superfamily is a large protein family that shares a similar three-dimensional structure but show low sequence similarity. The Bet v1 family is divided into eleven subfamilies, one of the subfamilies is the START domain proteins, to which Jassy shows the highest similarity. The hydrophobic cavity of most of the Bet v1 proteins functions in binding and metabolism of large and hydrophobic compounds such as hormones.

As shown in Figure 6, the JASSY protein contains a conserved START domain (from 120 to 230 aa) and a DUF (domain of unknown function) 220 domain (from 182 to 253 aa). These two domains have 48 aa overlapping (Fig. 6). The START domain is a lipid-binding domain (Schrick et al., 2004). However, the function of the Duf220 domain is still unknown. In mammals, the 30 kDa steroidogenic acute regulatory protein (StAR) has been reported to be involved in binding and transfer of cholesterol to the inner mitochondrial membrane (Schrick et al., 2004).

To analyze the function of the JASSY protein, we first searched for co-expressed genes from ATTED-II (http://atted.jp). The results suggested that JASSY was co-expressed with many genes involved in JA metabolism. According to this, we thought that JASSY might play a role in JA metabolic processes and respond to JA stimuli.

Combining all the *in silico* information obtained above, we concluded that JASSY might be involved in lipid transport across the chloroplast membrane and be related to the JA biosynthesis pathway.

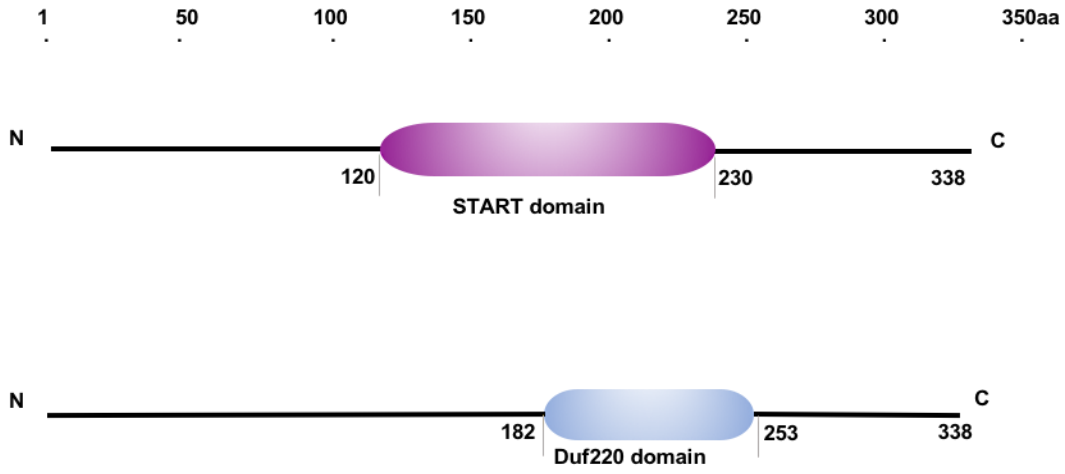


Figure 6. The domain structure of the JASSY protein from Arabidopsis. STATR domain: StAR-related lipid-transfer domain. Duf 220 domain: Domain with unknown function. aa: amino acid

3.2 JASSY is localized to the chloroplast outer membrane

Since the function of a protein is closely linked to its sub-cellular localization, we aimed to verify the sub-cellular localization of JASSY experimentally, since many public prediction programs have proposed a chloroplast localization of JASSY (ChloroP1.1 Server, http://www.cbs.dtu.dk/services/ChloroP/; TargetP 1.1 Server, http://www.cbs.dtu.dk/services/TargetP/).

As a first step, we fused a GFP-tag to the N- and C-terminus of JASSY (Fig. 7A), and these constructs were transformed into Agrobacteria (*AGL1*) and which were then used for transfection of tobacco leaves (Fig. 7B). Expression of the JASSY-GFP and GFP-JASSY fusion protein showed JASSY was truly localized in the chloroplasts as the GFP fluorescence was almost exclusively found to be overlapping with the chlorophyll autofluorescence, thus confirming the prediction of the chloroplast localization of JASSY.

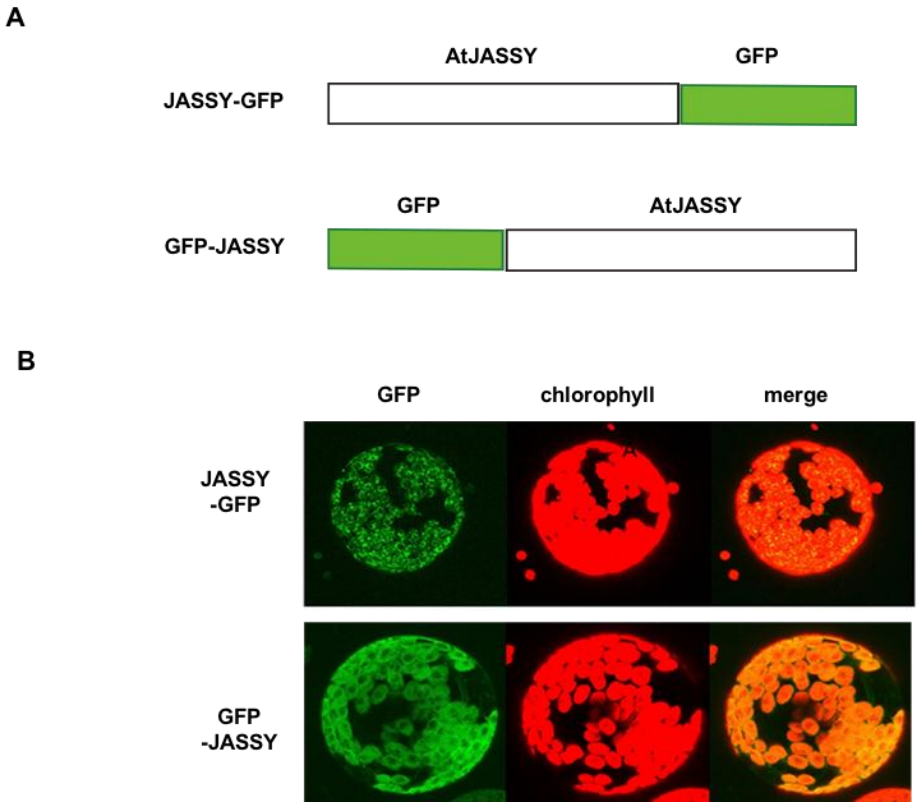


Figure 7. Subcellular localization of JASSY in the chloroplast. (A) The two fusion constructs used in the experiments: GFP was fused to the C-terminal or N-terminal of JASSY, respectively. **(B)** *In vivo* targeting of fusion proteins in tobacco protoplasts. GFP fluorescence was shown in green, and chlorophyll auto-fluorescence is shown in red.

To further investigate the localization of JASSY, we performed an *in vitro* import assay using radio-labeled JASSY. If JASSY were localized in the chloroplast stroma, we would expect a size shift before and after import due to cleavage of the transit peptide. When we compared the size of the translation product before and after the import reaction, we did not observe a size shift, indicating that the protein does not seem to contain a cleavable transit peptide (Fig. 8). To test whether JASSY is nevertheless protected by the chloroplast envelope membranes, we incubated the chloroplasts with thermolysin (a protein protease) after the import reaction. Thermolysin treatment removes proteins that are loosely attached to the chloroplast outer surface, but does not digest imported proteins or fully chloroplast envelope embedded proteins. The results showed that the radio-labeled JASSY was resistant to thermolysin treatment, indicating that JASSY is efficiently imported into the

chloroplast or fully embedded in the membrane (Fig 8, upper panel). FNR (the ferredoxin-NADP$^+$ reductase), a stromal chloroplast protein, was used as a control in this experiment. As anticipated, a size shift was observed for the FNR as the transit peptide was cleaved inside of the chloroplasts. Besides, the precursor of FNR localized outside of the chloroplast was digested by thermolysin treatment. In contrast, the mature FNR protein inside of chloroplast was not digested by thermolysin treatment because of the protection by the chloroplast envelope and the inaccessibility of the thermolysin. (Fig. 8, lower panel). Both experiments were performed in parallel, showing that thermolysin treatment was efficient. Therefore, JASSY has no transit peptide and is thus localized to the OE (outer envelope).

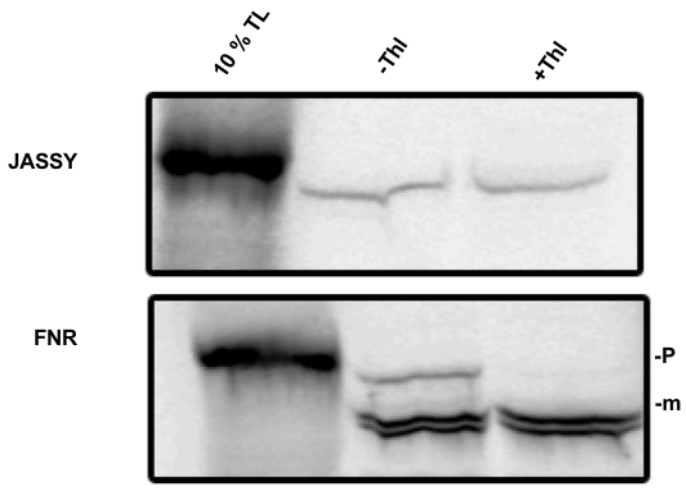


Figure 8. Import of JASSY into isolated chloroplasts from *P.sativum*: *In vitro* translation products of JASSY as well as FNR (control) were imported into isolated pea chloroplasts and treated without/with thermolysin (-/+ Thl). 10 % of the translation product used for the import reaction were loaded (TL). Signals were detected by phosphor plate imaging.

Interestingly, the GFP expression resulted in a ring-shaped signal, again indicating that JASSY might be associated to the chloroplast envelope (Fig. 7B). Moreover, JASSY was identified in a previous proteomics study in the outer envelope fraction of peas (Simm et al., 2013). To further investigate the chloroplast sublocalization we fractionated WT and *jassy* chloroplasts into envelopes, stroma and thylakoids. Fractions were subjected to SDS-PAGE and immunoblotting was performed with

antisera raised against the recombinant Arabidopsis JASSY protein. Indeed, a band at the expected size of 36.3 kDa was detected in the envelope fraction, which was absent in the mutant. The antibody recognized two additional bands in the stroma fraction; however, these were also present in the knockout mutant and therefore are expected to represent cross reactions of the antiserum (Fig. 9, upper panel). Treatment with antisera against Toc75 (Translocon of outer chloroplast envelope 75, outer envelope), FBPase (Fructose-1,6-bisphosphatase, stroma) and LHCII (light harvesting complex II, thylakoid) served as markers to show purity of the isolated chloroplast fractions (Fig. 9).

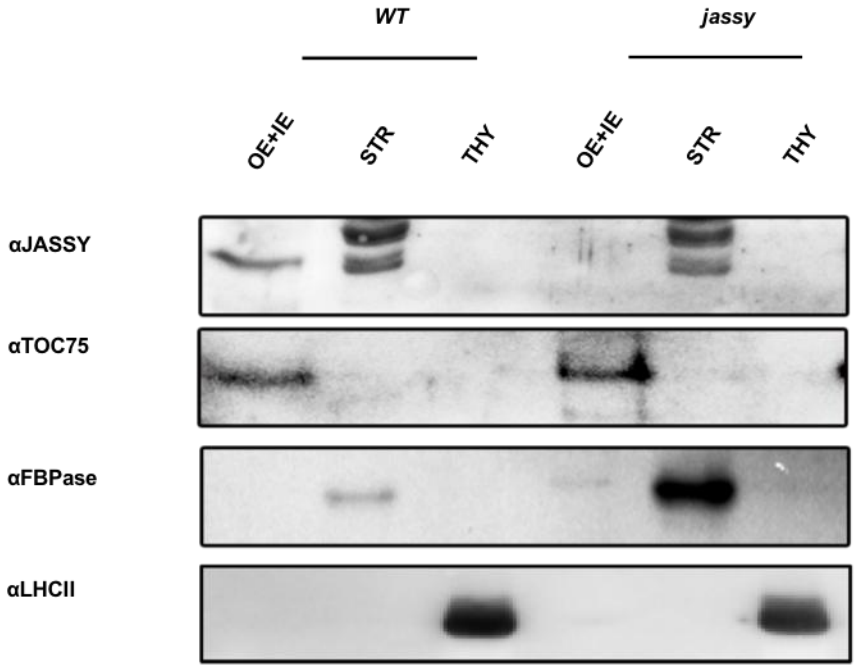


Figure 9. At-JASSY was localized in the chloroplast envelope. WT and *jassy* Arabidopsis chloroplasts were sub-fractionated into outer and inner envelopes (OE+IE), stroma (STR) and thylakoids (THY). Sub-fractions were separated by SDS-PAGE and subjected to immunoblotting with antisera against JASSY, Toc75 (OE marker), FBPase (stroma marker), LHCII (thylakoid marker).

Since in Arabidopsis we were not able to separate outer and inner envelopes, we repeated this experiment with pea chloroplasts and found that JASSY was exclusively localized in the OE subfraction (Fig. 10). Again, several controls were performed to ensure purity of the fractions. In addition to LHCII and the FBPase used above, antisera against OEP37 (the outer envelope protein 37) and Tic110 (the translocon of

the inner chloroplast envelope 110), were used to show purity of the outer and inner envelopes, respectively (Fig. 10).

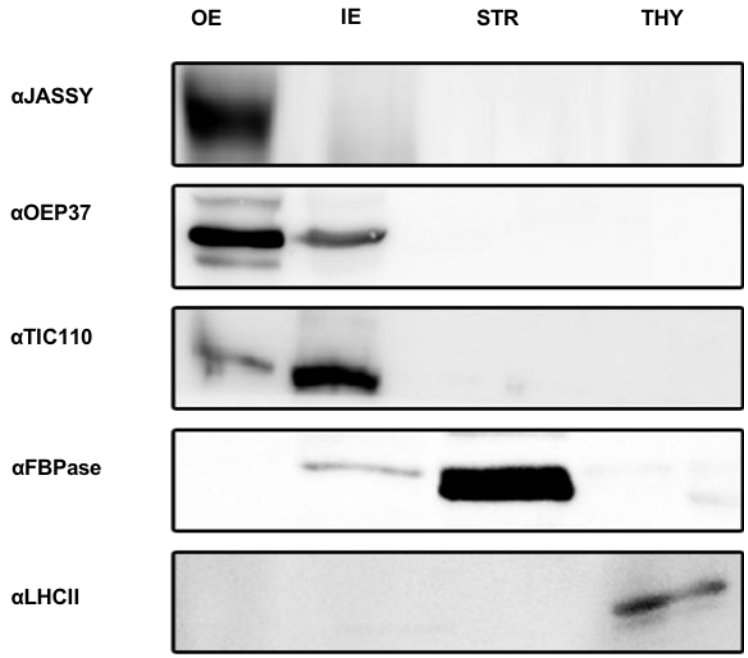


Figure 10. JASSY is localized in the OE envelope in pea. Pea chloroplasts were subfractionated into outer envelope (OE), inner envelope (IE), stroma (STR) and thylakoids (THY). Subfractions were separated by SDS-PAGE and subjected to immunoblotting with antisera against JASSY, OEP37 (OE marker), Tic110 (IE marker), FBPase (stroma marker), LHCII (thylakoid marker).

Since JASSY does not contain any predicted α-helical transmembrane domains, this finding was somewhat surprising. To further confirm the chloroplast outer envelope localization of JASSY, we treated pea outer membranes with several chaotropic reagents to analyze the mode of interaction (Fig. 11). Treatment with 1 M NaCl removes proteins loosely attached to the membranes, whereas 6 M Urea will solubilize partly integral membrane proteins. Treatment with 0.1 M Na_2CO_3 (pH 11.3) or 2 M NaBr could remove tightly associated peripheral proteins, whereas 0.1 M NaOH will partly solubilize the membrane proteins. SDS is a strong detergent and 1% SDS will totally solubilize the membrane proteins (Schweiger et al., 2012).

Neither upon treatment with 1 M NaCl, 6 M urea, 0.1 M Na_2CO_3, or 2 M NaBr, was JASSY found in the soluble fraction. Merely treatment with 0.1 NaOH released a portion of the JASSY protein from the membrane. As a control, JASSY was detected in the soluble fraction when the membranes were solubilized with 1% SDS (Fig. 11). The integral, beta-barrel protein Toc75 was used as control. As expected, Toc75 was only found in the supernatant after treatment with 1% SDS (Fig 11, lower panel). We therefore concluded that JASSY is inserted into the outer chloroplast membrane.

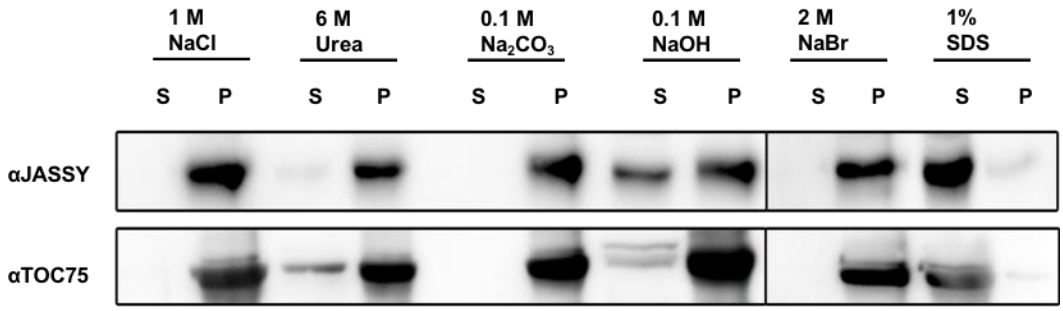


Figure 11. JASSY is recognized as an integral membrane protein. Chloroplast outer envelope equal to 10 mg of protein was treated with various reagents for 30 min. The soluble (S) and the pellet (P) fractions were separated by SDS-PAGE and immunostained with JASSY specific antibodies. Toc75 was used as an integeral membrane control.

Some soluble stroma proteins, which are sequestrated in envelope vesicles, are known to contaminate envelope fractions (e.g. large subunit of rubisco). These proteins can be released from envelope preparations by opening and closing of the membrane vesicle after sonication (Miras et al., 2002). We treated pea outer membranes with sonication to release the envelope vesicles-incorporated proteins, and subsequently the pellet was again treated with several reagents again to analyze the membrane association of JASSY. Additionally, 10 mM DTT was used to reduce potential disulfide bonds between the proteins.

As shown in Fig. 12A, JASSY was not solubilized after sonication of the membrane. Neither upon treatment with 1 M NaCl, 6 M urea, or 10 mM DTT, was JASSY found in the soluble fraction (Fig. 12B). Toc75 was used as a control and only found in the

supernatant after solubilized with 1% SDS treatment. We therefore conclude that JASSY is stably inserted into the outer membrane of the chloroplast.

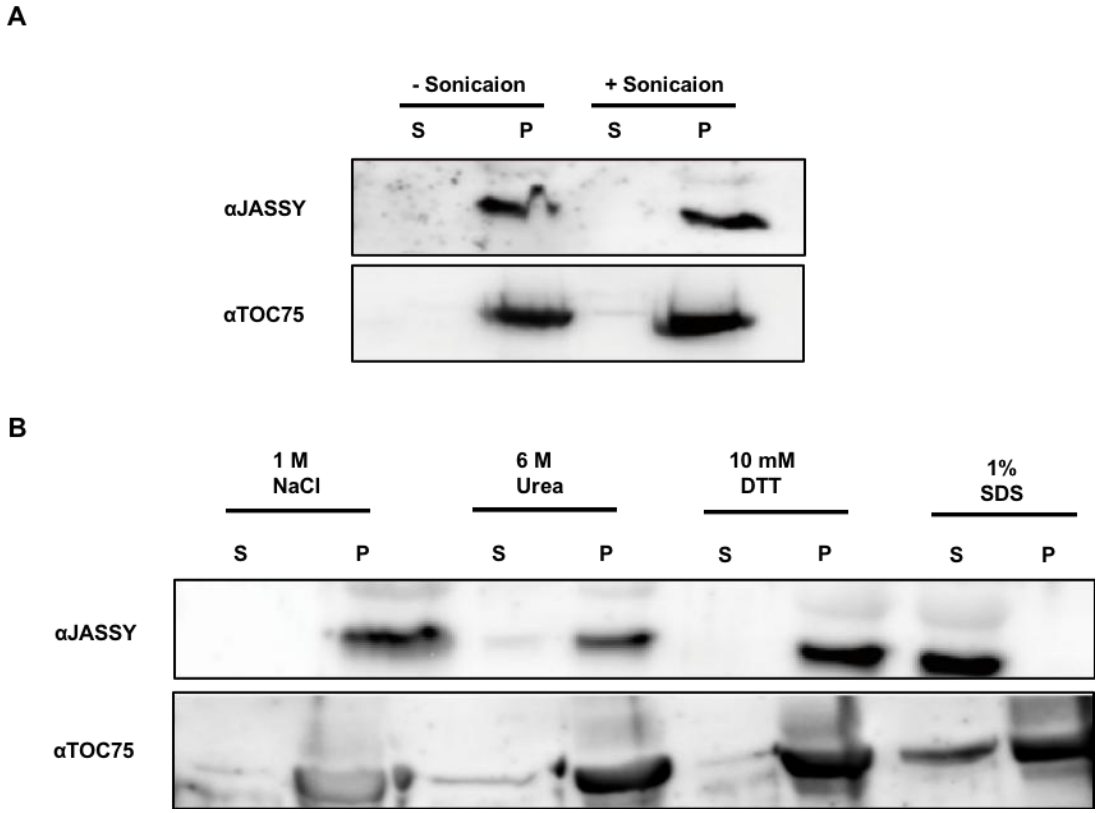


Figure 12. The JASSY protein is bound to the outer membrane of the chloroplast envelope. OE was sonicated for 5 s and soluble (S) and insoluble membrane protein (P) was separated by centrifugation **(A)**. Insoluble protein was then treated with various reagents for 30 min **(B)**. The soluble (S) and the pellet (P) fractions were separated by SDS-PAGE and immunostained with JASSY specific antibodies. Toc75 was used as an integeral membrane control.

3.3 Phenotypic analysis of the *jassy* mutant

3.3.1 Root growth in the *jassy* mutant is slightly decreased

To understand the function of JASSY, a T-DNA insertion line of the *JASSY* gene was used (Sail_N801719 (*jassy*)). After genotyping PCR (Fig. 13B) and sequencing the PCR product, T-DNA insertion site was identified in the 5th exon of the *JASSY* gene. (Fig. 13A). Next, we examined the morphological and developmental changes of the *jassy* mutant. As shown in Fig. 13C, no obvious morphological difference was found

between *jassy* and WT when grown under the normal conditions (23℃, 16 h light/8h dark) for 14 days, 21 days, 28 days or even 45 days (Fig. 13C).

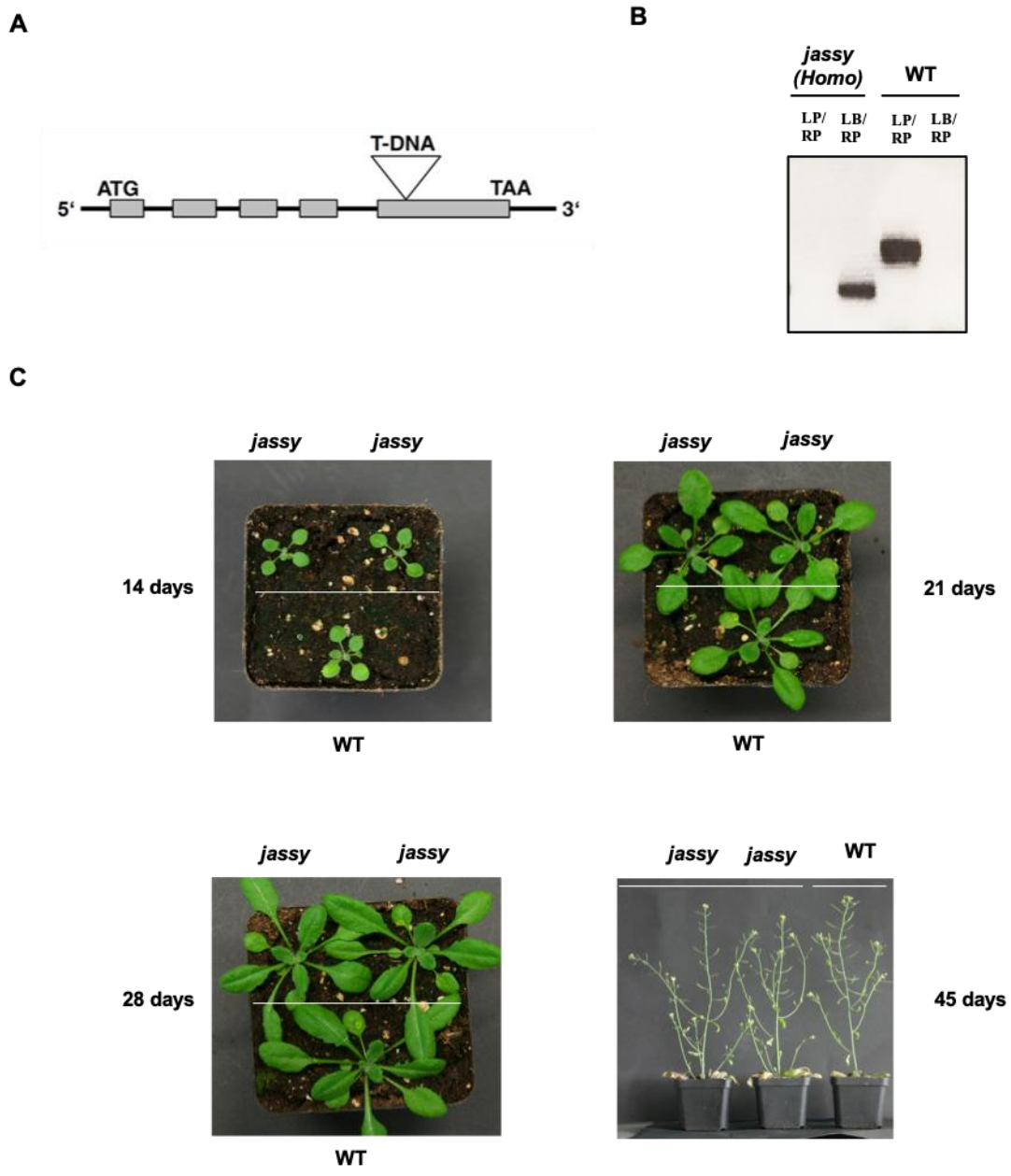


Figure 13. No obvious morphological difference can be detected between WT and *jassy* mutant. **(A)** Gene structure of *JASSY* and the position and orientation of the T-DNA insertion (SAIL_N 860179). **(B)** Genotype PCR of WT and *jassy* mutant. **(C)** Phenotypic analysis of WT, and the *jassy* mutant. Plants were grown for 14, 21, 28 and 45 days after germination in soil.

Since the *jassy* mutants did not show any leaf phenotype we proceeded to investigate whether the *JASSY* mutation affected the growth behavior of the roots. To ensure that the phenotype was caused by the lack of JASSY we complemented the mutant by expressing JASSY under the control of the 35S promoter. Moreover, RT-PCR results showed that expression of the *JASSY* transcripts were totally abolished in the *jassy* mutant and also that complemented, *jassy* 35S::JASSY, was shown to express the gene (Fig. 14C). Thus we compared the root length of WT, *jassy*, *CaMV35S::JASSY/jassy* and *CaMV35S::JASSY/WT* under normal growth conditions. After 8 days of growth, the root length of the *jassy* mutants was apparently shorter than the WT (Fig. 14A). In addition, the *CaMV35S::JASSY/jassy* showed the same root length as WT, and the root length of the *CaMV35S::JASSY/WT* transgenic plants were longer than the WT (Fig. 14A). Additionally, the root lengths of WT, *jassy*, *CaMV35S::JASSY/jassy*, *and CaMV35S::JASSY/WT* were quantified when plants were grown under normal growth conditions for 8 days (Fig. 14B). As is shown in Fig. 8E, after 8 days of growth, the root length of WT was 2.19 ± 0.5 cm, in *jassy* mutant 1.534 ± 0.4 cm were measured and the *CaMV35S::JASSY/jassy* reached a root length of 2.285 ± 0.7 cm, where the *CaMV35S::JASSY/WT* was at 3.135 ± 0.3 cm (Fig. 14B).

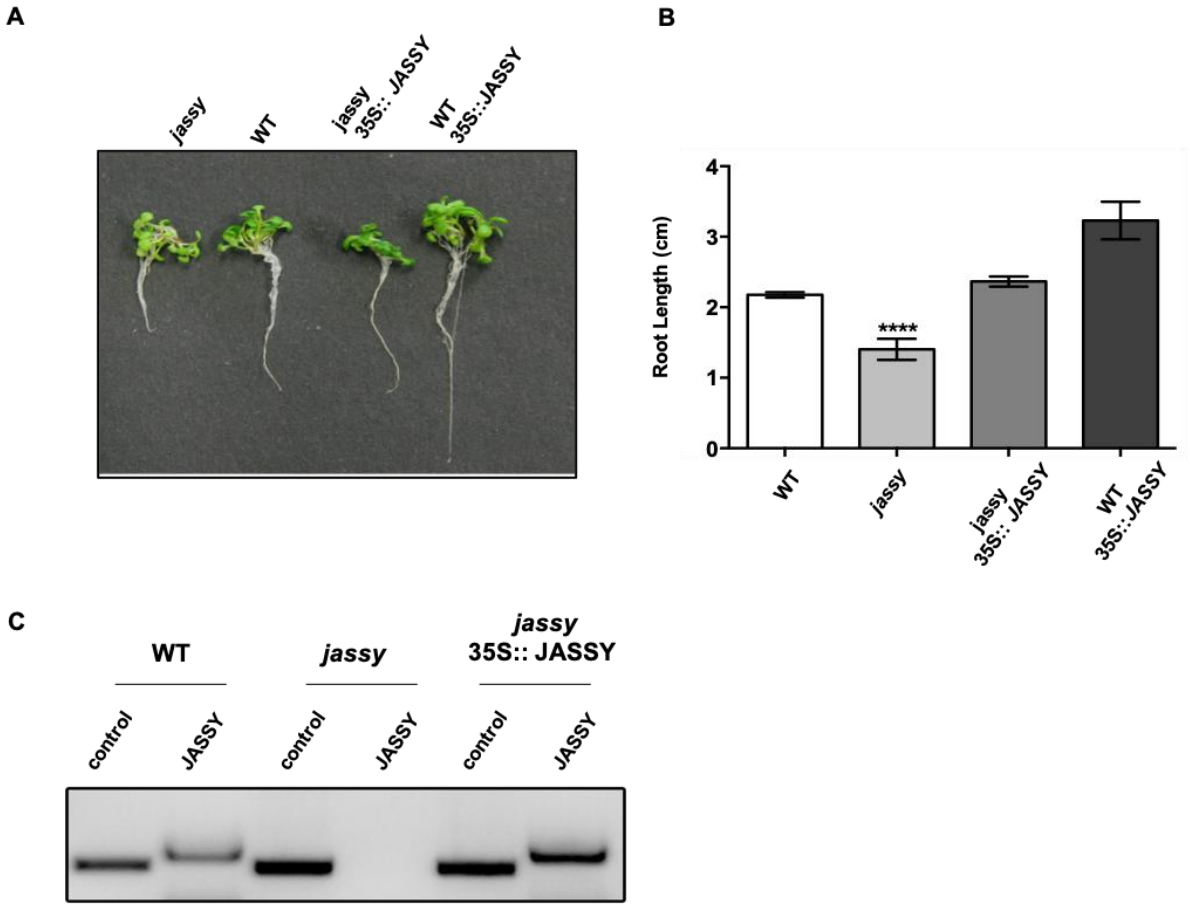


Figure 14. The *JASSY* mutation affected the growth of Arabidopsis root. (A) Growth of WT, the *jassy* mutant, *CaMV35S::JASSY/jassy*, and *CaMV35S::JASSY/WT* on ½ MS medium agar plates. Plants were grown vertically for 8 days after germination. **(B)** Root length quantification. n= 15 plants, error bars = SD. **(C)** RT-PCR was performed using cDNA from WT (Col-0), mutant (*jassy*), and *CaMV35S::JASSY/jassy*. *Yip1* was amplified as a control.

3.3.2 Loss of JASSY increases susceptibility to pathogen attack

Several of studies have demonstrated that JA is required for resistance against biotic stresses (pathogens attack, insect wounding, etc.) in plants (Fraire-Velazquez et al., 2011). To analyze the influence of the JASSY mutation on plant defense towards biotic stress (pathogens attack) we treated WT and the *jassy* mutant with the Arabidopsis pathogen *B. cinerea* (*Botrytis cinerea*) for two days. The reason for choosing the biotrophic pathogen *B. cinerea* was that this pathogen has a very narrow host range , it only intects the model plant Arabidopsis and is absolutely dependent on

nutrients acquired from the living plant cells (Glazebrook, 2005; Weiberg et al., 2013). After two days of treatment, the WT leaves showed a mild infection status, whereas the leaves of *jassy* showed large lesions (Fig. 15A). Quantification of the lesion size is shown in Fig. 15C. After five days of infection, WT leaves were yellowish in most parts of the leaves but still alive. In contrast, the *jassy* mutant was much more drastically affected (Fig. 15B). Since the leaves from the *jassy* mutant were largely damaged, a quantification was not performed after five days.

Conclusively, these data clearly showed that the *jassy* mutant exhibited enhanced susceptibility to pathogen attack as compared to the WT.

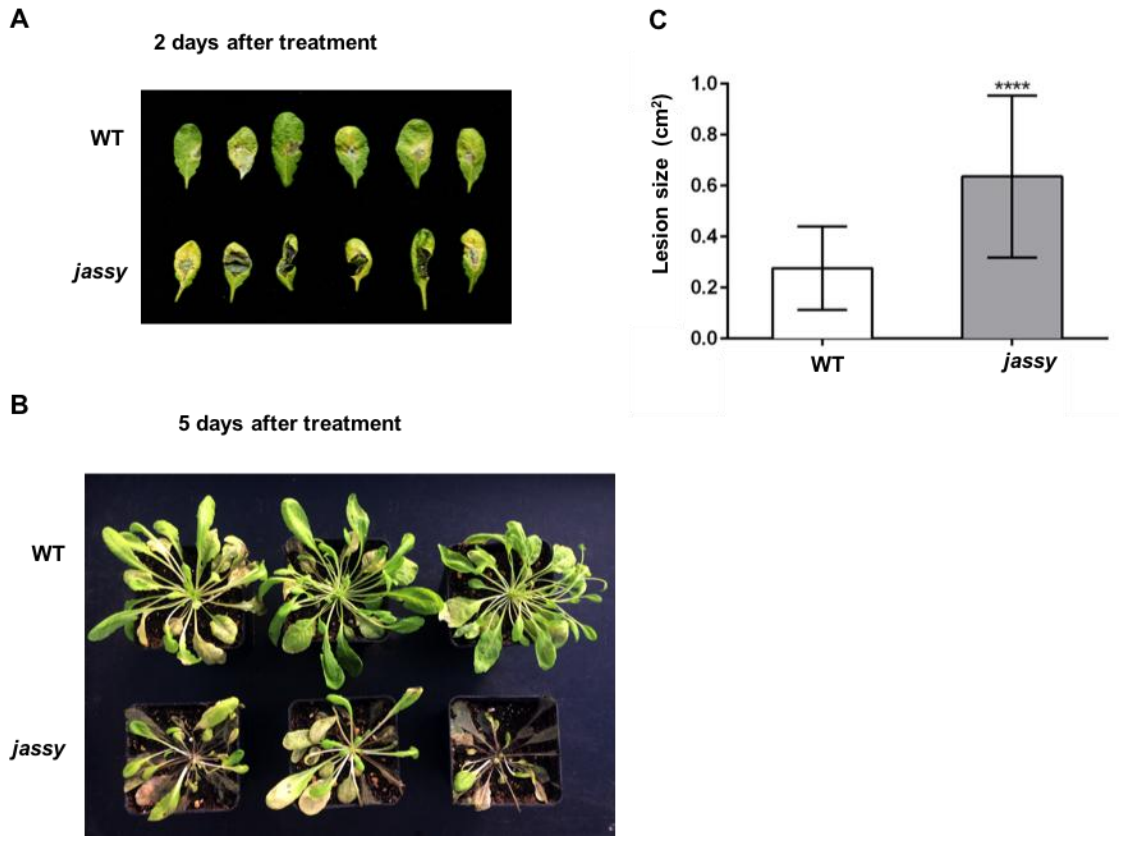


Figure 15: The *jassy* mutants showed enhanced susceptibility to pathogen attack. (A) Lesion formation in WT and the *jassy* mutant after *B. cinerea* treatment for two days or 5 days **(B)**. **(C)** Quantification of lesion size after two days of *B. cinerea* treatment. n > 100, p>0.001.

3.3.3 Loss of JASSY decreases cold tolerance in Arabidopsis.

Apart from its role in response to biotic stresses, JA is also an important player in the response to abiotic stresses, such as defense against cold (Hu et al., 2013) and wounding (Gfeller et al., 2011). To assess the role of JASSY in plants response to cold stress, WT and the *jassy* mutants were grown for 14 days under normal growth conditions and were then transferred to 4°C for 7 days. Compared to the WT control, the *jassy* mutants showed a significant reduction in growth, after the cold treatment (Fig. 16A). This was monitored by measurements of the leaf size as well as of the fresh weight, both of which were significantly reduced in the mutant after cold treatment (Fig. 16B and C). To the contrary, in the complemented line, *CaMV35S::JASSY/jassy*, both the leaf size and the fresh weight were recovered to a large extent (Fig. 16).

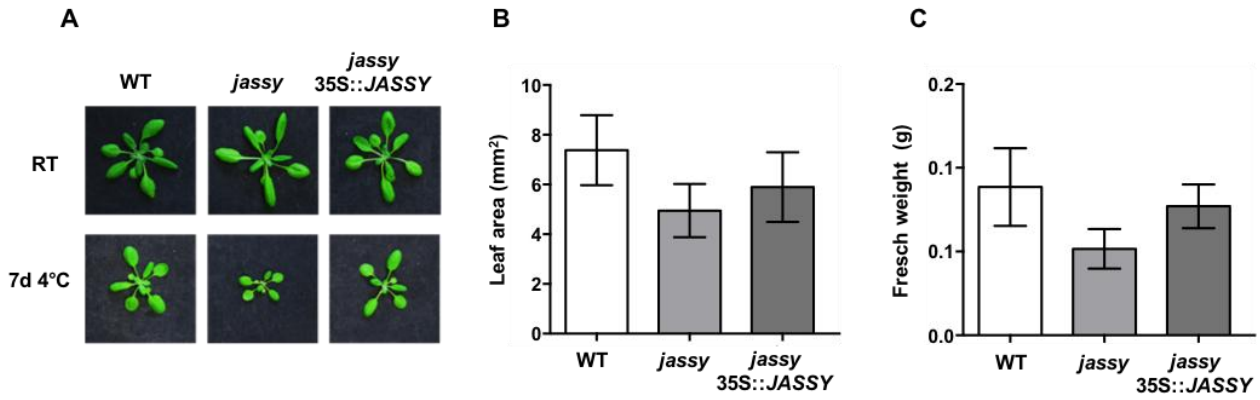


Figure 16. The *jassy* mutant showed reduced tolerance to cold treatment. (A) WT, *jassy* and *CaMV35S::JASSY/jassy* were grown under standard conditions for 14 days and then were transferred to 4°C for 7 days (lower panel) or continued to grow under standard conditions (upper panel). Measurements of leaf size **(B)** and fresh weight **(C)**.

3.4 JASSY is involved in the JA biosynthesis pathway

3.4.1 The expression of JA-responsive gene (*PDF1.2*) was not activated in the *jassy* mutant upon pathogen attack

As shown in Fig. 15, the *jassy* mutant was susceptible to pathogen attack. To study the relationship between JASSY and JA pathway during pathogen attack, the transcript level of *PDF1.2* (*Plant defensin 1.2*), was measured. *PDF1.2* is a well-characterized JA-responsive gene, specifically induced by JA upon pathogen treatment or wounding (Pieterse et al., 2012). We tested the expression of *PDF1.2* before and after pathogen treatment to determine whether the susceptibility of the *jassy* mutant to the pathogen attack was due to a defect in the JA signaling pathway. Indeed, although *PDF1.2* expression was increased to almost 2.5-fold in the WT upon two days pathogen treatment, no change of *PDF1.2* expression was observed in *jassy* (Fig. 17).

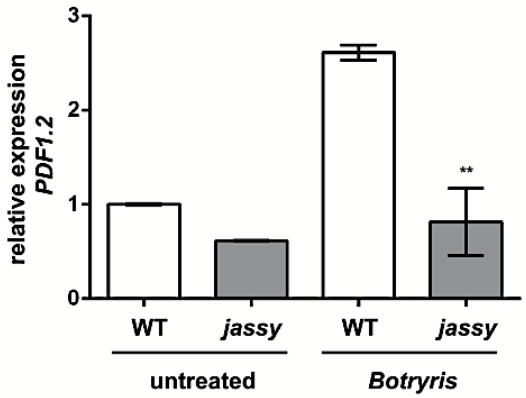


Figure 17. The induction of JA-responsive gene (*PDF1.2*) was suppressed in the *jassy* mutant upon pathogen treatment. Transcript levels of *PDF1.2* in untreated plants and pathogen treated (48 h) plants were measured by qRT-PCR. Values represented mean value of three independent replicates with standard error.

3.4.2 The expression of JA-responsive gene (*ICE1*) was not activated in the *jassy* mutant upon cold treatment

Previous studies have documented that JA deficient mutants were susceptible to cold stress (Sanders, 2000; Chinnusamy et al., 2007; Hu et al., 2017). Therefore, we treated the *jassy* mutant with similar cold stress. Indeed, we saw a strong reduced growth in the *jassy* mutant under the cold treatment (Fig. 16). Next, we analyzed the expression of the cold-responsive transcription factor *ICE1* (Inducer of *CBF* expression 1) by qRT-PCR (McConn, 1996; Creelman, 1997). *ICE1* is activated by JA and plays an important role in cold acclimation by inducing the expression of *CBF3*, which is a C-repeat binding factor (CBF). CBFs, in turn, activate downstream targets and mediated cold acclimation (Pieterse, 1998). In the WT, *ICE1* mRNA expression was upregulated almost eight-fold after 24 h cold treatment. As expected , expression levels of *ICE1* were below the limit of detection in the *jassy* mutant (Fig. 18).

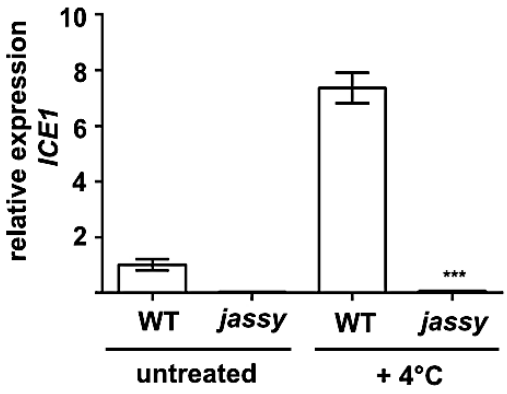


Figure 18. The expression of cold-responsive gene *ICE1* was not induced in the *jassy* mutant. Transcript levels of *ICE1* was measured by qRT-PCR. Plants were grown for 14 days at 21℃ and were grown at 4℃ for 1 week. For qRT-PCR, results represent mean values with standard error of three independent experiments and *ACTIN2* was used as an internal control.

3.4.3 The expression of JA responsive genes is not activated in the *jassy* mutant upon wounding

Plants grown under changing environmental conditions are highly vulnerable and easily susceptible to injury by wounding stress. In plants, under wounding stress, the JA biosynthesis pathway is triggered. Wounding stress causes large-scale changes in gene expression, such as transcription factors and JA-responsive genes (Rehrig et al., 2014). To further investigate whether loss of JASSY induces a defect in JA signalling, the expression of several JA-responsive genes was analysed under wounding stress (Fig. 19).

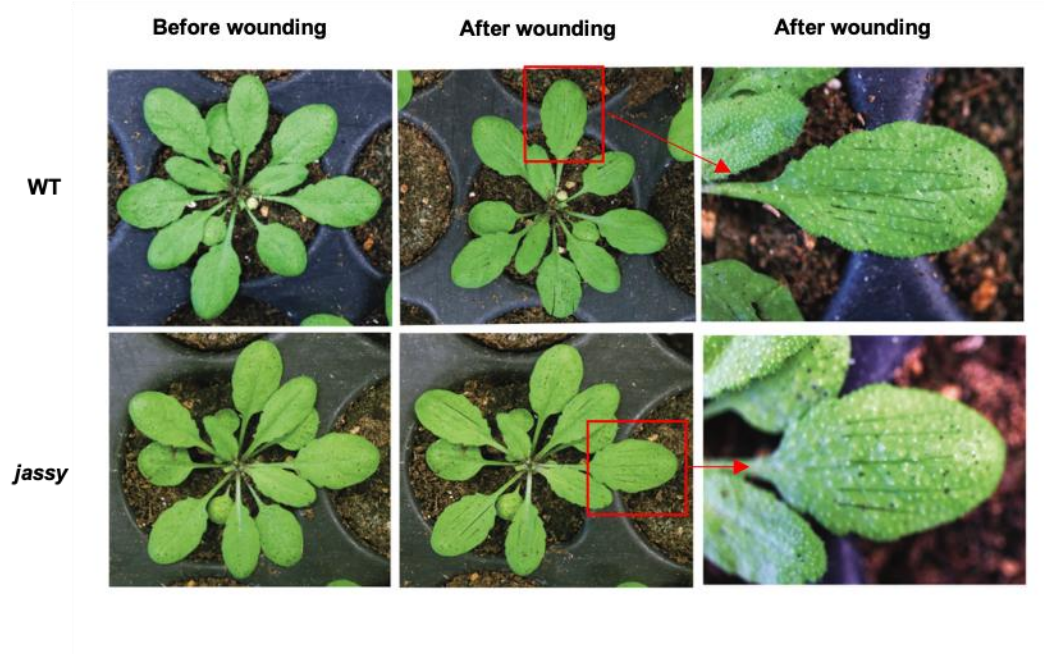


Figure 19. Wounding experiment setup: leaves of four-week-old plants (WT and *jassy* mutant) were wounded by cutting with sharp razor blades. Each leaf was wounded on each occasion for 5 times. No leaf was wounded more than once. Wounded plants were covered for 1.5 hours. Harvested leaves were immediately stored in liquid nitrogen.

Previous reports had shown that the genes involved in plant defence such as *PDF1.2 and MYC2* (Transcription factor *MYC2*) were upregulated upon wounding stress via participation of JA (Doerks et al., 2002). The accumulation of JA reaches its highest

level 90 min after wounding (Kienow et al., 2008). *PDF1.2* can be specifically induced by JA upon wounding and pathogen treatment. In the *jassy* mutant, the induction of *PDF1.2* was totally lacking 90 min after wounding (Fig. 20A). However, in WT, *PDF1.2* was highly inducted (roughly 15fold) by wounding (Fig. 20A). *MYC2* is a basic-helix-loop-helix transcription factor sensitive to JA. This protein is a well described master regulator of the JA signalling pathway (Pieterse et al., 2012). No increase of *MYC2* expression can be seen after wounding in the *jassy* mutant (Fig. 20B), similar to the expression of *PDF1.2* (Fig. 20A). Moreover, JASMONATE-ZIM DOMAIN (*JAZ*) repressor proteins were known to be upregulated upon wounding (Davies, 2010). The *JAZ* transcripts were upregulated for 30-fold in WT upon wounding. However, no upregulation was observed in the *jassy* mutant (Fig. 20C). According to the expression of these JA-responsive genes (*MYC2* (Fig. 20B), *PDF1.2* (Fig. 20A) and *JAZ* (Fig. 20C)), we concluded that JASSY is involved in the JA pathway.

Given that JASSY is a chloroplast envelope protein, we suspected that JASSY might be involved in the JA biosynthesis pathway. So, we measured the transcripts of two chloroplast localized enzymes (*LOX2* and *AOS*) which were involved in the JA biosynthesis (Wasternack, 2007). In the WT, the level of JA was increased upon wounding, which caused the degradation of JAZ and thus removed the suppression effects of JAZ. As a result, the expression of JA-responsive genes was upregulated. In line with our previous results, we observed that induction of *LOX2* and *AOS* were lacking in the *jassy* mutant (Fig. 20D and E). These data revealed that JASSY is involved in response to the mechanical wounding stress.

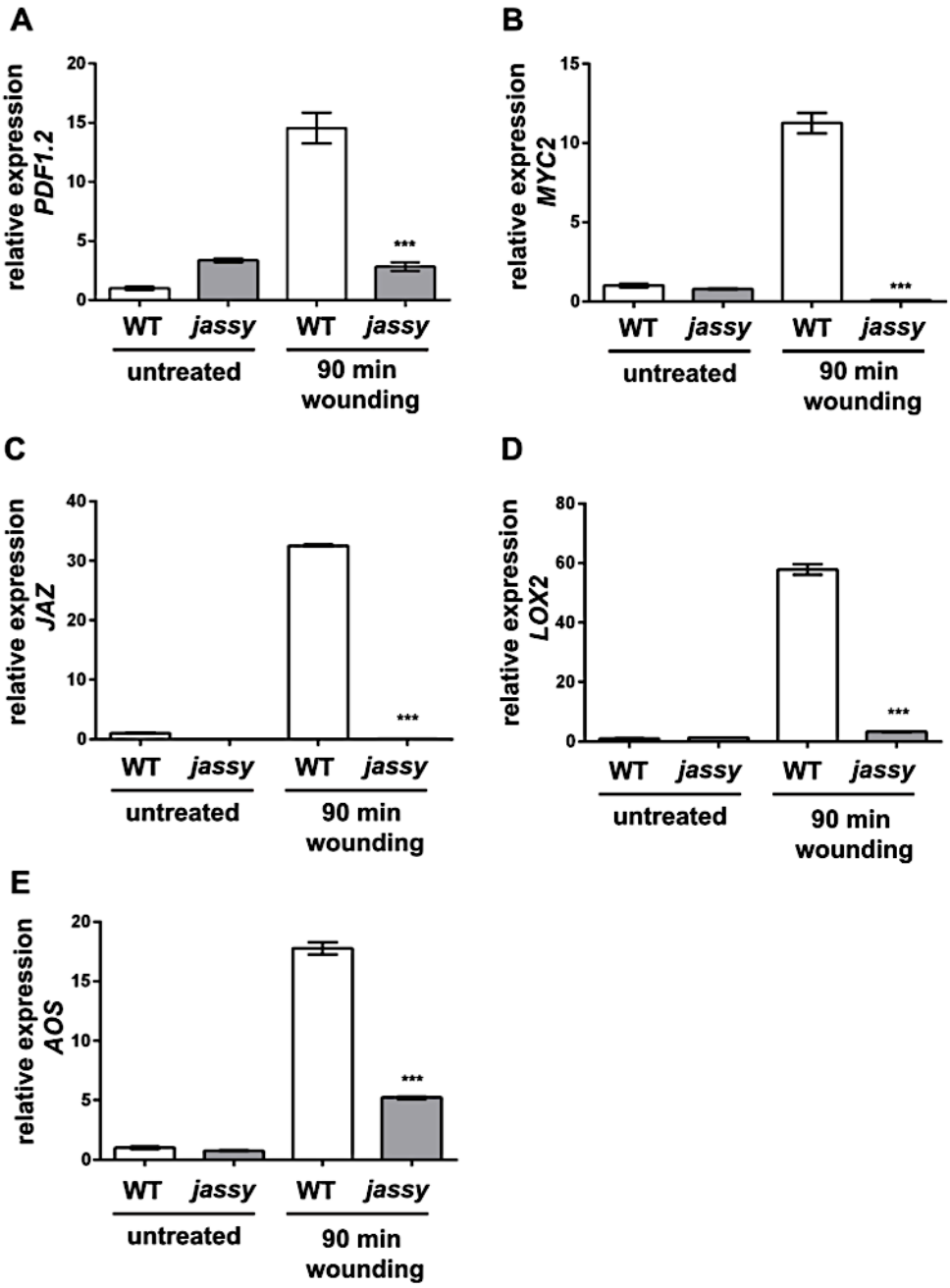


Figure 20. The induction of JA-responsive genes was abolished in the *jassy* mutant. Transcript levels of *PDF1.2* **(A)**, *MYC2* **(B)**, *JAZ* **(C)**, *LOX2* **(D)** and *AOS* **(E)** in unwounded plants and wounded (90 min) plants were measured by qRT-PCR. For qRT-PCR, results represent mean values with standard error of three independent experiments and *ACTIN2* was used as an internal control.

3.4.4 Feeding the *jassy* mutant with JA-Ile or JA biosynthesis intermediate, OPDA, restored the induction of JA-responsive gene *PDF1.2*.

To strengthen the hypothesis that the lack of JA was indeed responsible for the observed defects in gene expression, we fed WT and *jassy* mutants with JA-Ile. Interestingly, the expression of *PDF1.2* could be fully recovered in *jassy* after 30 min of feeding with JA-Ile (Fig. 21A). In the WT, the expression of *PDF1.2* was further induced 120 min after the feeding with JA-Ile. However, the expression of *PDF1.2* was reduced in the *jassy* mutant. A possible reason was that the JA-Ile used for feeding might be used up and the content of JA in the *jassy* mutant decreased, and thus led to the down regulation of *PDF1.2* expression. In the WT, however, a positive feedback is expected to be initiated, thus further enhancing expression. The observed induction clearly demonstrates that JASSY is not directly involved in the induction of JA-responsive genes, but rather participates in the biosynthesis of JA.

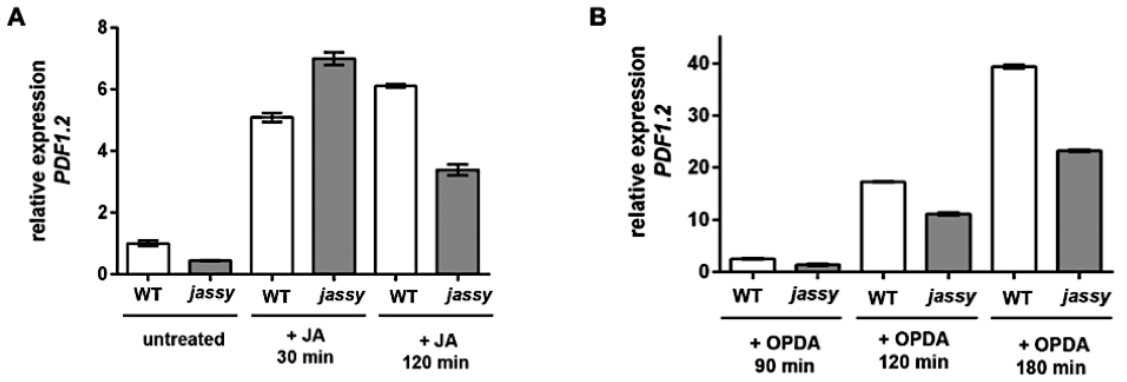


Figure 21. Feeding of the *jassy* mutant with JA-Ile or OPDA restored the induction of *PDF1.2*. (A). WT and the *jassy* mutants were sprayed with or without 200 µM JA-Ile (in 0.2% EtOH). The expression of *PDF1.2* was determined 30 and 120 min after the treatment. (**B).** WT and the *jassy* mutants were sprayed with or without 50 µM OPDA (in 0.05% EtOH). The expression of *PDF1.2* was determined 90, 120 and 180 min after the treatment.

Considering that JASSY localized to the chloroplast outer envelope, it is reasonable to assume that it could likely be involved in the export of OPDA. In this case, also external treatment with OPDA should restore the transcription of JA responsive genes. Therefore, OPDA was subjected to WT and *jassy* plants and expression levels of

PDF1.2 were determined 90, 120 and 180 min after the treatment. Indeed, it could again be observed that the expression of *PDF1.2* was induced in *jassy*, almost to WT levels (Fig. 21B). Taken together, these data strongly suggest that JASSY mediates OPDA export from the chloroplast.

3.4.5 Lack of JASSY prevented JA accumulation

Since lack of JASSY blocked the expression of JA-responsive genes, and feeding the *jassy* mutant with JA-Ile or the JA biosynthesis intermediate, OPDA, restored the induction of JA-responsive genes, we suspected that the *jassy* mutant might be unable to synthesize JA, which means that the content of JA in the *jassy* mutant should be very low or even undetectable. In here we used wounding to active the JA biosynthesis pathway, and then we determined the JA content using HPLC. In the WT, 90 min after wounding, a clear accumulation of JA was observed (Fig. 22). Whereas in the *jassy* mutant, no increase was detected, only a basal level of JA could be detected (Fig. 22).

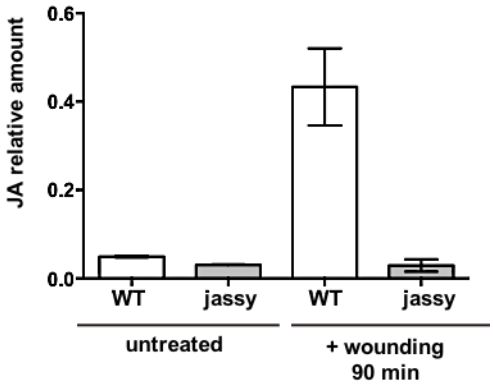
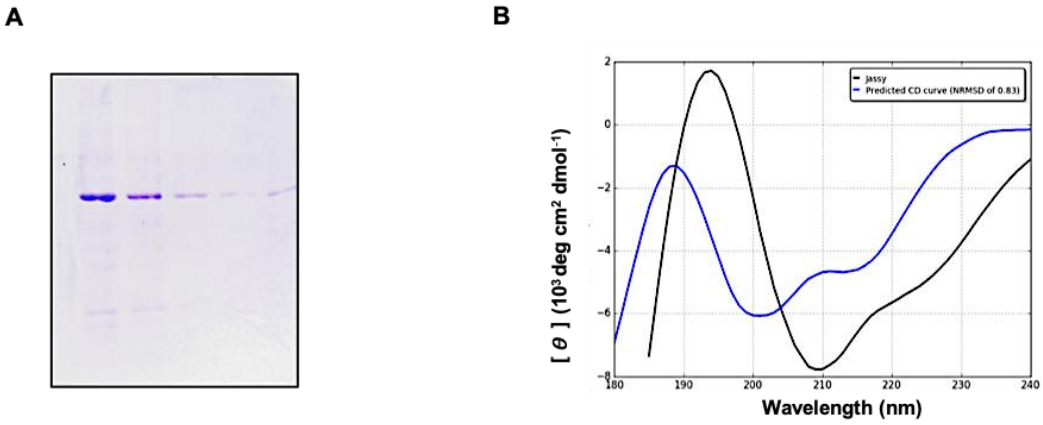


Figure 22. No JA accumulation is observed in the *jassy* mutant after wounding. The Arabidopsis WT and *jassy* mutant plants were wounded 5 times with a sharp blade, and the whole plants were harvested 90 min later. Plants were immediately frozen, and stored in liquid nitrogen for subsequent extraction and analysis by HPLC. The data showed the mean and SD of at least three independent determinations.

3.5 The purified JASSY protein was properly folded

To estimate of the overall secondary structure of JASSY, we first cloned the cDNA (without start and stop codon) of *JASSY* into the expression vector pET-51b(+). Thereby the C-terminus of the JASSY protein was fused with a Strep tag and the N-terminus was fused with a His tag. The recombinant protein was expressed in *E.coli Rosetta II*, and purified via Ni-NTA and Strep beads (Fig. 23A). The secondary structure of JASSY was characterized via circular dichroism spectroscopy (CD spectroscopy).



C

Prediction via Chou-Fasman-algorithm
(Only based on provided amino acid sequence)

Irregular	ß-strand	helix
0.41	0.27	0.31

Prediction via basis spectra

Irregular	ß-strand	helix
0.55	0.36	0.02

Figure 23. Purification of the JASSY protein and its secondary structure determination. (A) The JASSY protein was purified via Ni-NTA and Strep beads and its purity was verified using 12% SDS-PAGE. **(B)** The secondary structure of the purified JASSY protein was determined by CD spectroscopy (black line) or predicted via Chou-Fasman-algorithm (blue line). The wavelength started from 185 nm to 260 nm and the step length is 1 mm. **(C)** The structure prediction based on the obtained spectrum

The recorded spectra of JASSY, displaying two minima at 210 and 220 nm and a large peak of positive ellipticity cantered at 193 nm, was a typical characteristic of ß-strand

protein (Fig. 23B). These spectra indicated that the secondary structure of the JASSY protein consisted of 2% α-helix, 36% ß-strand and 55% irregular structure. This result was different with the secondary structure predicted by the Chou-Fasman-algorithm (http://www.biogem.org/tool/chou-fasman/) based on the amino acid sequence of JASSY (Fig. 23C). But the similarity of the curve between the CD spectroscopy and the structure prediction demonstrated that the protein was properly folded after purification. (Fig. 21B)

3.6 JASSY binds to OPDA

As mentioned above, the JA biosynthesis takes place in both chloroplast and peroxisomes. OPDA is the final product of JA biosynthesis in the chloroplast, then OPDA is exported from the chloroplast and goes into the peroxisome to finish the JA biosynthesis. Considering that JASSY is a chloroplast outer envelope protein and the *jassy* mutant is unable to accumulate JA, we believe that JASSY might be involved in transport of OPDA out of the chloroplast. To test this idea, we performed an interaction assay between JASSY and OPDA. Since JASSY belongs to the Bet v1-like superfamily, which comprises domains known to function in binding hydrophobic components, we analyzed a potential direct interaction between OPDA and JASSY using microscale thermophoresis (MST) (Fig. 24). The assay results indicate that a binding event occurs between JASSY and OPDA, even if rather high concentrations of OPDA had to be applied, resulting in an equilibrium dissociation constant (KD) of ~ 1 mM (Fig. 24). The KD value is used to evaluate the strengths of bimolecular interactions. The larger the KD value, the weaker interactions; the smaller the KD value, the stronger interactions. The KD value of 1 mM implies a weak but real binding between JASSY and OPDA (Fig. 24). In contrast, no binding curve could be detected between JASSY and JA (Fig. 24).

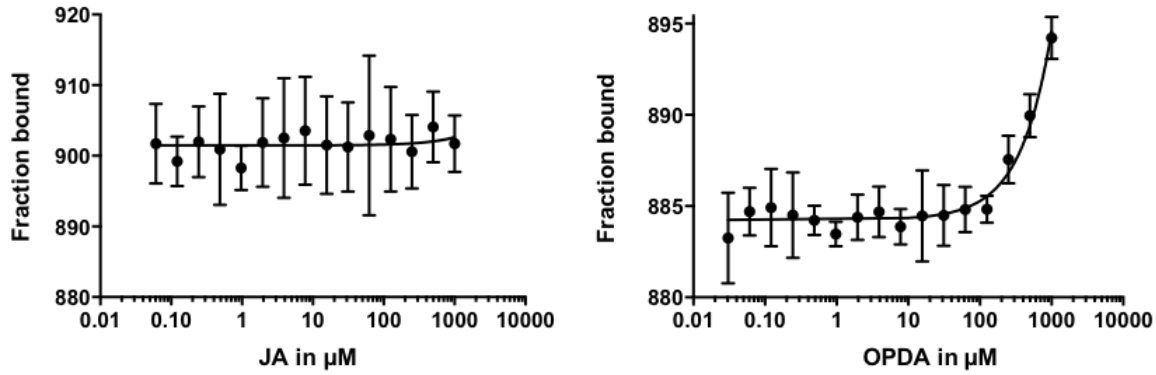


Figure 24: JASSY transiently interacts with OPDA but not JA. Thermophoretic mobility was monitored upon OPDA or JA titration (from 30 nM up to 1mM) was applied to a constant fluorescence labelled JASSY (25 nM). A weaker interaction between JASSY and OPDA was observed with a KD value of 0.8 mM, and no interaction between JASSY and JA was observed. This binding assay was determined by MST. This experiment was performed by Ahmed Eisa.

3.7 JASSY functions as a membrane channel

Since JASSY was able to bind OPDA, we wondered whether JASSY has the capacity to form a membrane channel and transfer OPDA out of the chloroplast. Thus, we performed a liposome leakage assay. To this end, carboxyfluorescein (a fluorescent dye) containing liposomes were generated, which were then reconstituted with recombinant JASSY protein. After the reconstitution, fluorescence emission of the carboxyfluorescein dye was recorded. As carboxyfluorescein is a large molecule with a formula mass of 376.3, and is unable to transfer freely through the liposome, the only possibility for the dye carboxyfluorescein to exit the liposome is via the reconstituted protein. If the JASSY protein can form a channel embedded in the liposome, the fluorescent dye carboxyfluorescein could be able to passthrough this channel and to be released into the buffer outside of liposome. As a result, the fluorescence in the buffer will increase as the dye carboxyfluorescein starts to accumulate in the buffer over time. We do see a fluorescence increase in the buffer when JASSY protein is incorporated in the liposome (Fig. 25). This means that JASSY indeed forms channel in the liposome. TIC110 is a known protein that forms a channel in the chloroplast envelope, and is used as a positive control in this experiment (Fig. 25).

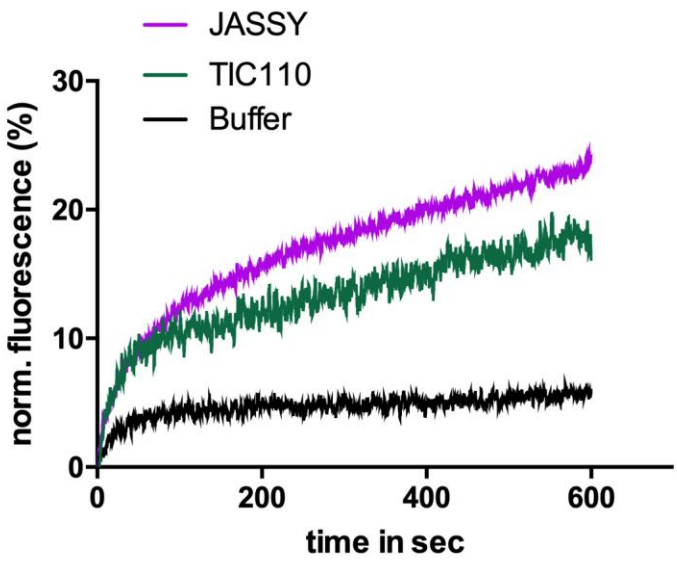


Figure 25: JASSY functions as a channel. 5 µg of 1 mg/ml of JASSY or TIC110 was added to 1 mg of liposome (200 µl). Its fluorescence emission at 515 nm excited by a wavelength of 494 nm was recorded every 1 ms. Initial fluorescence was set to 0, whereas total liposome quenching resulted from adding of 0.5 % Triton X-100 was set as 100 %. 1 x PBS buffer (pH7.4) was added to the liposomes as a negative control.

Since the liposome leakage assay indeed suggested that JASSY is able to form membrane pores, we used the planar lipid bilayer technique for an electrophysiological characterization to investigate this observation further. To this end, we inserted JASSY into preformed liposomes via detergent-mediated reconstitution. To assess incorporation success, we analyzed co-migration of the protein with liposomes in a density flotation assay and found the JASSY containing floating proteoliposomes. We confirmed that incorporated JASSY was resistant to carbonate extraction and as such behaves as an integral membrane protein (Fig. 26A). In a next step, JASSY containing vesicles and identically treated liposomes with a purified sample of an empty plasmid mock expression were separately fused with planar lipid bilayers and subjected to high-resolution electrophysiological characterizations. While JASSY containing proteoliposomes readily fused with bilayers and showed stable channel activity (Fig. 26B and C), the mock sample did not lead to channel insertion even after prolonged incubation times of several hrs (Fig. 26B). JASSY channels exhibited frequent voltage-dependent gating with dynamic conductance states between 100 and 600 pS at 250 mM KCl (Fig. 26B and C). The channels displayed a reversal potential of 31.4 ±

2.0 mV at a 12.5-fold KCl-gradient (Fig. 26D). Using the Goldman-Hodgkin-Katz equation this translates to a mild cation preference of about 5:1 over anions.

As we have shown that JASSY shows binding affinity to OPDA, we probed possible substrate specific alterations of OPDA on the channel properties. Ethanol solubilized OPDA was added to preinserted channels in situ and channel characteristics prior and after OPDA addition were determined. OPDA addition led to significantly increased gating frequencies of incorporated JASSY channels (Fig. 26F – H). Gating frequency analysis suggested that all conductance states were enhanced similarly; with an additional increased abundance of a high conductance state above 600 pS (Fig. 26G). While OPDA addition led to a 6-fold increase in gating frequency on average, no significant change was observed after addition of the solvent (Fig. 26H).

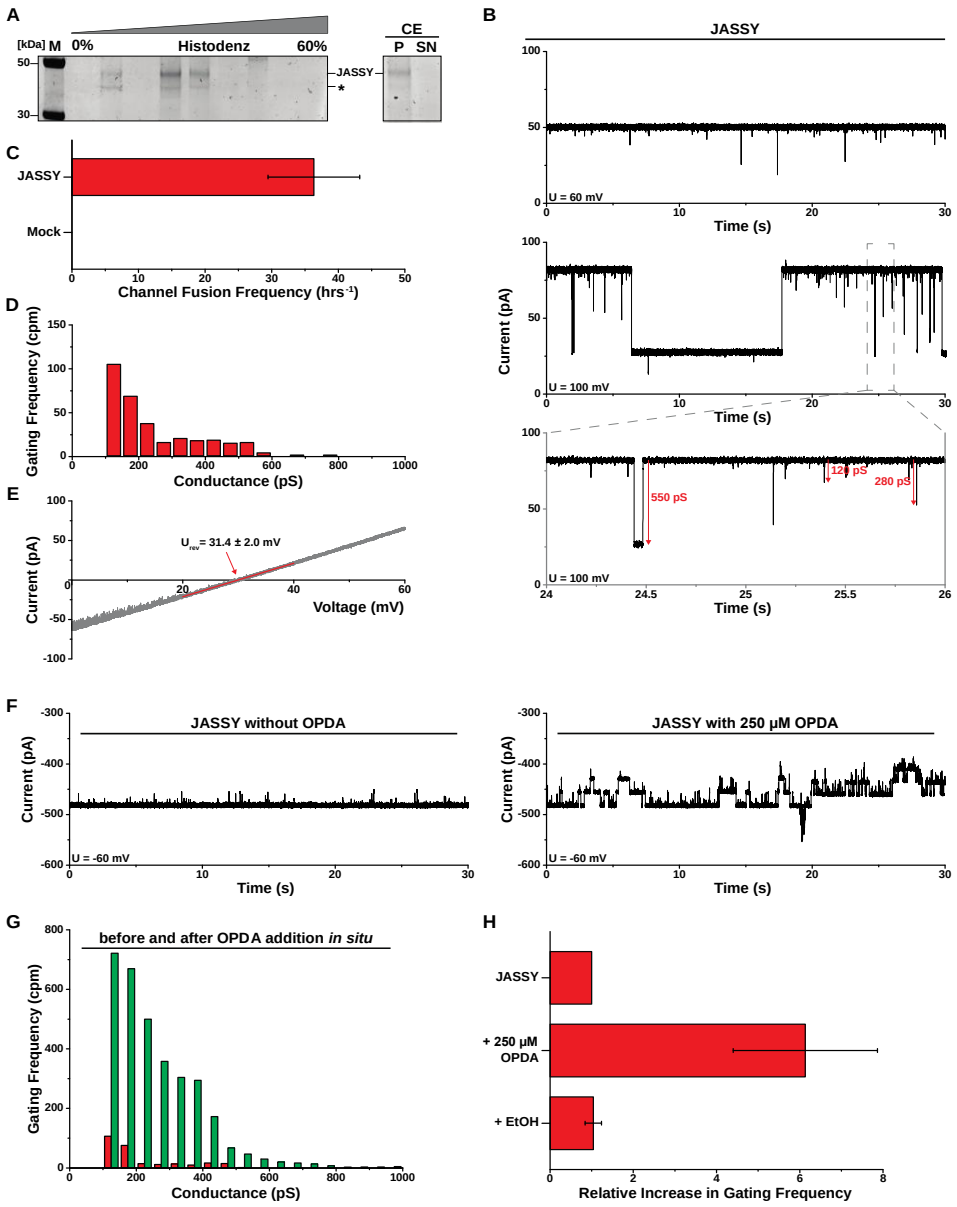


Figure 26: JASSY forms an OPDA-sensitive membrane channel. (A) JASSY-proteoliposomes were subjected to density gradient flotation and subsequent sodium carbonate extraction (CE). Asterisk denotes JASSY degradation band. (**B)** Current flux through channels formed by JASSY were recorded at various indicated membrane potentials. Conductance changes of selected gating events are indicated. (**C)** Frequency of channel appearance due to proteoliposome fusion with the lipid bilayer was determined for both JASSY- and mock-expressed incorporations (n = 3, two hrs per experiment, error: SEM). (**D)** Gating event frequency was calculated from current recording sets as shown in B (n = 6). (**E)** Current-voltage relation was recorded at asymmetric buffer conditions (250 mM (cis) to 20 mM (trans) KCl), and the reversal potential was extracted via linear regression for ion selectivity quantification (n = 3). (**F)** Current trace of multiple JASSY channels was recorded before (left) and after (right) *in situ* incubation with OPDA. (**G)** Frequency of gating events before (red) and after (green) incubation of JASSY with OPDA was calculated from three independent experiments, as shown in D. (**H)** Average increase in gating frequency was quantified for OPDA and EtOH (n = 3, error: SEM). This experiment was performed by Niels Denke and Michael Meinecke (University of Göttingen).

4. Discussion

Hormones play a critical role in plant growth and development. For example, Auxin can promote or inhibit the abscission of leaf and fruits depending on the different timing. GA (Gibberellins) can cause seed germination and stem elongation. Ethylene plays a key role in ripening of fruits, and BR (Brassinolide) is needed for plant fertility. Hormones not only play a role in growth and development but also in response to different stresses. For example, ABA (Abscisic acid) promotes shoot growth and also response to drought stress. When facing different biotic and abiotic stresses, plants rely on specific hormones, to cope with these different stresses (Davies, 2010). Among those various hormones, JA derivates are particularly necessary for plant's survival under extreme environments. JA-Ile, the most common active form of JA in the plant kingdom, is best described in root elongation, sterile and leaf sensitivity (McConn, 1996; Creelman, 1997; Pieterse, 1998; Overmyer, 2000), Moreover, JA-Ile appears to be involved in response to special environmental signals like wounding (Howe, 2004) , cold (Sharma and Laxmi, 2016), and pathogen attack (Zhang et al., 2017).

Whereas the enzymatic steps involved in JA biosynthesis have been investigated and studied in detail during the past decades, slow progress has been made concerning the transport of JAs and its precursors. How different JA-related precursors transport into the different organelles became a popular topic in recent years. Recently, JAT1 was identified as a transporter for JA-Ile into the nucleus (Li et al., 2017). An interesting finding in this study was that JAT1 controls the partition of JA in cytoplasm and nucleus by mediating the efflux and influx of JA-Ile (Li et al., 2017). This work illustrates that the entry of small hormone molecules into the nucleus by the transporter JAT1 is presents a novel mechanism to mediate JA signal pathway. As to OPDA, the ATP binding cassette (ABC) transporter COMATOSE (CTS) has been suggested to at least partially be involved in OPDA import into peroxisomes. CTS is thought to transport a wide range of peroxisomal fatty acids or acyl-coenzyme A coupled fatty acids. Levels of JAs are reduced in the mutants, but not absent, thus it is expected that other pathways for OPDA entry into peroxisomes exist in parallel (Theodoulou, 2005; Footitt et al., 2007; Bussell et al., 2014). Unfortunately, the

mechanism of OPDA export out of the chloroplast is still a remaining gap which needs to be closed.

In this study, we provide evidence that JASSY is a novel chloroplast OE protein, which fulfils the function of transporting OPDA out of the chloroplast. To test this hypothesis, we used a JASSY knockout line in comparison to WT plants. On the one hand, due to the defect in OPDA export, JA accumulation in Arabidopsis leaves is impaired entirely in the mutant, leading to a disturbed response to wounding, pathogen attack as well as cold acclimation. On the other hand, these effects were not only observed on the phenotypical level, however we could also show that gene expression in the respective JA responsive signaling cascades is not induced in mutants lacking JASSY. These results provide evidence that JASSY is involved in the JA signaling pathway. Experiments depicting that gene expression could be re-activated by feeding JA-Ile as well as OPDA clearly demonstrate that JASSY functions upstream of JA perception in the nucleus as well as upstream of OPDA to JA conversion in the cytosol and peroxisome. This finding seems to be in partial agreement with the data obtained by Seth J. Davis, who analyzed the transcription level of *JAZ* and *PDF1.2* (JA-responsive marker) by exogenous MeJA treatment in WT and *myc2* mutant. They found that exogenous applied MeJA could recover the JA signaling pathway in *myc2* mutant (Shin et al., 2012).

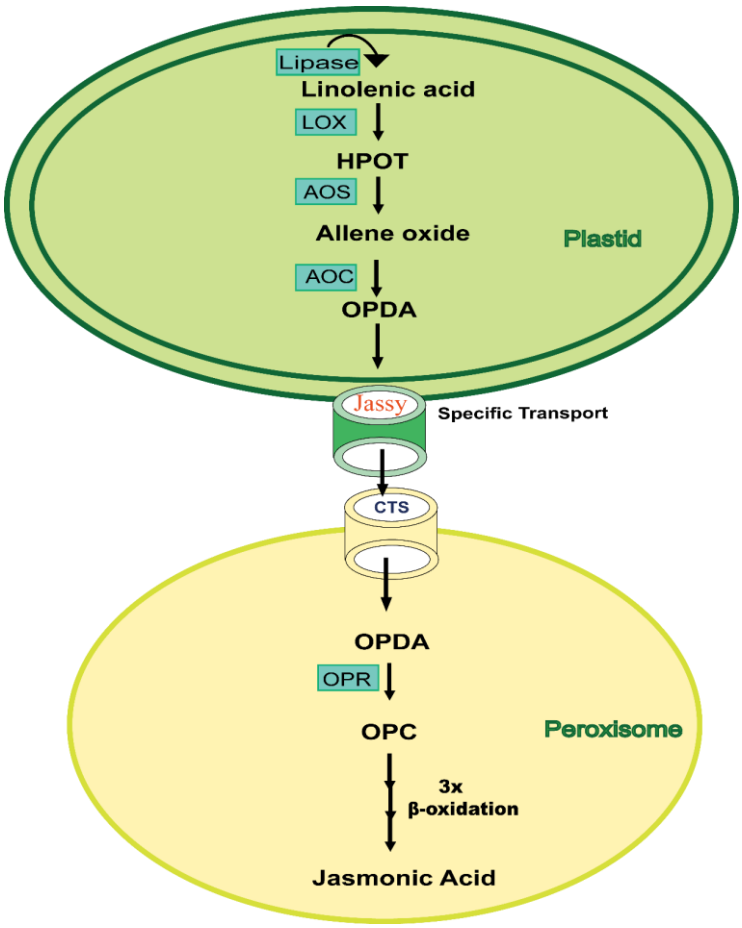


Figure 27: Model of JA biosynthesis and the hypothesized function of JASSY. JASSY is involved in the JA biosynthesis pathway via transporting OPDA from the chloroplast across the outer envelope. Abbreviations: LOX, 13-lipoxygenase; 13-HPOT, 13S-hydroperoxyoctadecatrienoic; AOS, Allene oxide synthase; AOC, allene oxide cyclase; OPDA, 12-oxo-phytodienoic acid; OPR, OPC: 3-OXO-2(2' 2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid reductase.

Over the last few years, a number of JA related mutants have been analyzed helping to elucidate the JA biosynthesis pathway as well as the role of JA during stress response and development. For example, the *fad3/fad7/fad*8 mutant is deficient in JA synthesis due to lack of α-LeA, the OPDA precursor in the chloroplast (Routaboul et al., 2000; Vijayan, 2002). Moreover, this mutant, just like many other mutants involved in JA biosynthesis or perception, such as *dad1* (Hatakeyama et al., 2003), *aos* (Park et al., 2002), *opr3* (Feys, 1994), and *coi1* (Yan et al., 2009), is male sterile. Interestingly, we did not observe problems with either male or female sterility in *jassy*. This may be explained by the fact that the Arabidopsis genome harbors a second gene (At1g23560, JASSY-2) with 46% identity on the protein sequence level to JASSY. JASSY-2, however, is solely transcribed in flower buds in contrast to JASSY, which is ubiquitously expressed in all developmental stages (www.bar.utoronto.ca). JASSY-2

might therefore take over the function of JASSY in flowers and play a role in flower maturation. Moreover, root growth inhibition is also one of the first and the best physiological response described of JA (Wasternack, 2007). For example, under normal growth conditions, the root length of Arabidopsis JA perception mutant *coi1* is relatively shorter compared with the WT control. Exogenous applied MeJA can inhibit the root growth of WT, but this root growth inhabitation was not detected in the JA perception mutant *coi1*(Yan et al., 2009). Based on these previous studies, we compared the root length of WT and the *jassy* mutant under normal growth conditions. Consistent with our anticipation, the *jassy* mutant did show a shorter root compared with the WT control (Fig. 13D). However, there was no significant difference could be seen when we compared the leaf size between WT and *jassy*. The results are exactly in line with the phenotype of *coi1*(Xie et al., 1998).

JASSY belongs to the Bet v1-like superfamily, a large protein family containing proteins with a similar three-dimensional structure but low sequence similarity. The common structure is characterized by a β-α2-β6-α fold, forming a U-shaped incomplete β-barrel wrapped around a long α-helix, thus forming a large hydrophobic binding cavity. The Bet v1 family is divided into eleven subfamilies, including the pathogenesis-related protein 10 (PR10), steroidogenic acute regulatory protein-related lipid transfer (START) domain proteins and oligoketide/cyclase/dehydrases (Radauer et al., 2008).

JASSY shows highest similarity to the START domain proteins. Moreover, a domain of unknown function (DUF220) is predicted in JASSY, partially overlapping with the START domain (Fig. 6). The hydrophobic cavity of most of the Bet v1 proteins functions in binding ligands, such as lipids, sterols or secondary metabolites (Radauer et al., 2008). In line with this, we observed that JASSY is able to bind OPDA, an interaction that is likely mediated by this conserved domain and may be important for the transport process.

Moreover, we found that JASSY is not only able to bind on membranes, but forms a voltage-gated channel in planar lipid bilayers. Our results suggest that JASSY is important for facilitating export of OPDA across the OE. Importantly, we found that JASSY indeed is an integral OE membrane protein and is able to form pores in

liposomes and planar lipid bilayers. These channels are substrate sensitive and show an OPDA-dependent activation, implicating a mode of transport of JA precursors across the OE through JASSY. Although the relatively high OPDA concentrations applied in these experiments may not reflect the overall OPDA concentration in chloroplasts, local concentrations of OPDA in the vicinity of JASSY may be significantly elevated *in vivo*. Moreover, it remains to be elucidated whether additional chloroplast proteins enhance binding efficiency under *in vivo* conditions.

Although the activity of some of the JA-related transporters like JAT1 (JA-Ile transporter in nuclei) and CTS1 (OPDA transporter in peroxisome) rely on the presence of ATP, the activity of JASSY does not. Moreover, we performed CD spectroscopy and our data demonstrates that JASSY is rich in ß-strand and irregulate folds. But this result is unexpected and contrary to the prediction from the amino acid sequence which suggesting a dominant of α-helix folded. This might be because of the expression with His and Strep tag, which promotes the discrepancy of the measurement. Or possibly, some of the α-helix are found as irregular folded protein during CD measurement. Additionally, the START domain that always forms the α-helix and ß-strand structure also supports the prediction of the structure instead of the measurement (Alpy et al., 2009). Whether the presence of His and Strep tags in the recombinant JASSY protein might affect the measurement of its structure is waiting for further characterization.

Whereas the function of JAs has been well established in all higher land plants, the presence of JAs in lower land plants and algae is still controversial. JAs have been detected in a broad range of bryophytes, however not in the moss *Physcomitrella patens* (Oliver et al., 2009; P.K.G.S.S. et al., 2009; Ponce De León et al., 2012). It was also demonstrated that JA and MeJA can be detected in most of the green algae and rhodophytes (Krupina and Dathe, 1991; Fujii et al., 1997) but not in the red algae *Chondrus crispus* (Bouarab et al., 2004). Interestingly, MeJA was detected after red algae *Chondrus crispus* incubation with linolenic acid (Bouarab et al., 2004). Whether JA and MeJA exists in the brown algae is still a unclear. It is possible that there is another JA biosynthesis pathway exists in algae. Moreover, JA and JA-Ile were found in many of the fungus species, like *Fusarium oxysporum* (Günther et al., 1993;

Miersch et al., 1999; Wasternack, 2007). Few years later, the results of genomic-wide similarity search clearly demonstrated that all the homologs of JA biosynthesis membranes can be found in higher land plants and some but not all the chlorophytes and charophytes. So, it is possible that the canonical JA biosynthesis machinery existed even before the appearance of the land plants but have been lost many times during the plant evolution (Han, 2017). OPDA can be detected in many of the species, such as *P.patens* (Stumpe et al., 2010) and *K. Flaccidum* (Yasumura et al., 2007). However, there is no JA present in these plants. Whether OPDA is present in chlorophytes is still unclear, but it is known that OPDA biosynthesis originated in the algal lineage even before the emergence of the land plants. In lower plants, OPDA seems play an import role in fertility, wounding responses and seeding germination. In higher land plants, OPDA can be cooperative with JA to regulate the expression of defense genes (Yasumura et al., 2007; Stumpe et al., 2010).

Interestingly, JASSY is conserved among land plants, bryophytes and green algae, supporting the notion that OPDA functions as a signaling molecule in *Physcomitrella* (Fig. 28).

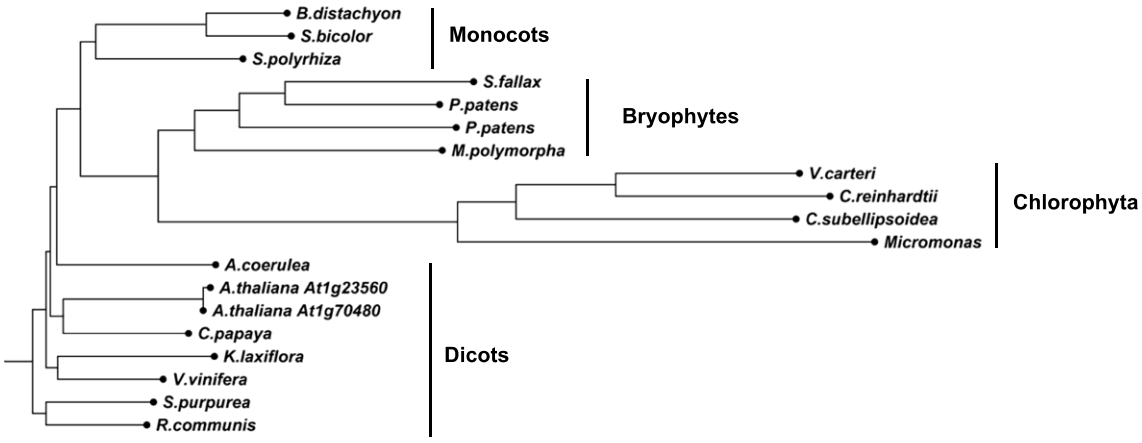


Figure 28: Phylogenetic analysis of JASSY Phylogenetic tree based on degrees of homology between protein sequences of JASSY from green algae to higher plants. Representative members of each clade are shown. The phylogenetic tree was constructed by the distance-based method with the neighbor-joining algorithm using a bootstrap value of 100.

With our experiment results and discussion above, we conclude that JASSY is localized in the outer envelope of the chloroplast and forms a channel to transport the

precursor of JA, OPDA, out of chloroplast outer envelope. The model (Fig. 27) illustrates the role of JASSY in the biosynthesis of JA.

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Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt wurde.

München, den 27.03.2019

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Erklärung

Hiermit erkläre ich, dass ich zuvor nicht versucht habe, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen. Die vorliegende Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

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Danksagung

Die Danksagung wurde aus Datenschutzgründen entfernt.