The role of cytokinin receptors in plant defense response of *Nicotiana attenuata*

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List of Abbreviations

- BSA bovine serum albumin
- cDNA complementary desoxyribonucleic acid
- CK(s) cytokinin(s)
- CKR(s) cytokinin receptor(s)
- cm centimeter(s)
- cZR cis-zeatin riboside
- DEPC diethylpyrocarbonate
- DTG diterpene glycoside
- dNTP didesoxyribonucleosidetriphosphate
- EV empty vector
- FAC fatty acid-amino acid conjugate
- fig figure
- GA_3 gibberellic acid
- h hour(s)
- HAMP herbivory-associated molecular pattern
- HK histidine kinase
- HPLC high performance liquid chromatography
- JA jasmonic acid
- MAPK mitogen-activated protein kinase
- min minutes
- MJ methyl jasmonate
- MES 2-(N-morpholino)ethanesulfonic acid
- mm millimeter(s)
- OD optical density
- PCR polymerase chain reaction
- PDS phytoene desaturase
- PI proteinase inhibitor
- qPCR quantitative PCR
- RNA ribonucleic acid
- ROS reactive oxygen species
- RT room temperature
- SA salicylic acid
- tab table
- tZ trans-zeatin
- VIGS virus-induced gene silencing
- WT wild type

1 Zusammenfassung

Um sich gegen herbivore Insekten verteidigen zu können, haben Pflanzen komplexe Mechanismen entwickelt, die in direkte und indirekte Verteidigungsreaktionen unterteilt werden können. Die indirekte Verteidigungsreaktion beinhaltet beispielsweise die Anlockung von natürlichen Feinden der Herbivoren, z.B. durch Abgabe von volatilen Duftstoffen. Zusätzlich werden chemische Stoffe produziert, die den Insekten Schaden direkt zufügen können: Beispiele sind verdauungshemmende Proteinase-Inhibitoren (PI) oder Sekundärmetabolite, die toxisch oder abstoßend wirken können. All diesen Reaktionen liegt ein streng reguliertes Signalnetzwerk zu Grunde, das durch die Wahrnehmung Herbivoren-assoziierter molekularer Muster aktiviert wird. Obwohl dieses Netzwerk noch nicht vollständig aufgeklärt ist, ist bereits bekannt, dass Zellmembrandepolarisation, Calcium-Ströme, reaktive Sauerstoff-Spezies, mitogen-aktivierte Proteinkinasen und Phytohormone, v.a. Jasmonsäure und -derivate, daran beteiligt sind. Schließlich führen diese Signalwege zu umfassenden Veränderungen der Transkription und des Metabolismus, sodass eine lokale wie auch systemische Resistenz gegen Herbivore induziert wird.

Unsere Intention war es, die Rolle von Cytokininrezeptoren (CKRs) in der Verteidigungsreaktion von Pflanzen näher zu beleuchten. Diese Rezeptoren binden Cytokinine, die in erster Linie für ihre regulative Rolle in der Organellenentwicklung und der Wachstumsförderung bekannt sind. Kürzlich konnte jedoch in *Arabidopsis* auch gezeigt werden, dass Cytokinine über die Rezeptoren HK2 und HK3 eine Resistenz gegenüber Pathogenen vermitteln. Um herauszufinden, welche Funktion CKRs bei der Verteidigung in *N. attenuata* ausüben, haben wir die CKRs CRE1, HK2 und HK3 jeweils mit Virus-induziertem Gensilencing (VIGS) herunterreguliert und die Effizienz der Transkriptionsreduktion anhand von qPCR-Analyse überprüft. Anschließend haben wir die basalen (nach Kontrollapplikation von Lanolin) und induzierten (nach Methyljasmonat (MJ)-Behandlung) Level der PI-Aktivität und bestimmter phenolischer Sekundärmetabolite mit dem radialen Diffusionstest bzw. mit HPLC bestimmt. Wir konnten zeigen, dass die MJ-Behandlung zu erhöhter PI-Aktivität und einer Akkumulation von Caffeoylputrescin, Dicaffeoylspermidin und Diterpenglykosiden führt und konnten damit eine MJ-induzierte Abwehrreaktion nachweisen. Weiterhin konnten wir beobachten, dass das Ausschalten von HK3 erniedrigte Level in der induzierten PI-Aktivität zur Folge hat und dass das Silencing von HK2 zu erhöhten Basalleveln in nahezu allen analysierten Parametern führt. Dies

lässt vermuten, dass HK2 ein negativer Regulator bei der konstitutiven Abwehrreaktion ist, oder zeigt eine Redundanz zwischen den Rezeptoren auf: Insbesondere HK2 und HK3 könnten in der Lage sein, sich gegenseitig zu ersetzen, da beide CKRs in den Blättern exprimiert werden und die Expression eines Rezeptors hochreguliert war, wenn das Transkriptlevel des jeweils anderen durch VIGS gesenkt worden war. Allerdings bedarfdue es weiterer Untersuchungen und besserer Konstrukte, mit denen ein Silencen aller CKRs gleichzeitig möglich ist, um die Funktion von CKRs in der pflanzlichen Abwehrreaktion gegen Herbivore aufzuklären.

2 Abstract

To defend themselves against herbivore insects plants have evolved sophisticated defense mechanisms including direct and indirect responses. Indirect defense is the attraction of the herbivore's natural enemies, e.g by emitting volatiles. Additionally, many chemical compounds are produced that affect the insects directly: Antidigestive PIs or secondary metabolites that can be toxic or repellant. All these reactions are the result of a strictly regulated signaling network, whose initiation is the perception of HAMPs. Currently it is known that cell membrane depolarization, calcium-fluxes, ROS, MAPKs and phytohormones, especially JA and its derivates, are involved into the complex signal transduction and finally lead to an extensive alteration of the transcriptome and metabolome to induce local and systemic resistance against herbivores.

We wanted to investigate the role of CKRs in the defense response. These receptors perceive CKs, which are primarily known for their regulative role in organ development and growth promotion. However, recently it was shown that CKs systematically induce resistance against pathogens in *Arabidopsis* via CKR-signaling of HK2 and HK3. In order to analyze the function of the CKRs in *N. attenuata* we silenced the CKRs CRE1, HK2 and HK3, respectively, by VIGS and verified the knock-down by qPCR. We determined the basal (after control application of lanolin) and the induced (after MJ treatment) levels of PI activity and secondary metabolites in leaves by radial-diffusion assay and HPLC, respectively. We could show that MJ treatment leads to elevated levels of PI activity and accumulation of caffeoyl putrescine, dicaffeoyl spermidine and DTGs and hence confirmed an MJ-induced defense reaction. Moreover, we could reveal that silencing HK3 results in a decrease of induced PI activity and, interestingly, silencing HK2 causes elevated basal levels of nearly all analyzed parameters. This suggests HK2 being a negative regulator of constitutive defense reaction or indicates a cross talk between the receptors: especially HK3 and HK2 are likely to have redundant roles since they are both expressed in leaves and one's transcription level was upregulated when silencing the other. But further studies and new constructs for silencing several CKRs simultaneously are needed to elucidate the involvement of CKRs in plant's defense response against herbivores.

3 Introduction

In addition to abiotic stress factors like drought or heat, plants also have to cope with plenty of biotic stress. Those biotic factors are not only pathogenic microbes but also herbivores, like insects. 45% of all insect species are phytophagous [1], indicating that plants have to develop mechanisms to resist herbivory. In 350 million years of coevolution [2] both insects and plants have evolved sophisticated mechanism to defend themselves. The plants' defense reaction can be divided into direct and indirect response. Chemical compounds belonging to the direct response can be toxic, repellant or anti-digestive to herbivores [3, 4], for example alcaloids, glucosinolates and Proteinase Inhibitors (PIs). But also physical barriers such as cuticles, trichomes and thorns [5] are means of direct protection. On the other hand, plants can also defend themselves indirectly by attracting natural predators of herbivores. This can be achieved by producing extrafloral nectar or emitting volatiles, for example linool and bergamotene [6]. Regarding the energy and nutrient demand of producing these defense compounds it is not surprising that plants have a strictly regulated induced (active) defense beside the constitutive (static) one [4]. The stimulation to induce a defense reaction is mediated by the recognition of herbivory-associated molecular patterns (HAMPs), which consist of chemical elicitors originating from the oral secretions or oviposition fluids of insects. Elicitors already identified are Inceptin, which is a proteolytic product of the plant's chloroplastic ATP-synthase, fatty acid-amino acid conjugates (FACs) like Volicitin, and Caeliferins [3]; their application on leaves causes a reaction similar to a herbivore attack. However, plants also recognize ovipostion via chemical compounds: The application of Bruchins for example, which were isolated from pea weevil, results in neoplasm [7], which is one way plants repel oviposition. Other reactions observed are the production of ovicidal substances and volatile emission to attract parasitoids [8]. Volatile emission occurs also after damaging lima beans with MecWorm that mimics the timing of herbivory feeding [9]. This indicates that plants are able to identify herbivores not only regarding the mode tissue is removed, but also according to the frequency and time period of feeding.

But how does the stimulation lead to a defense reaction like the production toxic secondary metabolites? One of the earliest cellular events occuring within minutes is membrane depolarization. Interestingly, there are distinct depolarization profiles in different distances to the bite zone [3]. This depolarization is caused by calcium influx 30 to 200 µm away from

the damaged area and is proposed to happen due to the channel-forming properties of the FACs in the insect's oral secret. This might be an explanation for the higher calcium-fluxes after herbivory than after wounding [10]. Calcium signaling is also associated with ROS that accumulate not after wounding but after herbivory [11] and oxidizes proteins which alter gene expression downstream. The NADPH oxidase is responsible for this reactive oxygen species (ROS) production and is phosphorylated by a calcium-dependent protein kinase and thus gains higher activity [3]. Other phosphorylation events occur during the mitogen-activated protein kinase (MAPK) signaling pathway; especially the salicylic acid-induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK) are shown to have a critical role in defense reaction [12]. Moreover, the transcription of many phytohormones such as jasmonic acid (JA) or ethylene are MAPK dependent: Silencing MAPK's leads to a decrease of JA production in tomato and tobacco [13]. A central role in regulating wound- and herbivory-induced reactions is assigned to JA [3]. Within 5 min after damaging the highly active JA derivate JA-isoleucine accumulates in the plant as well as JA-valine and-leucine; additionally a *de novo* synthesis of JA is observed [14]. JA-isoleucin binds to COI1, which is part of the ubiquitin-ligase SCF^{COI1}. That leads to the degradation of the repressor protein JAZ (protein containing the jasmonate ZIM-domain) and therefore results in the release of transcription factors (e.g. MYC2) regulating JA-responsive genes positively [3]. Due to this, different responses like altering gene expression and hormone levels (within minutes), producing toxins, deterrents and volatiles (within hours) and trichome initiation (after several days) take place [14]. Furthermore, emitted or applied MJ also have effect on plants: Emitted MJ from clipped *Artemesia tridentata* leaves causes defense and priming reactions in adjacent plants [3]. Similar observations were made on maize cell membranes that gain a higher binding capacity to FAC's after MJ treatment, which indicates a priming reaction. But JA is also believed to have a key role in transducing systemic signals, which means the transmission into distal, intact leaves or into roots. It has been shown that JA is required for the long distance transmission in systemic responses [16], as well as the vascular system is involved. But the identity of the mobile signal molecule remains unknown [4]. Nevertheless, a systemic production of PIs and other secondary metabolites is approved. Isoprenoids like diterpene glycosides (DTGs) or alcaloids such as putrescine and nicotine [17], which is known for it's inhibiting effect on herbivore's acetylcholine receptors [18] are examples for secondary metabolites.

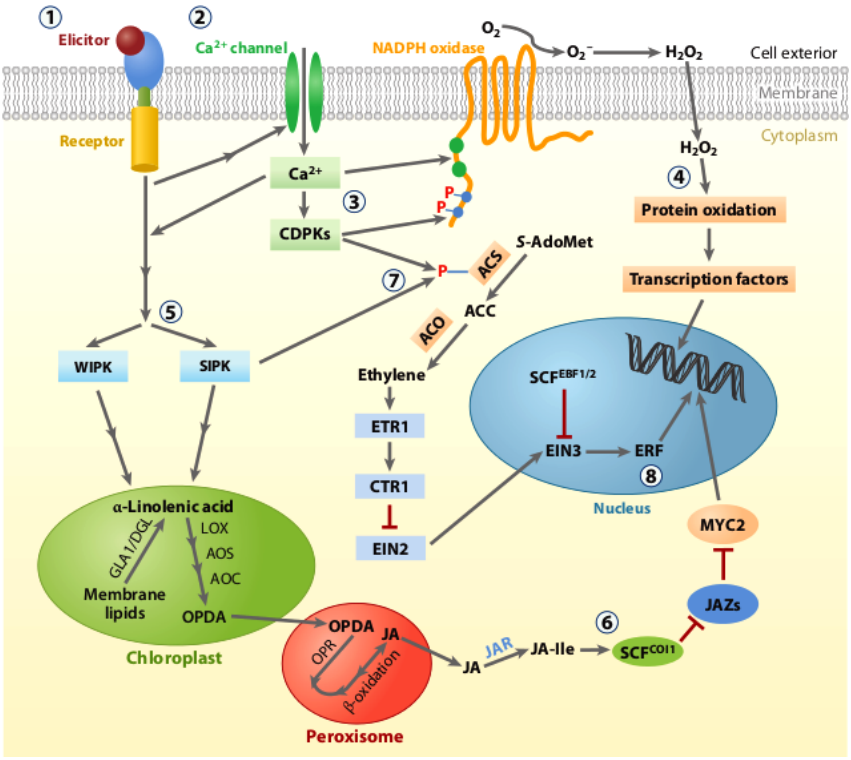

Figure 3.1: **Early signaling events after herbivore attack:** 1: Elicitors are perceived by receptors in the cell membrane. 2: The perception triggers the activation of calcium channels and/or FACs form channels which leads to the influx of calcium. 3: Binding of calcium and phosphorylation by calcium-dependent protein kinases (CDPKs) increases the activity of NADPH oxidase and thus enhances the ROS production. 4: Proteins are oxidated by ROS and regulate the activity of ROS-dependent transcription factors. 5: The MAPKs SIPK and WIPK are also early activated and trigger the biosynthesis of JA, which is converted into JA-isoleucine (JA-Ile). 6: JA-Ile binds to COI1 which leads to the degradation of the repressor protein JAZ and results in the release of transcription factors like MYC2. 7: SIPK causes an increase of the ethylene biosynthesis by enhancing the stability of ACC synthase (ACS). 8: Through a signaling pathway ethylene responsive transcription factors such as ERF are activated. All these signaling events will be translated into a defense reaction including e.g. the accumulation of toxic metabolites or volatile emission. Picture taken from: [4].

CKs are phytohormones known to be involved in many processes referring to organ development and growth promotion. This includes maintenance of meristematic cells, vascular differentiation, leaf senescence and nutrient mobilization [19]. Additionally, these hormones regulate the response to extrinsic factors like light, nutrients or water, but also to beneficial and pathogenic microbes. In terms of chemistry CKs are N^6-substituted adenine derivates [19]; cis-Zeatin and trans-Zeatin (see fig. 3.2.) belong to the most important CKs. The hormones are bound with distinct affinities by the CK receptors (CKRs) HK2, HK3 and HK4, also known as CRE1 [20]. This binding initiates a phosphotransfer cascade similar to the two-component-system in microbes. First, the conserved histidine in the kinase domain autophosphorylates and then transfers a phosphate to the receiver

domain. Histidine phosphotransfer proteins and responsive regulators (RRs) that are divided into type A and type B [19] are further acceptors of the phosphorelay. Type B response regulators positively effect the CK signaling and concurrently activates the transcription of type A RRs [21], which are repressors. One of these type A RRs is RR5 which can be used as a possible marker for CK-dependent reactions [22]. An increase of the RR5 transcript levels in local and systemic leaves, as well as in roots of *N. attenuata* was observed in previous experiments after the application of oral secretion from the herbivore *Manduca sexta* (data not shown). Hence, we suggest that herbivores mediate CK levels in the plant systemically. But not only type A RRs are important for CK signaling but also type B RRs: ARR2 in *Arabidopsis* for example, which is activated after CK perception by AHK2 and AHK3, is supposed to activate the salicylic acid (SA) signaling during the interaction with the bacterial pathogen *P. syringae* pv tomato (*Pst* DC3000) [23]. However, ahk2 ahk3 double knock out mutants show an enhanced susceptibility to *Pst* DC3000. Recently it was found that ARR2 also binds to the promoters of PR1 and PR2 and thus systemically induces plant resistance against pathogens. Interestingly, the application of SA enhances the binding of this RR to the PR1 promoter. Endogenous SA-levels, in turn, were observed to increase after CK treatment; this might be due to the transcription of the SA biosynthetic gene SID2, which is transiently activated by CKs [21]. As a consequence, CKRs augment the resistance to pathogens via SA signaling. But it remains an open question which role CKRs play in fending off herbivores. In this work we will examine if CKRs are important in the defense reaction of *N. attenuata.* Therefore we used virus-induced gene silencing (VIGS) to knock down the transcript levels of the CKRs CRE1, HK2 and HK3 and then triggered a defense reaction by application of MJ onto the leaves. This led to the accumulation of PIs and phenolic secondary metabolites, which we finally analyzed. We could show that silencing HK2 leads to an increase in the transcript level of HK3 and elevated basal levels of MJ-induced PI activity and secondary metabolites.

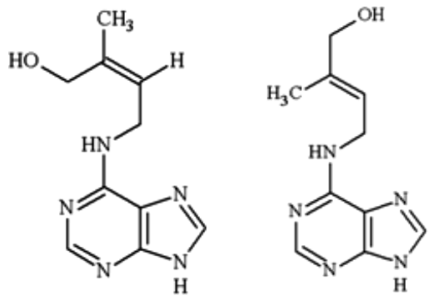

Figure 3.2: **Examples for CKs:** Cis-zeatin (left) and trans-zeatin (right).

4 Materials and Methods

4.1 Chemicals

The chemicals used for all experiments are obtained from Sigma-Aldrich (USA), Merck (Germany), Roth (Germany), or VWR (USA) if not mentioned otherwise in the text.

4.2 Plants

We use *Nicotiana attenuata* WT plants which were originally collected in Utah (USA) and inbred for 30 generations in the glasshouse.

4.2.1 Germination

Media and solvents:

- Sterilization medium
- Gibberellic acid
- Smoke solution
- Germination medium

Procedure:

For sterilization the seeds are incubated in the sterilization solution for 5 min. Afterwards, the solution has to be decanted and the seeds are washed in sterile water 3 times. The seeds are then incubated with 50 µl of Giberellic acid and 50 ml of smoke solution for 1 h. After this incubation the seeds are washed again in sterile water for 3 times. For germination, about 50 seeds per petri dish are placed onto the germination medium with a sterile Pasteur pipette, and then the plates are sealed with Parafilm. Having completed this the petri dishes are incubated for 10 days under the following conditions: 27°C/16 h 100% light, 24°C/8 h dark.

4.2.2 Cultivation

Media and solvents:

- Plug soil (Klasmann-Deilmann GmbH, Geesten, Germany)
- Soil

The seedlings are potted into plug soil and into 1 l pots with soil 10 days later. Fertilization is done with 3.0 g Borax on the day of potting, 2.0 g Borax and 3.0 g Peter's Allrounder (Scotts International, Heerlen, Netherlands) from day 7, 1.0 g Borax and 40 g Peter's Allrounder from day 14, 25 to 30 g Peter's Allrounder from day 21; each solved in 400 l water.

The plants are watered with 300 ml from day 1 to 5 and later with 100 ml.

4.2.3 VIGS

The VIGS system is used to silence the accumulation of transcripts of HK2, HK3 and HK4 (CRE1).

Media and solvents:

- YEP
- Kanamycin
- Inoculation solution

Constructs:

We used different *Agrobacteria* strains with plasmids that contain the constructs shown in tab. 4.1.

As positive controls constructs containing the complementary sequence of the phytoene desaturase (PDS) were used. After successful VIGS, leaves inoculated with PDS are bleached due to the impairment of the chlorophyll and carotinoid biosynthesis [24].

An emtpy vector (EV) was used as negative control as it should have no effect on any transcript levels.

The coat proteins necessary for the virus (tobacco rattle virus) are encoded in pBINTRA. The constructs were made as described in [25].

Construct	Sequence
HK2	GCGGCGGTCGACAAGCATGTGTGACGAACGAGCGCGAA TGTTGCAGGACCAGTTCAACGTAAGCATGAACCATGTTC ATGCATTGGCTATTCTTGTTTCCACATTTCACCACGGAA AGCAACCTTCTGCGATAGACCAGAAAACTTTTGAAGAAT ATACCGAGAGAACAGCTTTTGAGAGGCCACTTACAAGT GGTGTTGCCTATGCTTTAAGGGTTCGTCACTCAGAGAG AGAAGAGTTTGAGAAGCTGCATGGGTGGACAATCAAGA AAATGGAATCCGAGGACCAAACTTTAGCGGGATCCCGC CGC
HK3	GCGGCGGTCGACTTAGAAGGCATGAGATGCGCTGCAGA TTCAAACAGAAACCACCATGGCCTTGGCTAGGCATCACA ACTGCCACAGGAATTCTTGTAATTGCATTGCTTATTGGG CAAATATTTCATGCAACAATAAACAGAATAGCCAAAGTT GAGGATGATTATCATGAGATGATGGTGCTAAAAAAGCG TGCTGAGGATGCTGACGTTGCAAAATCACAGTTTCTCGC TACTGTTTCCCACGAGATCAGGGGATCCCGCCGC
CRE1	GCGGCGGTCGACCCTTTGAAAGCACAAGATCGAGTTTT CACGCCATTTATGCAGGCAGACAGTTCAACCTCTAGAAA CTATGGAGGAACGGGTATTGGATTAAGCATTAGCAAGT GTCTCGTCGAGCTGATGGGTGGTCAAATAAGTTTCATT AGCCGTCCTCAGATTGGAAGCACATTTTCTTTCACTGTT AACTTCCTAAGATGTGAGAAATATTCTGTTGGCGATCTG AAAAAACCTCATTATGATGAATTGCCTGGATCCCGCCGC

Table 4.1: **Sequences of the constructs used for VIGS:** HK2, HK3 and CRE1; inserted into the pBINTRA vector system in *Agrobacterium*.

Procedure:

The whole experiment is carried out in a climate chamber with 20°C and a 16 h day/8 h night light regime unless mentioned otherwise in the description. All works with *Agrobacterium* are performed under sterile hood until the centrifugation step.

3 days before the plants are potted, the different *Agrobacterium* strains are streaked onto YEP agar plates with 50 mg/l Kanamycin and are incubated for 2 - 3 days at 26°C. Thereafter, the plates are stored at 4°C.

The day before VIGS, pre-cultures of *Agrobacterium* are started in 30 ml YEP with 50 mg/l Kanamycin. After overnight incubation the pre-cultures are added to 300 ml YEP in an Erlenmeyer flask and incubated for 5 - 6 h at 20°C until an OD_{600} of ca. 0.5 is reached. They are then centrifuged for 5 min at 600 g and the pellets are resuspended in inoculation solution (1/5 of the volume of the culture); the OD_{600} should be 2.0 - 3.0. The final inoculation solution is achieved by mixing half of the inoculation solution containing the construct and half of the inoculation solution containing pBINTRA.

The inoculation starts 3 - 5 days after the plants were potted and the plants were watered on the morning of the day starting VIGS. The final solution is inoculated by pressure-injection with a 1 ml-syringe without a needle. 3 leaves per plant are inoculated by 3 - 6 inoculations on the underside of every leaf until it is ca. 75% saturated.

After inoculation, the plants are covered with a plastic bag and kept in dark. After 2 days the plastic bags are removed and the plants put into light. 2 weeks later the bleaching of the PDS inoculated plants indicates a successful VIGS experiment (see fig. 4.1) and further experiments can be started.

Figure 4.1: ***N. attenuata* plants 3 weeks after VIGS experiment:** Bleached leaves in PDS-silenced plants (right) indicate a successful silencing. The silencing constructs used in the VIGS experiment are denoted below the respective plants.

4.2.4 Treatments

To induce a defense reaction, 3 leaves from each plant are treated either with 10 µl of MJ (MJ, 15 µg/µl, dissolved in heat-liquified lanoline) or with pure lanoline (LAN) as control.

The samples are harvested after 3 days and stored at -80°C; from each plant 5 samples are taken as replicates.

4.3 RNA analytics

To figure out whether the VIGS was successful the transcript levels of CRE1, HK2 and HK3 are determined.

4.3.1 RNA-Isolation

Media and solvents:

- Trizol-Reagent
- Chloroform
- Isopropanol
- Ethanol (96%, in DEPC water)
- DEPC water

Procedure:

To 150 mg of ground, frozen tissue 1 ml Trizol-Reagent is added and vortexed briefly until the tissue is saturated. Thereafter, the samples are shaken for 5 min at RT and 300 µl chloroform are added. The samples are shaken again for 1 min and centrifuged for 15 min at 16,100 g at 4°C. The upper phase is transfered into a new 1.5 ml-tube and 500 µl isopropanol are added to it. To mix the composite gently it is inverted 8 times (not vortexed). After an incubation for 15 min at RT the samples are centrifuged for 10 min at 16,100 g at 4°C. While the supernatant is discarded the pellet is washed in 1 ml ethanol and vortexed briefly. Subsequently the samples are centrifuged for 10 min at 16,100 g at 4°C and the supernatant is discarded again. To remove remaining ethanol the samples are shortly spinned down and the liquid is pipetted out. After drying the pellet for ca. 5 min at RT it is dissolved in 20 - 50 µl DEPC water, depending on the pellet's size. Finally, the isolated RNA is stored at -20°C.

4.3.2 RT-PCR

A reverse transcription (RT-PCR) is used to synthesize cDNA, which is needed for the subsequent quantitative PCR (qPCR).

Media and solvents:

- Denaturation mix
- Reaction Mix

Procedure:

Before starting the RT-PCR the RNA concentration is measured by Nanodrop and the RNA diluted to 500 ng/µl. First 1 µl of RNA is incubated with 4 µl denaturation mix for 5 min at 65 °C. Then, the samples are cooled on ice for 1 min. Afterwards, 5 µl reaction mix is added and the reverse transcription gets completed with the following program:

- 60 min at 42°C
- 15 min at 70°C
- (hold at 20°C)

After the reaction is finished 15 µl water are added and the cDNA stored at -20°C.

4.3.3 qPCR

To analyze the transripts quantitatively a qPCR is carried out using the Mx3005P from Stratagene (USA). The Kit used is taken from Fermentas.

For one sample the following solvents are mixed together in one 1.5 ml-tube:

- 2 µl 10 x Buffer mix
- 1.4 µl 50mM Magnesium chloride
- 0.8 µl 5mM dNTP
- 0.1 µl Hotgoldstar enzyme 5 U/µl
- 1.2 µl Forward primer (5µM)
- 1.2 µl Reverse primer (5µM)
- 0.6 µl SYBR green I (diluted)
- 11.7 µl Water
- 0.08 µl ROX (undiluted)
- 1 µl cDNA
    - Total: 20.08 µl

The following primers are used to analyze the transcripts of HK2, HK3, CRE1:

Construct	Forward primer	Reverse primer
HK2	CCTTGGTGTTGTTCTTACA TTTGC	ACAATAGTTTGCTTGCTTG CAAGC
HK3	TGCTCTCCGGAGAGGAAGA TC	TTAGAAGGAAGATCGGTTT TGTAAACT
CRE1	GAATGAGCAATTTGACTCA AAGAG	CTCCTTCTGATTAGCATCCA TAG

Table 4.2: **Sequences of the primers used for qPCR:** HK2, HK3 and CRE1.

As reference the transcripts of actin are measured. We also used references with known cDNA-concentrations (2000 ng/µl and dilutions af that concentration: 1:4 , 1:16, 1:64, 1:128).

4.3.4 Analysis

For converting the Ct (Cycle threshold)-values into cDNA-concentrations a reference curve is necessary. The relative transcript levels are calculated and shown in a diagram (the calculations are shown exemplarily for actin):

- Decadic logarithm of the cDNA concentration from the references:

$$\lg(\mathrm{cDNA}_{\mathrm{conc1}}) = \log\left(\frac{2000\frac{ng}{\mu l}}{1\frac{ng}{\mu l}}\right) = 3.301029996$$

$$\lg(\mathrm{cDNA}_{\mathrm{conc2}}) = \log\left(\frac{500\frac{ng}{\mu l}}{1\frac{ng}{\mu l}}\right) = 2.698970004$$

$$\lg(\mathrm{cDNA}_{\mathrm{conc3}}) = \log\left(\frac{125\frac{ng}{\mu l}}{1\frac{ng}{\mu l}}\right) = 2.096910013$$

$$\lg(\mathrm{cDNA}_{\mathrm{conc4}}) = \log\left(\frac{31.25\frac{ng}{\mu l}}{1\frac{ng}{\mu l}}\right) = 1.494850022$$

$$\lg(\mathrm{cDNA}_{\mathrm{conc5}}) = \log\left(\frac{16\frac{ng}{\mu l}}{1\frac{ng}{\mu l}}\right) = 1.204119983$$

- Reference curve with log(cDNA) on the abscissa and Ct-values on the conc4ordinate:

log (cDNA)	Ct-values
3.301029996	18.87
2.698970004	21.02
2.096910013	23.33
1.494850022	25.75
1.204119983	27.95

Table 4.3: **Values for the reference curve used for qPCR:** log (cDNA) and Ct-values from the references are shown.

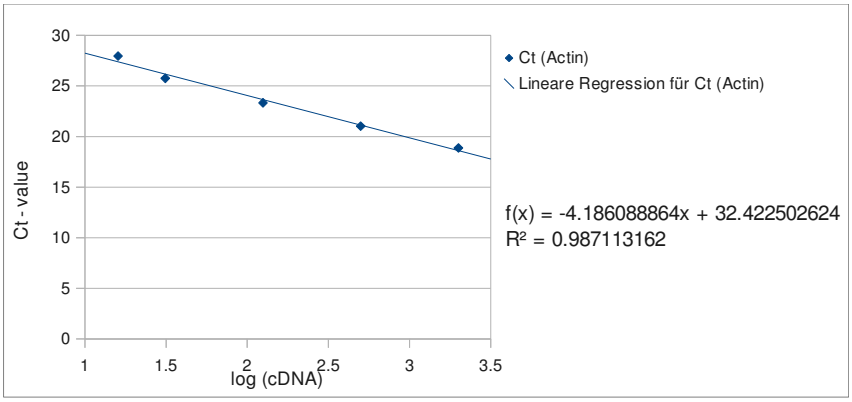

Figure 4.2: **Reference curve for extrapolating cDNA concentrations:** log (cDNA) on the abscissa and Ct-values on the ordinate; regression equation is shown on the right.

- Regression equation: f(x)=ax+b
- Calculate the concentrations with the parameters from the regression equation (example: Ct_{Actin}-value from WT + MJ = 18,64):

  $\log(\text{cDNA})=\frac{Ct-b}{a}=\frac{18,64-32,422502624}{-4,186088864}=3,2924534265$

- Change the logarithmic value back into concentration :

  $\text{cDNA}=10^{log(cDNA)}*1\frac{ng}{\mu l}=10^{3,2924534265}*1\frac{ng}{\mu l}=1960,8908794438\frac{ng}{\mu l}$

- To get a relative transcript level, the cDNA-concentration of CRE1, HK2 and HK3 has to be divided by the value for actin
- The average and standard error of the results of all 5 replicates are calculated and used for the diagrams

4.4 Analyzing PI activity

To determine if proteinase inhibitors are produced leaves are used for making an extract which is tested with a radial-diffusion assay.

4.4.1 Extraction

Media and solvents:

- Extraction buffer

Procedure:

Approximately 100 mg ground tissue are aliquoted into 1.5 ml tubes and 300 µl extraction buffer added. After shaking for 15 min the samples are centrifuged for 20 min at 12,000 g at 4°C. The supernatant is transfered into a new tube.

4.4.2 Measurements

To determine the protein concentration of the extracts a Bradford-assay is accomplished.

Media and solvents:

- Tris (0.1 M)
- BSA (0.5 mg/ml, in Tris)
- Bradford Reagent (1:5 dilution of dye reagent concentrate in Tris)

Procedure:

10 µl of the extract (diluted 1:10 in distilled water) and 10 µl of each reference (BSA 0.5 mg/ml and the following dilutions: 1:2, 1:4, 1:8, 1:16 in Tris) are added to 190 µl Bradford reagent in wells of an ELISA plate and incubated for 10 min at RT. Subsequently, the absorption at 595 nm is measured with the TECAN ELISA reader. The Magellan 3 software (from TECAN) is used to convert the absorption into concentration by means of a reference curve.

4.4.3 Radial-diffusion assay

To quantify the PI activity we use the radial-diffusion assay described by Jongsma 1994 [26].

Media and solvents:

- Agar mixture
- Trypsin inhibitor (from soybean)
- Staining solution

Procedure:

The agar mixture is poured into plates and holes with a diameter of 4 mm are punched out and completely filled with the extracts and the references (trypsin inhibitor in different concentrations, see tab. 2.4). After an incubation overnight at 4°C the staining solution is poured onto the agar and the plates are incubated for 1 h at 37°C. Finally the diameters of the PI inhibition zones (see fig. 4.3) around every hole are measured with a caliper.

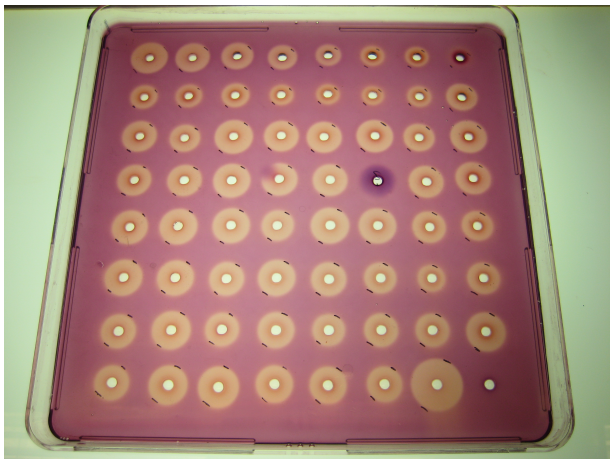

Figure 4.3: **Plate for PI assay:** PI inhibition zones, which appear as bright circles, are measured with a caliper.

4.4.4 Analysis

The PI molarity is extrapolated by means of a reference curve created from the trypsin inhibitor reference solutions (logarithmic regression): The molarity is assigned on the abscissa and the averages of the diameters on the ordinate:

Concentration (mg/ml)	PI molarity (µM)	Diameter (cm)
0.7	10.71	2.1800
0.3	4.59	2.0600
0.15	2.30	1.9333
0.075	1.15	1.7466
0.0375	0.57	1.6200

Table 4.4: **Values for the reference curve used for PI assay:** Concentrations, PI molarities and diameters of the reference solutions are shown.

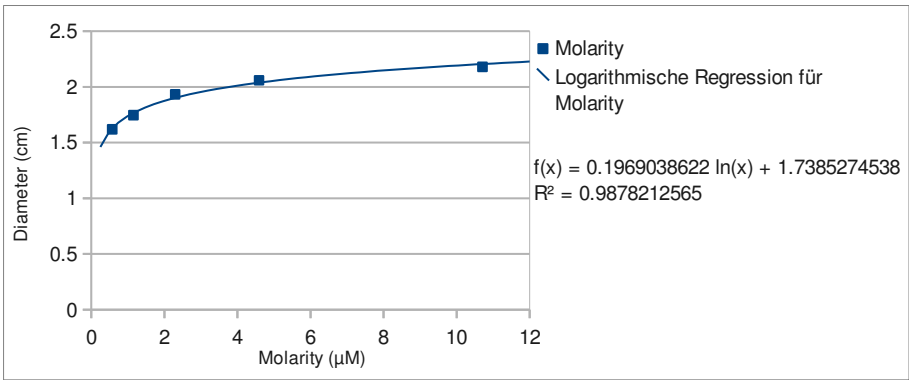

Figure 4.4: **Reference curve for extrapolating the PI concentration:** Molarity in µM is plotted against diameter in cm; regression equation is shown on the right.

- Regression equation: f(x)=a ln(x)+b
- Calculate the molarities with the parameters from the regression equation (example: diameter from HK3 + MJ = 2.2 cm):

  $\ln(\text{concentration}) = \frac{\text{diameter}-\text{b}}{\text{a}} = \frac{2.2\text{cm}-1.7385274538}{0.1969038622\text{cm}} = 2.343643954$

- Convert logarithmic value into molarity:

  $\text{molarity} = \text{e}^{\ln(\text{concentration})} * 1\mu\text{M} = e^{2.343643954} * 1\mu\text{M} = 10.41913432\mu\text{M}$

- For getting a relative PI activity the PI molarity is divided by the protein concentration:

  $\text{PIactivity}_{\text{rel}} = \frac{\text{molarity}}{\text{concentration}} = \frac{10.41913432\mu\text{M}}{2.412533\frac{\text{mg}}{\text{ml}}} = 4.318753078\frac{\frac{\mu\text{mol}}{\text{l}}}{\frac{\text{mg}}{\text{ml}}} = 4.318753078\frac{\mu\text{mol}}{\text{g}}$
  (amount of substance is equimolar to concentration or molarity)

- The averages of the relative PI activities from all 5 replicates and the standard errors are used for the diagrams.

4.5 HPLC

4.5.1 Extraction

Media and solvents:

- Extraction buffer (0.1% Acetic acid in 40/60 (v/v) methanol/water)

Procedure:

The frozen tissue is aliquoted to 50 mg and the exact weight has to be noted. After grinding the tissue with 2 metal balls in the GenoGrinder 2000 (Spex, USA) for 1 min at 250 strokes/min 300µl extraction buffer are added and the samples are stored at 4°C. The extraction is done in the GenoGrinder by grinding twice for 45 s at 50 strokes/s. Subsequently, the samples are centrifuged for 20 min at 4°C at 16,100 g and 400 µl of the supernatant are transfered into a new tube. The centrifugation step is repeated and 200 µl of the supernatant filled into vials which get crimped.

4.5.2 HPLC

The HPLC is performed using water (with 0.1% formic acid and 0.1% ammonium hydroxide, pH=3,5) as buffer A and methanol as buffer B. The sample elution steps are shown in tab. 4.5. The injection volume is 1 µl, the flow rate 0.8 ml/min at 40°C and the starting pressure 108 bar rising to 160 bar during gradient.

Time (min)	Step
0.5	0% B
6.5	80% B, linear gradient
3	80% B, isocratic gradient
5	reconditioning
15	total runtime

Table 4.5: **HPLC elution steps:** Rising gradient of buffer B, isocratic for 3 min, and reconditioning time are listed.

The external calibration is done with the following standards, each with a concentration of 250 µg/ml:

- Nicotine (solved in methanol)
- Chlorogenic acid (solved in water)
- Rutin (solved in dimethylsulfoxid)

The precolumn used is an Gemini NX RP18, 3 µm, 2 x 4.6 mm and the column is Chromolith FastGradient RP18e, 50 x 2 mm (Lot No. HX 802433); the software is Agilent Chemstation with the program Chrom. The detection channels are listed in tab. 4.6.

Wave length (nm)	Compounds
210	DTG's
254	general aromatics
260	nicotine
320	caffeoyl putrescine, caffeoyl spermidine, Chlorogenic acid
360	rutin

Table 4.6: **Recorded detection channels**: Compounds and the wave lengths they are detected with are listed.

4.5.3 Analysis

The peak integration areas are determined from the chromatograms using Chromelion software. The quotient of area per mass is calculated for each sample and the averages of all 5 replicates and the standard errors are shown in the diagrams.

5 Results

5.1 Silencing efficiency

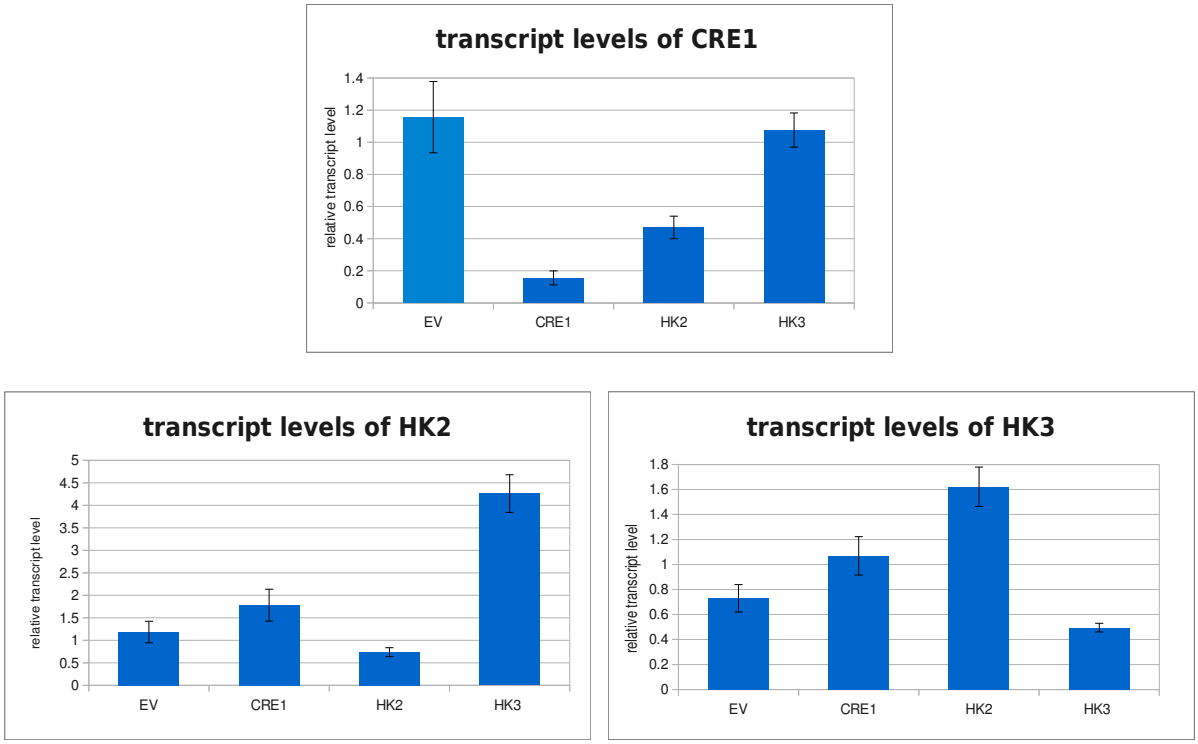

Figure 5.1: **Transcript levels of CRE1, HK2 and HK3:** Plants were treated with VIGS using CRE1-, HK2-, HK3-silencing constructs and EV. Relative to transcript level of actin. Mean value and standard error (n= 4 - 5).

For determining the silencing efficiency after VIGS treatment the relative transcript levels of all three receptors CRE1, HK2 and HK3 were measured with qPCR. The results are shown in fig. 5.1.

The transcript levels of CRE1 are equal in EV treated and HK3-VIGSed plants, but slightly reduced in plants treated with HK2-silencing constructs. The lowest level of CRE1-transcripts can be observed in plants that are silenced with the VIGS-construct for CRE1.

The results of the HK2-transcript level analysis show a slight decrease in HK2-VIGSed

plants compared to EV-treated plants. The highest level is seen in HK3-silenced plants: It is about 4-fold higher than in EV-plants. CRE1-silenced plants are equal to the controls.

Interestingly, HK3 transcripts are elevated in HK2-VIGSed plants; it is about twice as high as the EV-treated plants (EV-treated plants: ca. 0.8, HK2-VIGSed plants: 1.6). The level in plants treated with CRE1-silencing constructs lies between that of HK2-VIGSed and EV-treated plants. In plants that were silenced with VIGS for HK3 the transcript level lies significantly under that of EV-treated plants.

5.2 Defense reaction parameters

5.2.1 PI activity

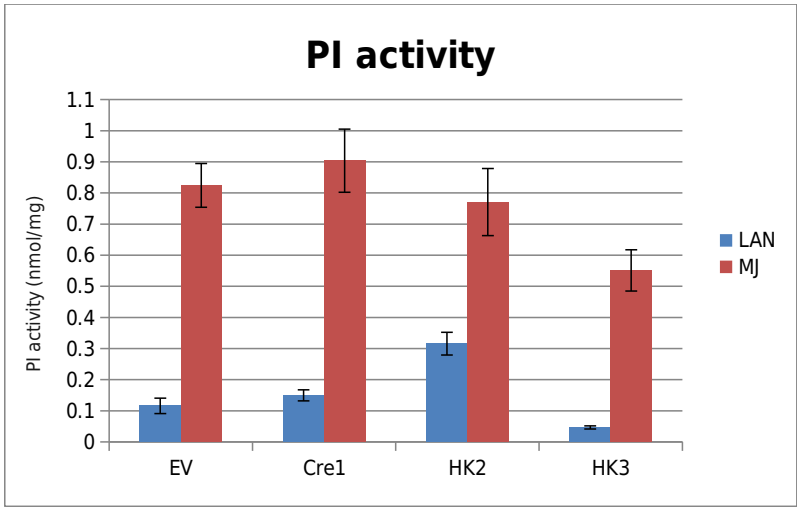

Figure 5.2: **PI activity:** The receptors CRE1, HK2, HK3 were silenced by VIGS, EV was used as control. Leaves were wounded and treated with lanolin as negative control (LAN) and MJ triggering a defense reaction (MJ), respectively. Mean value and standard error (n= 4 - 5).

The activity of PIs is one of the parameters that indicates a denfense reaction in plants. It was measured with a radial-diffusion assay and the results are shown in fig. 5.2.

In both EV-treated and CKR-silenced plants it is obvious that the PI activity is higher after MJ treatment than after lanolin application (control): The PI activity is double (HK2-VIGSed plants: ca. 3.5 nmol/mg control and ca. 7.5 nmol/mg MJ) up to 10-fold (HK3-VIGSed plants: 0.05 nmol/mg control and ca. 5.5 nmol/mg MJ) higher in MJ-treated than in control plants. The plants treated with HK2-silencing constructs show a 3-fold higher PI activity compared to EV-treated plants after lanolin application. The lowest activity in control as well as after MJ treatment is observed in HK3-VIGSed plants.

5.2.2 Secondary metabolites

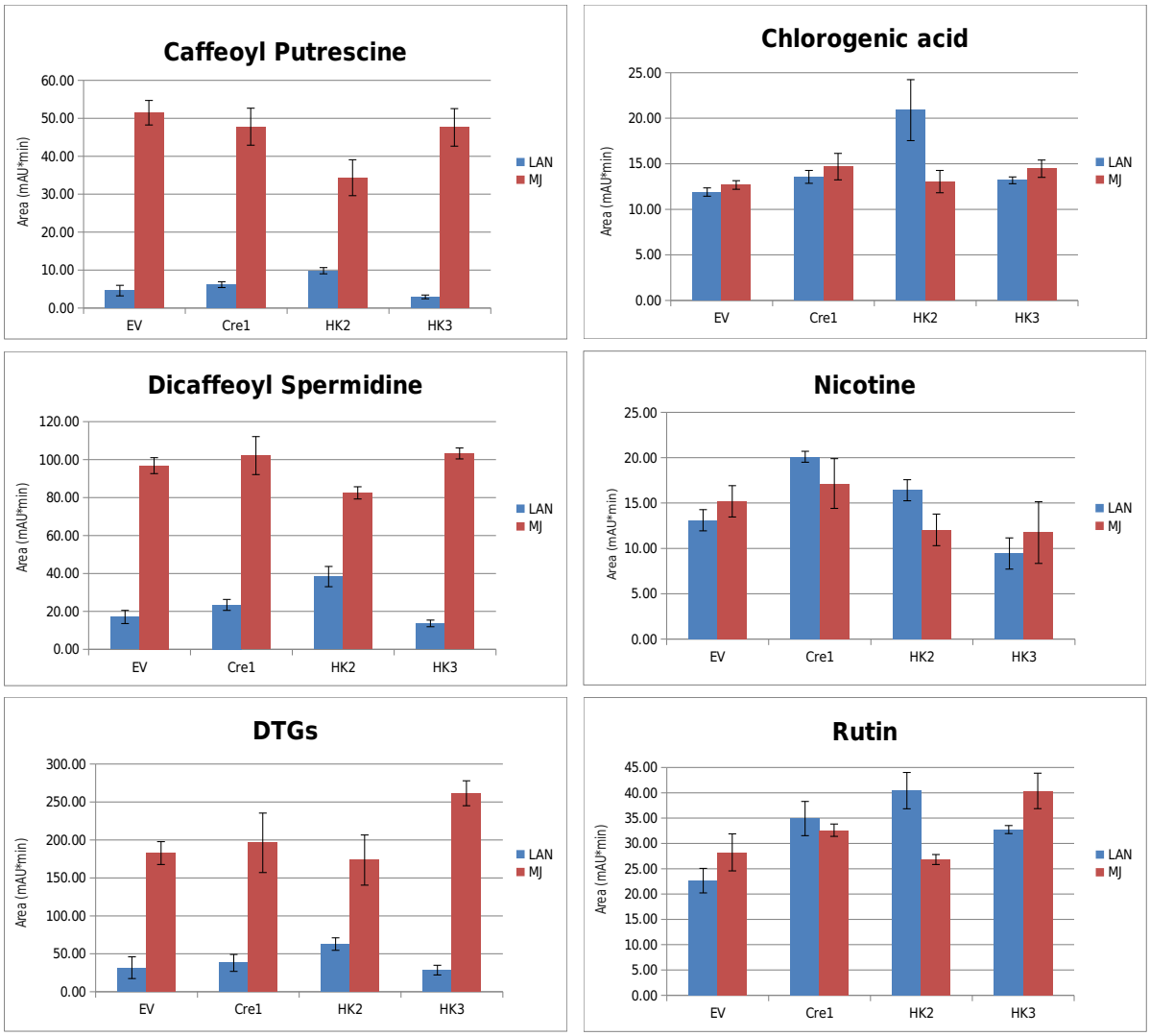

Figure 5.3: **Secondary metabolites:** Plants were silenced by VIGS-constructs for CRE1, HK2, HK3 or treated with the EV-construct. Leaves were wounded and lanolin (LAN) or MJ (dissolved in lanolin) was applied. Intensity of caffeoyl putrescine, dicaffeoyl spermidine, diterpene glycosides (DTGs), chlorogenic acid, nicotine, rutin was measured with HPLC. Mean value and standard error (n= 3-5).

Another indicator for plants' defense reaction is the biosynthesis of secondary metabolites. The accumulation of caffeoyl putrescine, dicaffeoyl spermidine, DTGs, chlorogenic acid, nicotine and rutin was measured exemplarily. The results can be seen in fig. 5.3.

Concerning the effect of MJ application in comparison to lanolin control the secondary metabolites can be divided into two groups: Caffeoyl putrescine, dicaffeoyl spermidine and DTGs clearly show elevated levels after MJ treatment in all plants while the intensity of chlorogenic acid, nicotine and rutin after application of MJ only slightly differs from the control.

Having a look at caffeoyl putrescine, dicaffeoyl spermidine and DTGs, the smallest difference between control and MJ-treated plants is observed in HK2-silenced plants due to the higher level in the control plants (lanolin application). The greatest difference between control and MJ treatment can be seen in plants silenced with VIGS for HK3: The intensity of all three metabolites mentioned above is about 10-fold higher after the application of MJ.

The intensities of chlorogenic acid, nicotine and rutin show no significant difference between control and MJ application in plants that are CRE1-, HK3-silenced or EV-treated, except for HK3-VIGSed plants which show a slight but significant increase of rutin after MJ treatment. However, compared to EV-treated plants HK2-silenced plants show a significant decrease in the intensities of the three named metabolites when treated with MJ.

6 Discussion

CKs are known for their important role in organ development and growth promotion as well as for their regulative function in the response to extrinsic factors. But recently it was shown that CKs are also able to systematically induce the resistance against pathogens and augment plant immunity via the accumulation of salicylic acid: For example, ahk3 and ahk4 knock out mutants did not generate pathogen-dependent deformations [21].We wanted to elucidate the role of the CKRs CRE1, HK2 and HK3, which were shown in heterologous expression to bind CKs specific and saturable [27], in the resistance against herbivory. In order to do this, we first silenced the receptors CRE1, HK2 and HK3 in *N. attenuata* by VIGS, respectively, and then triggered a defense reaction by MJ-treatment or applied lanolin onto the leaves as positive control to survey the basal levels. Finally, we analyzed JA-induced defense response parameters: PI activity and accumulation of secondary metabolites.

Having analyzed the transcript levels of the CK receptors we were sure that the gene silencing of CRE1 and HK3 had been successful: The transcript levels of the respective receptors in the silenced plants were significantly decreased in comparison to the transcript levels in plants treated with EV. However, HK2-transcript levels in HK2-VIGSed plants were only slightly decreased when compared with the controls. It is possible that the basal level was already low and as a consequence of this it was difficult to detect a silencing in an unelicited state. To verify the silencing of HK2 in future experiments, improved methods for the qPCR and the cDNA-synthesis can be used. The location of expression could be another explanation for the low difference in the HK2-transcript levels: HK2 could be localized in specific tissues of the leaf. Considering the fact that the entire leaf was used for the RNA analytics, the silencing effect might be difficult to detect. To assure this hyopthesis, different structures of the leaf should be isolated and analyzed separately in further studies.

The PI assay as well as quantifying the accumulation of caffeoyl putrescine, dicaffeoyl spermidine and DTGs confirmed the induction of a defense reaction by MJ: The induced levels after MJ treatment were highly elevated up to the 10-fold of the control levels (lanolin application). However, there was nearly no effect of the silencing on the induced levels of PIs or secondary metabolites. Only HK3-VIGSed plants showed significantly decreased PI activity after MJ treatment when compared to EV-constructs; another slight effect was

detected in HK2-silenced plants: The induced levels of caffeoyl putrescine and dicaffeoyl spermidine were lower compared to EV-plants. Opposite to the secondary metabolites mentioned above, chlorogenic acid, nicotine and rutin are not highly induced by MJ treatment in all plants. Nevertheless, they are important compounds the plant needs for defending itself against herbivores. Interestingly, in all of the analyzed parameters HK2-silenced plants showed elevated basal levels: With the exception of nicotine, whose basal level is just slightly increased in HK2, the control levels of all secondary metabolites and of the PI activity are double as high in HK2-silenced plants as in EV-treated plants. This suggests HK2 being a negative regulator of the constitutive defense levels and as a result silencing this receptor causes an increase of the basal PI activity and secondary metabolite accumulation. Redundancy or cross talk between the CKRs might be other reasons for these results: If one CKR is not functioning anymore due to the VIGS, the other CKRs are highly upregulated and lead to an elevated basal level of defense parameters. Especially HK3 is likely to be able to replace HK2: The transcript level of HK3 in HK2-silenced plants was twice as high as in plants treated with EV-constructs and on the other hand, the HK2-transcript level were extremely upregulated in plants that were silenced in HK3. This would be consistent with the expression patterns of the receptors found in *Arabidopsis*: While CRE1 is highly expressed in roots but has low expression levels in the leaves, HK2 and HK3 are expressed moderately or highly expressed in leaves, respectively[27]. As we took only leaves for the experiments this might be an explanation why CRE1-silenced plants were equal or similar to the EV-treated control plants: The basal CRE1-level is already low so that knocking down the transcript rate of it does not lead to significant effects. For obtaining a better insight into the CK signaling samples from roots should be taken, too. Higuchi *et al.* also showed that a *hk2 hk3* double knockout in *Arabidopsis* leads to an altered phenotype in the shoot while single mutants developed normally and showed normal or just slightly decreased sensitivity to the hormone. Triple knockout mutants were even completely insensitive to CKs regarding for example the expression of RRs [27]. Considering these findings and our results it is likely that it was not possible to affect the CK signaling pathway and interrupt CK-dependent reactions entirely. Future experiments should be done using double- or triple-constructs in VIGS, which knock down combinations of two or all CKRs. Alternatively, transgenic *N. attenuata* lines that are silenced in CKRs or CKR combinations or which are deficient in CK biosynthesis should be used in further studies.

A better understanding of CK signaling and the role of CKR in defense against herbivory is needed since CK regulation has large potential: Maybe crop plants with increased immunity against herbivores as well as enhanced growth might be cultivated in future, and modulating CK-dependent pathways will be a pivotal step in developing such plants [21]. But further studies are needed to elucidate the complex signaling network of CKR and their effect on defense reactions.

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7 Appendix

7.1 Media and solvents used for the plants and VIGS

- Sterilization medium:
  - 0.1 g Dichloroisocyanuric acid (DCCS)
  - 5 ml Distilled water
  - 50 µl of 0.5% Tween-20 detergent stock solution
- Gibberellic acid:
  - 0.1 M GA_3 (dissolved in ethanol), sterile filtered
- Smoke solution:
  - 50x Diluted liquid smoke (House of Herbs, Passaic NJ) in distilled water, autoclaved and stored at RT
- Germination medium:
  - 1x Strength Gamborg's B5 (3.16 g/l) diluted in 980 ml distilled water (pH = 6.80)
  - 6 g Phytagel
    - ▷ medium is autoclaved for 121^{o}C for 20min
    - ▷ pouring into 100x20 mm petri dishes in the sterile bench
- Soil:
  - 0.75 g Superphosphat
  - Multimix 14:16-18 (Haifa Chemicals Ltd., Haifa Bay, Israel)
  - 0.35 g Magnesium sulfate
  - 0.05 g Micromax (Scotts Deutschland GmbH, Nordhorn, Germany)
- YEP:
  - 10 g/l Yeast extract
  - 10 g/l Peptone (from soybean)
  - 5 g/l Sodium chloride

- Kanamycin:
  - 1000x Stock solution, in MilliQ water, sterile filtered
- Inoculation solution:
  - 5 mM Magnesium chloride, autoclaved
  - 5 mM MES (pH = 5.5-6.0), autoclaved

7.2 Media and solvents used for RNA analytics

- Trizol-Reagent:
  - 0.4 M Ammonium thiocynate
  - 0.8 M Guanidium thiocyanate
  - 0.1 M Sodium acetate trihydrate
  - 5 % (v/v) Glycerol
  - 38 % (v/v) Phenol
- Denaturation mix:
  - 0.5 µl dNTP
  - 0.5 µl Oligo dT
  - 3 µl DEPC water
- Reaction Mix:
  - 2 µl 5x RT buffer
  - 0.24 µl RiboLock (40 U/µl, Fermentas)
  - 0.075 µl RevertAid (200 U/µl, Fermentas)
  - 2.75 µl DEPC water

7.3 Media and solvents used for analyzing PI activity

- Extraction buffer:
  - 0.1 M Tris-Cl (pH = 7.6)
  - 2 g/l Phenylthiourea
  - 5 g/l Diethyldithiocarbamate
  - 18.6 g/l Sodium Ethylenediaminetetracedic acid
  - 50 g/l Polyvinylpyrrolidon

- Agar mixture:
  - 0.9 g Plant agar
  - 50 ml Tris (0.1 M)
- Staining solution:
  - 12 mg Fast Blue (solved in 20 ml Tris (0.1 M))
  - 6 mg Acetyl phenylalanine naphtyl ester (solved in 5 ml Dimethylformamide)

Eigenständigkeitserklärung

Hiermit versichere ich, Franziska Eberl, dass ich die vorliegende Bachelorarbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Jena, den 06. Juli 2011

Franziska Eberl

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