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The role of cytokinins in the regulation of flavonoids and poplar defense against rust fungus *Melampsora larici-populina*

Master Thesis

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List of Abbreviations

µM	Micromolar
ABA	Abscisic acid
ADP	Adenosine 5’ diphosphate
AMP	Adenosine 5’ monophosphate
ANOVA	Analysis of variance
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
ATP	adenosine 5’ triphosphate
BA	benzyladenine
CHASE	Cyclases/Histidine kinases associated sensory extracellular
CHI	Chalcone isomerase
CHS	Chalcone synthase
CKs	Cytokinins
CKX	Cytokinin oxidase/dehydrogenases
CYP735A	Cytochrome P450 mono-oxygenases
cZ	*cis*-zeatin
cZG	*cis*-zeatin glucoside
cZR	*cis*-zeatin riboside
cZRMP	c*Z* riboside 5′-monophosphate
cZROG	*cis*-zeatin riboside-*O*-glucoside
DFR	Dihydroflavonol 4-reductase
Dpi	Days post inoculation
DTT	Dithiothreitol
DW	Dry weight

dZ	Dihydrozeatin
dZR	DZ riboside
dZRMP	DZ riboside 5′-monophosphate
F3H	Flavanone-3-hydroxylase
HPT	Histidine phosphotransferase protein
iP	N^6-(Δ^2-isopentenyl)-adenine
IPA	Isopentenyladenine
IPR	Isopentenyladenine riboside
iPRMP	iP riboside 5′-monophosphate
IPTs	Isopentenyltransferases
JA	Jasmonic acid
JAG	Jasmonic acid glucoside
LAR	Leucoanthocyanidin reductase
LOG	Cytokine riboside 5'monophosphate phosphoribohydrolase enzyme
LPI	Leaf plastochron index
MEP	Methylerythritol phosphate
mg	Milligram
mT	*meta*-topolin
ng	Nanogram
NPR1	Non-expressor of PR genes 1
ns	Not significant
OH-JA-Ile	Hydroxylated jasmonyl-L-isoleucine
OPDA	*cis* (+)-12-oxophytodienoic acid
oT	*ortho*-topolin
PAL	Phenylalanine ammonia lyase

PAs	Proanthocyanidins
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time PCR
RR	Response regulator
SA	Salicylic acid
SAG	Salicylic acid glucoside
SPE	Solid phase extraction
Ti	Time
Tr	Treatment
tZ	*trans*-zeatin
tZG	*trans*-zeatin glucoside
tZR	*trans*-zeatin riboside
tZRDP	tZ riboside 5′-diphosphate
tZRMP	tZ riboside 5′-monophosphate
tZROG	*trans*-zeatin riboside-*O*-glucoside
tZRTP	tZ riboside 5′-triphosphate
UBQ	Ubiquitin
UFGT	UDP glucose flavonoid glycosyl transferases

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1. INTRODUCTION

Poplars (*Populus* spp) are deciduous, woody tree species naturally present in the Northern hemisphere with great ecological and economic values. Hybrid poplars are commercially cultivated worldwide for wood, plywood, paper production and also provide enormous environmental benefits such as phytoremediation, soil carbon sequestration and biofuel production (Stanton *et al.*, 2002). Besides, poplars are considered as an ideal model system for tree biology research mainly because the genome sequenced *Populus trichocarpa* is available, the feasibility of clonal reproduction, their fast growth and the relative ease of genetical modification (Jansson and Douglas 2007). However, under natural conditions poplars are subjected to a range of biotic and abiotic stresses (Newcombe *et al.*, 2001).

One major biotic stress of poplars is the poplar rust fungus (*Melampsora larici populina*), an obligate biotrophic pathogen belonging to the division Basidiomycota (Duplessis *et al.*, 2011). This leaf rust fungus is responsible for causing severe infections in poplar plantations as well as in natural forests (Newcombe *et al.*, 2001). It is one of the most devastating and widespread pathogens, and can cause decreased photosynthesis, premature defoliation and increased susceptibility to other pests and diseases, resulting in decreased biomass production (Gérard *et al.*, 2006).

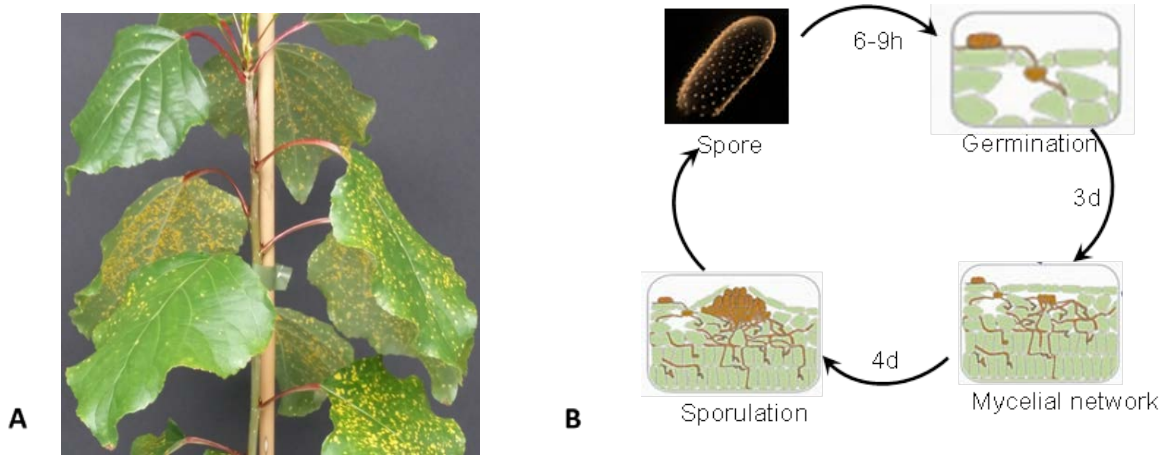

Figure 1: ***Melampsora larici populina*** A) A 3-month-old black poplar sapling infected by the rust fungus *Melampsora larici populina* (Photo credit: Dr. Chhana Ullah) B) *M. larici populina* life cycle (asexual, adapted from Hacquard *et al.*, 2011).

Melampsora larici populina requires larch (*Larix* spp) as an alternate host to complete its heteroecious lifecycle, which consists of five different types of spores namely teliospores, basidiospores, pycniospores, aeciospores and urediniospores (Duplessis *et al.*, 2011;

Hacquard *et al.,* 2011). On infected poplar leaves, the asexual stage is represented by the yellow pustules formed on the abaxial surface of leaf producing numerous urediniospores infecting new poplar hosts (Figure 1A). The urediniospores germinate (Figure 1B) and penetrate the poplar leaf through stomatal openings establishing specialized infection structures known as haustoria (Duplessis *et al.,* 2011; Hacquard *et al.,* 2011).

Poplar trees protect themselves from rust infection by synthesizing and accumulating phenolic secondary metabolites such as flavan-3-ols which include catechin and proanthocyanidins (PAs) (Ullah *et al.,* 2017). Typically, the structural variability of flavan-3-ols depends on the stereochemistry of the asymmetric carbons on the C ring, the B ring hydroxylation pattern and the degree of polymerization (Dixon *et al.,* 2005).

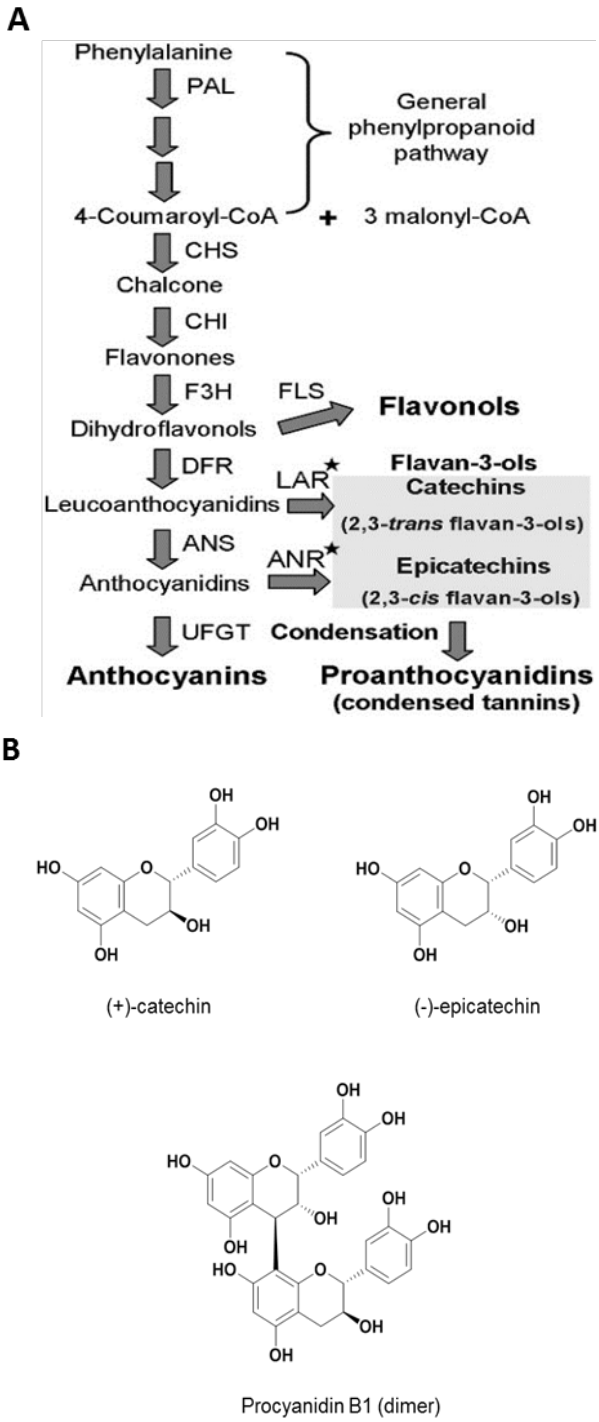

Figure 2: Biosynthesis of flavan-3-ols via flavonoid pathway. A) Biosynthetic pathway B) Structures of catechin, epicatechin and procyanidin B1. PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP glucose flavonoid glycosyl transferases.

Catechin and PAs are plant polyphenols, which are produced in the last steps of the flavonoid biosynthetic pathway (Dixon *et al.*, 2005; He *et al.*, 2008). The biosynthesis of catechins and

PAs (condensed tannins) is shown above (Figure 2). In brief, the LAR enzyme catalyzes the reduction of leucoanthocyanidins to form (+)-catechins. For the synthesis of (-)-epicatechins, leucoanthocyanidins are converted to anthocyanidins by ANS and then further reduced by ANR (Dixon *et al.,* 2005). In poplar, previous studies demonstrate that monomeric flavan-3-ols and PAs were induced upon fungal infection and protect trees against these pathogens (Miranda *et al.,* 2007; Wang *et al.,* 2013; Ullah *et al.,* 2017; Ullah *et al.,* 2019a; Ullah *et al.,* 2019b).

Plant hormones such as auxins, cytokinins (CKs), abscisic acid (ABA), salicylic acid (SA) and jasmonic acid (JA) play a major role in modulating plant growth and development as well as defense against stresses (Soliman *et al.,* 2018).In Arabidopsis, SA is a vital hormone in plant defense for protecting against biotrophic and hemi-biotrophic pathogens whereas JA signaling is activated when plants encounter necrotrophic pathogens or chewing herbivores (Pieterse and Dicke 2007). Furthermore, SA and jasmonate signaling pathways are usually considered to be antagonistic to each other (Pieterse *et al.,* 2012). Interestingly, poplar trees activate both the SA and JA signaling pathway in leaves after the biotrophic rust infection (Ullah et al., 2019a) and in stem-internodes after a hemi-biotrophic *Plectosphaerella populi* infection (Ullah et al., 2019b) suggesting that these two hormones are probably not interacting negatively with each other in poplar. Previous study also revealed that SA is a key defense signal in poplar infected by rust resulting in enhanced production of flavan-3-ols, which are considered to be chemical defense compounds (Ullah *et al.,* 2019a). ABA plays an important role in plant immunity by inducing stomatal closure to prevent the entry of bacterial or fungal pathogens and also controls water loss under drought stress conditions (Lim *et al.,* 2015). ABA levels increased in rust infected poplar leaves (Eberl *et al.,* 2018; Ullah *et al.,* 2019b) and in trees exposed to mild drought stress (Ullah *et al.,* 2019b). Exogenously applied or drought-induced ABA enhanced tree resistance to rust suggesting that ABA also plays an important role in poplar defense against this pathogen (Ullah *et al.,* 2019a).

SA can be synthesized from chorismate through the shikimate pathway (Lee *et al.,* 1995). In many herbaceous plants including Arabidopsis, SA signaling is controlled by the protein NON-EXPRESSOR OF PR GENES 1 (NPR1), which interacts with several transcription factors such as a WRKY protein and TGA for the regulation of the defensive response against the pathogen (Dong 2004; Wang *et al.,* 2006). JA biosynthesis starts from α-linolenic acid that is eventually converted to *cis* (+)-12-oxophytodienoic acid (OPDA) through a few

enzymatic steps in the chloroplast. OPDA is then exported to the peroxisome and directly acts as the precursor molecule for the biosynthesis of JA (Turner *et al.*, 2002) which is further converted to other forms of jasmonates including JA-Ile.

CKs are plant growth-promoting hormones which regulate cell proliferation and differentiation as well as defense against biotic stresses (Sakakibara 2006). The major classes of CKs include isoprenoid and aromatic types. Isoprenoid CKs including N^6-(Δ^2-isopentenyl)-adenine (IPA), *trans*-zeatin (tZ), *cis*-zeatin (cZ) and dihydrozeatin (dZ) are ubiquitous in plants. Aromatic CKs are *ortho*-topolin (oT), *meta*-topolin (mT), their methoxy-derivatives (meoT and memT, respectively) and benzyladenine (BA). Aromatic CKs are found rarely in plants but were identified in poplar and *Arabidopsis* (Sakakibara 2006). Among the well-studied plant defense hormones, the role of CKs in poplar defense against pathogen infection is much less understood than SA, JA or ABA.

1.1 Biosynthesis of cytokinins

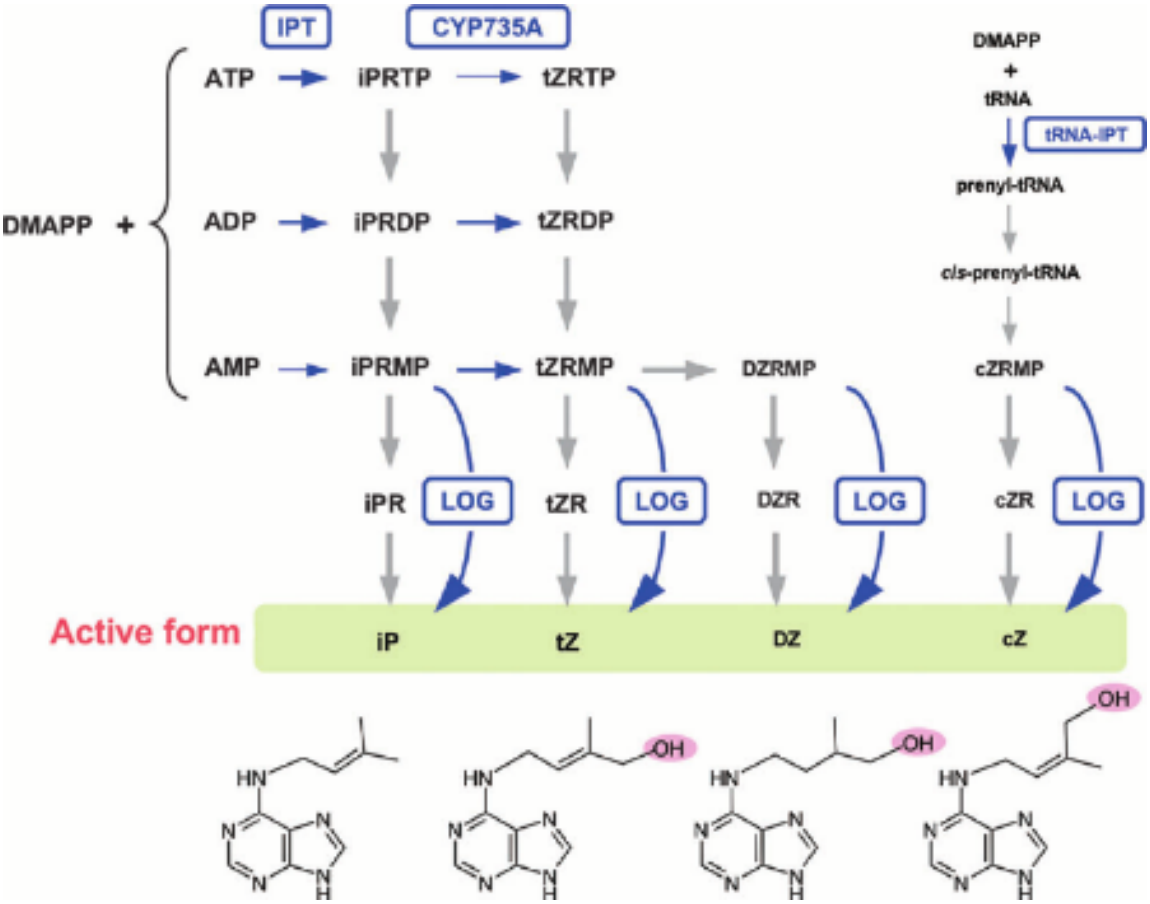

Figure 3: Biosynthetic pathway for Cytokinins. [Adapted from (Hirose *et al.*, 2007)]. iPRMP, iP riboside 5′-monophosphate; tZRTP, tZ riboside 5′-triphosphate; tZRDP, tZ riboside 5′-diphosphate; tZRMP, tZ riboside 5′-monophosphate; DZRMP, DZ riboside 5′-monophosphate; cZRMP, cZ riboside 5′-monophosphate; DZR, DZ riboside; cZR, cZ riboside; LOG, lonely guy enzyme, cytokine riboside 5'-monophosphate phosphoribohydrolase.

The biosynthesis of CKs occurs in the chloroplast via the methylerythritol phosphate (MEP) pathway. In brief, the first step of iP and tZ biosynthesis (Figure 3) is the addition of a prenyl group derived from dimethylallyl diphosphate (DMAPP) to the N^6 position of the adenosine 5' phosphates (AMP, ADP, or ATP) or tRNA to form iP nucleotides. This reaction is catalyzed by isopentenyltransferases (IPTs) (Kakimoto 2001; Kakimoto 2003). The iP nucleotides can then be hydroxylated to form tZ nucleotides by the cytochrome P450 mono-oxygenase CYP735A (Takei *et al.*, 2004). In addition, the LONELY GUY (LOG) enzyme (cytokine riboside 5'-monophosphate phosphoribohydrolase) is responsible for catalyzing the conversion of inactive CK nucleotides to free active CK bases (Kuroha *et al.*, 2009). CK

bases are irreversibly degraded by the enzymes known as cytokinin oxidases/dehydrogenases to maintain the homeostasis of cellular CKs (McGaw and Horgan 1983; Sakakibara 2006).

1.2 Cytokinin metabolism and signaling

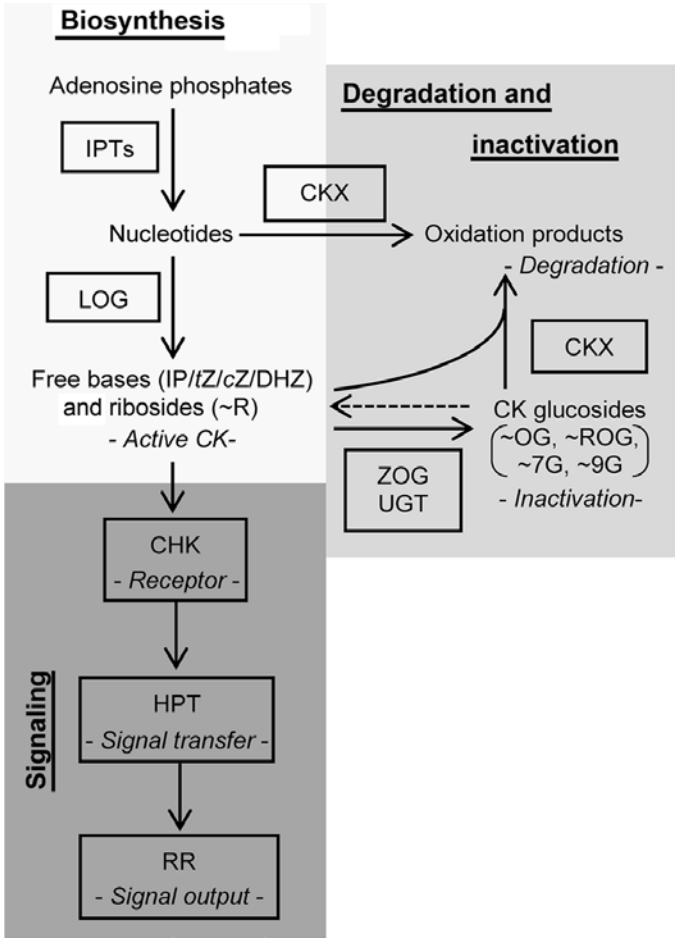

Figure 4: Cytokinin metabolism and signaling [Adapted from (Schäfer *et al*., 2015)]

CK signaling in plants involves a multistep two-component histidine-aspartate phosphorelay pathway. The well-characterized CK receptors have a CHASE (Cyclases/Histidine kinases associated sensory extracellular) domain-containing histidine kinase (CHK). CK binds to the CHASE domain inducing auto-phosphorylation followed by transfer of a phosphoryl group to the histidine phosphotransferase protein (HPT). Furthermore, HPT phosphorylates type B response regulators (type B RRs). These proteins function as transcription factors activating the transcriptional response of CK primary response genes including type-A RRs whereas type-A RRs act as negative feedback regulators of CK signaling (Hwang *et al.,* 2012; Muller *et al.,* 2007; To and Kieber 2008).

CKs have been previously shown to be positive regulators of plant defense against pathogens (Choi *et al.,* 2010; Großkinsky *et al.,* 2011), a role that is associated with activation of the hormone signaling pathways. For instance, CK triggers defensive responses via the SA signaling pathway in Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 Pst. In addition (ARR2), one of the type-B RRs that modulate the expression of CK genes interact with the SA mediated transcription factor TGA3 resulting in elevated plant defense response against pathogens (Choi *et al.,* 2010). On the other hand, CKs might be effective in defense against pathogen infection by inducing accumulation of anti-microbial compounds independent of the SA signaling pathway (Großkinsky *et al.,* 2011). In wild tobacco (*Nicotiana attenuata*), CK modulates the JA signaling pathway activating many downstream metabolites which are responsible for plant defense response against herbivore attacks (Schäfer *et al.,* 2015a; Schäfer *et al.,* 2015b). On the contrary, CKs play a crucial role in the development of club root disease caused by the pathogen *Plasmodiophora brassicae* (Siemens *et al.,* 2006). A study by Pertry *et al.,* (2009) revealed that biotrophic actinomycete *Rhodococcus fascians*, which is responsible for causing leafy gall disease, produces cytokinins acting through histidine kinase CK receptors in Arabidopsis (AHK2 and AHK3), which are important for the development of disease symptoms.

CKs also play a vital role in regulating cambial development (Nieminen *et al*., 2008) and leaf phyllotaxy (Besnard *et al.,* 2014) in poplar. *Populus* PtRR13, a type-B response regulator, was shown to be a negative regulator of root formation and development (Ramírez-Carvajal *et al*., 2009). Furthermore, recent studies showed that CK activity decreased in poplar (*Populus* × *canescens*) that were exposed to drought stress conditions (Paul *et al.,* 2016; Paul *et al.,* 2017). To date, the role of CKs in trees in defense against pathogens has not been reported. Interestingly, a recent study in our group found that *Plectosphaerella populi* infection increased the accumulation of CKs in poplar stems suggesting a role of CKs in tree-pathogen interactions (Ullah *et al.,* 2019b). This study also indicated that exogenous CKs decreased flavonoid accumulation in poplar stems. However, the role of CKs in woody plant defense against biotrophic rust infection remains unexplored. In addition, there is little information regarding CK interactions with other phytohormones in defense against pathogens.

The aim of my thesis was to study the role of CKs during poplar-rust interaction. Therefore, the main objectives of this research were to answer the following questions: (i) Do the contents of CKs and the downstream signaling change in black poplar leaves after rust

infection? (ii) Do the altered CK accumulation regulated by CK synthesis or degradation? (iii) Do CKs regulate the reduced flavan-3-ol accumulation? (iv) Do CKs decrease resistance to the biotrophic rust *M. larici-populina*? (v) What is the mechanism of CK action? Do CKs affect the levels of other hormones such as SA and JA?

1.3 Scope of work

Previous studies have reported that CKs participate in plant defense in herbaceous species by interacting with defense hormones such as SA and JA as well as by modulating secondary metabolism. However, the role of CKs in tree defense remains unknown so far. Previous experiments using a slow-growing hemi-biotrophic fungus (*Plectosphaerella populi*) suggest that fungal infection induces the accumulation of CKs in poplar stems, which is likely to affect flavan-3-ol defenses negatively (Ullah *et al.,* 2019b). To further investigate the role of CKs in tree defense, I used the poplar-rust system In this study, I inoculated poplar trees using rust spores and quantified different forms of CKs over a course of infection. Then I analyzed the expression of genes involved in CK biosynthesis and signaling in the rust-infected as compared to control samples. Then to manipulate poplar CKs, an active CK (tZ) was exogenously sprayed onto the leaves before infection to determine its effect on rust infection and the accumulation of flavonoids and defense hormones. Furthermore, I analyzed the transcripts of flavonoid biosynthetic genes in CK-treated poplar trees with and without rust infection. I also analyzed the transcript accumulation of CK biosynthesis, metabolism and signaling genes in the presence or absence of rust infection. The results provide a better understanding of the part played by CKs in anti-fungal defense in poplar.

2. MATERIALS AND METHODS

2.1 Plants and pathogen used in this study

Black poplar (*Populus nigra*, clone f. 65, originally collected by Sybille Unsicker in Küstrin-Kietz, Germany) and the hybrid poplar *Populus* × *canescens* were used in this study. The plants were grown in the greenhouse and York chamber as described by Ullah et al., 2019b. Plants with a height of approximately 80 cm were chosen for different experiments. *Melampsora larici-populina* urediniospores were used for inoculation experiments. The rust spores were collected and preserved as described previously (Ullah *et al.*, 2017). In brief, the rust spores used were multiplied from a single uredium. The urediniospores were collected by using a fine brush. After drying in dry silica gel beads for 2-3 days, they were stored at -20°C.

2.2 Exogenous spraying of cytokinins before rust inoculation

To manipulate CK levels externally, *trans*-Zeatin (Sigma-Aldrich Chemie GmbH, Germany) solutions at different concentrations were uniformly sprayed on both the surface of poplar (*Populus* × *canescens*) leaves and stem internodes. The mock-treated plants were sprayed similarly with 0.2% methanol in water. After one day and three days, six leaves from leaf plastochron index LPI 5-10 on each plant were harvested by removing the midrib and immediately frozen in liquid nitrogen.

2.3 Inoculation of poplar leaves with rust fungus *M. larici populina*

M. larici populina urediniospores were used for inoculation. Poplar saplings were transferred from the greenhouse to the climate chamber (22°C/19°C day/night temperature, natural light conditions, 60% relative humidity) before the onset of the experiment. Plants were watered about 150 ml every day until the end of the experiment. All mock-treated plants were sprayed with water. The rust spores were prepared at a concentration of 10^5 spores ml^{-1} water. The rust spore suspension was uniformly sprayed onto the lower surface of the leaves. After spraying, the plants were wrapped around with polyethylene terephthalate bags (Bratschlauch, Toppits, Minden, Germany) and tied at both ends in order to maintain sufficient humidity for the germination of spores. The bags were opened from the top for aeration on the next consecutive day after 12 hours of darkness in the climatic chamber. Different sampling time points at 6 hours, 3-, 7- and 14 days after inoculation were chosen

for harvesting leaves. At different time points, six fully expanded leaves from LPI 5-10 on each plant were harvested as described previously.

2.4 Extraction and purification of cytokinins

Harvested poplar leaves were ground to a fine powder with liquid nitrogen and then freeze-dried. About 10-12 mg of freeze-dried leaf tissue were weighed and transferred to 96-well biotubes. Extraction was conducted in 96-well biotubes (1.1 mL individual tubes, Arctic White LLC, Bethlehem, PA, USA) and Nunc 96-Well Deep Well Plates (Thermo Scientific, Waltham, MA USA). CKs were extracted twice with extraction buffer containing deuterated CKs followed by two solid-phase extraction (SPE) purification steps. An extraction buffer of MeOH: H_2O: HCOOH (15:4:1) was used containing a solution of deuterated internal CK standards (10 µl per sample, which contained 1 ng D_5-tZ, 0.2 ng D_6-IPR, 0.2 ng D_5-tZR, 4.0 ng D_5-tZROG, 2.0 ng D_5-tZ7G and 0.2 ng D_6-IPA). SPE was performed on a Multi 96 HR-X column (reversed phase matrix, Macherey-Nagel, Düren, Germany, 96 x 25 mg) in the first step. The second SPE step was performed on a Multi 96 HR-XC column (cation exchange matrix, Macherey-Nagel, 96 x 25 mg). Both the steps were conducted in the Chromabond Multi 96 vacuum manifold chamber (Macherey-Nagel). The Evaporator system (Glas-Col, Terre Haute, IN, USA) was used for evaporation under constant nitrogen flow. For a step-by-step protocol, see (Schäfer *et al.,* 2014) except that panshaker replaced the genogrinder in the complete protocol.

2.5 Extraction of other phytohormones and phenolics

Approximately 10-12 mg of freeze-dried leaf tissue were weighed out for each sample. An extraction buffer of 1 ml methanol was used which contained 5 µg apigenin-7-glucoside, 3 µl phytohormone standard containing the following internal standards per ml: [30 ng of D_4-SA (Santa Cruz Biotechnology, Santa Cruz, U.S.A.), and 30 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), 6 ng D_6-JA-Ile (HPC Standards GmbH)], 30 ng trifluoromethylcinnamic acid as internal standards. One ml methanolic extraction solvent was added to each sample, vigorously vortexed and then incubated for 25 min at 20°*C i*n a shaker at 1000 rpm. After centrifugation of the extracts at 12000 rpm at 4°C for 5 minutes, the supernatant of about 900 µl was transferred to a new micro centrifuge tube. The samples were analyzed for flavan-3-ols, other flavonoids and phytohormones by LC-MS.

2.6 Quantification of cytokinins

CK analyses were performed using a high-performance liquid chromatography system (Agilent 1260) coupled to a triple quadrupole mass spectrometer (API5000, AB SCIEX, Darmstadt, Germany) equipped with turbospray ionization source in positive mode. Zorbax Eclipse XDB-C18 column (50 mm x 4.6 mm, 1.8 µm, Agilent) was used as a stationary phase. The mobile phases used are A, 0.05% formic acid in water, and B, acetonitrile, at a flow rate of 1.1 ml/min and a column temperature of 25 ^{0}C. The elution profile was; 0-0.5 min, 5% B; 0.5-6.0 min 37.4% B; 6.0-7.5 min 100% B; 7.6-10.0 min, 100% B. The instrument specifications and multiple reaction monitoring mode settings are described in (Schäfer *et al*., 2014). Settings were as follows: ion spray voltage, 5,500 V; turbo gas temperature, 700°C; nebulizing gas, 70 psi; curtain gas, 25 psi; heating gas, 60 psi; and collision gas, 6 psi. Analyst 1.6 software (Applied Biosystems) was used for data acquisition and processing. Peak areas for tZ, tZR, tZG, tZROG, IPA, IPR and *oT* peak areas were quantified using their respective deuterated internal standards peak areas, whereas peak areas for cZR, cZROG and cZG were quantified to their respective *trans* forms.

2.7 Quantification of other phytohormones

Other phytohormones like SA, JA, JA-Ile, ABA, OPDA were analyzed on the same LC-MS/MS systems used for CK quantification except that the turbospray ionization source was employed in the negative mode. The hormone metabolites were separated on a Zorbax Eclipse XDB-C18 column (stationary phase) using mobile phases A, 0.05% formic acid in water, and B, acetonitrile, at a flow rate of 1.1 ml/min and a column temperature of 25 ^{0}C. The parent ion and the corresponding fragments of SA, JA and ABA were detected by multiple reaction monitoring mode settings described in (Vadassery *et al*., 2012). The elution profile is also described in (Vadassery *et al*., 2012). SA, JA, JA-Ile and ABA concentrations were quantified using their corresponding deuterated internal standards whereas OPDA, OH-JA, Sulfo-JA and JA-glucoside (JAG) concentrations were determined according to D_6-JA by applying a theoretical response factor of 1.0.

2.8 Quantification of flavan-3-ols monomers and PA dimers

Flavonoid measurements were performed on an HPLC (Agilent 1200) coupled to an API 3200 tandem mass spectrometer (AB SCIEX, Darmstadt, Germany) equipped with turbospray ionization source in negative mode. For flavan-3-ol separation, a Zorbax Eclipse XDB-C18 column was used as a stationary phase. The mobile phases used were A, 0.05% formic acid in water, and B, acetontrile, at a flow rate of 1.1 ml/min and a column temperature of 25 ^{0}C. The parent ion and the corresponding fragments ion of (+)-catechin, (-)-epicatechin, (+)-gallocatechin, PA dimers, other flavonoids and apigenin-7-glucoside were analyzed by multiple reaction monitoring mode settings described by (Ullah *et al.*, 2017). Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Flavan-3-ols and other flavonoids were quantified using apigenin-7-glucoside as an internal standard.

2.9 Isolation of total RNA

Isolation of total RNA from freshly ground tissue was done using the previously optimized method as described by (Ullah *et al.,* 2017). Briefly, approximately 900 µl lysis buffer RP was added to around 75 mg of fresh tissue, vigorously vortexed, then incubated for 30 min in a shaker. Samples were then centrifuged at 13500×g for 5 min. The supernatant was transferred to the prefilter and centrifuged for 1 min at 9000 rpm. Approximately 350 µl of absolute ethanol was added to the filtrate and mixed by inverting the tubes a few times. The content was then transferred to the RTA spin filter, centrifuged for 2 min and the flow-through was discarded. The first washing step using 300 µl of wash buffer R1 was done on the RTA spin filter. Then, DNase (30 Kunitz units in an 80 µl volume of 10 µl of RNase-free water and 70 µl of buffer RDD) was added onto the column and incubated at room temperature for 15 min. An additional washing step using 300 µl of wash buffer R1 was conducted. Two repetitive washings using wash buffer R2 (700 µl) were conducted and the spin filter centrifuged at maximum speed to remove traces of ethanol. After transfer of the filter to a new elution tube followed by adding 40 µl of RNase-free water, RNA was eluted and the concentration and quality of the RNA in samples were measured using Nanodrop 2000 Spectrophotometer (ThermoScientific).

2.10 cDNA synthesis

cDNA synthesis was performed in a 20 µl reaction volume which contained 1 µg of total RNA, oligo $(dT)_{12-18}$ primer (1 µl), and dNTP mix (1 µl). The mixture (13 µl volume) was heated at 65^0C and quick chilled on ice for 5 min. Then 5x first strand buffer (4 µl), 0.1M DTT (2 µl) and Superscript II Reverse Transcriptase (1 µl) were added to the reaction mixture, further incubated at 42^0C for 50 min and the enzyme was inactivated by heating at 70^0C for 15 min. The cDNA was diluted using 80 µl of sterile water and its quality was checked by PCR using plant UBQ forward and reverse primers.

2.11 qRT-PCR

The qRT-PCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad) using a two-step amplification protocol (cycling parameters: 3 min at 95°C followed by 39 cycles of 10 s at 95°C and 30 s at 58°C) followed by melting curve determination. The reaction mixture in a 20 µl volume contained 6.5 µl of sterile water, 0.75 µl of forward and 0.75 µl of reverse primers, 10 µl of Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) and 2 µl of diluted cDNA. Five biological replicates and two technical replicates for each biological sample were used. The mean of two technical replicates was used to quantify transcript abundance. Poplar UBQ was used to normalize the transcript abundance of all the flavonoid biosynthetic genes. The formula used for determining the relative expression level of genes was $2^{\Delta Ct}$ (where Ct is the number of cycles required to be above background) A sterile water sample was used as a reaction control. Primer sequences for *LAR*, *ANR* and plant *UBQ* were according to (Ullah *et al.*, 2019a). Relative colonization of rust was determined as described previously (Ullah *et al.*, 2019b). Primer sequences for *IPT*, *CKX* and *RR* are provided below (Table 1). The poplar *actin* gene was used to normalize the transcript abundance of all the CK genes.

Table 1: List of CK primers used in this study

PtIPT2 qRT-F	CGTGGGCATAGAAAACGAATCT
PtIPT2 qRT-R	GACCTCCTGCTCCACTAAGGAAT
PtIPT3 qRT-F	CCTACTGTGGACTTTACAGCAACAA
PtIPT3 qRT-R	CCTCGAGTCGAAATTGATTCG
PtIPT5a qRT-F	CGAATCAATCGTCACACGTGAT
PtIPT5a qRT-R	GCCTCCACGTACGAATTGGA
PtIPT6b qRT-F	GGAGCAACAGGGTGTGGAA
PtIPT6b qRT-R	GTATTTTATCGGAGTTGATGATTTCG
PtCKX1 qRT-F	ACCCTTATAGCCAGAAATTCG
PtCKX1 qRT-R	TCGTTTCATCAAAGCCTCCA
PtCKX4 qRT-F	TGGGACCAGACACATTGTTGA
PtCKX4 qRT-R	TCATGGAAAACACCCTTGTTG
PtCKX6 qRT-F	TGGGAAGATGTGTTAAAACGA
PtCKX6 qRT-R	GCACCAAAAAACAATTCACTG
PtRR7 qRT-F	ACCAGATGTTTGGAGGAGGGA
PtRR7 qRT-R	TCTTACTGGCTTCAAGAAAAA
PtRR13 qRT-F1	GTGAATTTAGTAGTGTCTCTCTTTTGTTTAG
PtRR13 qRT-R1	CATAAAGGCAGGTCCTAAGCATC
PnActin qRT-F	CCCATTGAGCACGGTATTGT
PnActin qRT-R	TACGACCACTGGCATACAGG

2.12 Statistical Analysis

All data were tested for statistical assumptions using the R package (version 3.4.0). Initially, normality of the data and homogeneity of variances were analyzed using Shapiro-Wilk and Levene's tests, respectively. Data were analyzed using one-way or two-way ANOVA followed by Tukey's post-hoc test (for one-way ANOVA only) to compare the differences among different groups. A student's t-test was used to analyze flavan-3-ol metabolites and hormones in CK vs mock-treated trees with and without rust infection.

3. RESULTS

3.1. Rust infection modulates the levels of cytokinins in poplar leaves

To analyze the contents of CKs during poplar-rust interaction, we conducted a time-course infection experiment. Young black poplar saplings (*P. nigra*) were inoculated with the rust fungus *M. larici populina* and leaves sampled at different time points after inoculationand compared to uninfected control leaves. *Trans*-zeatin (tZ) concentrations significantly changed after rust infection. At 6 hr, tZ accumulated to significantly higher levels than in uninfected controls, while at 3 dpi and 7 dpi the levels were decreased with respect to controls. Levels of *trans*-zeatin riboside (tZR) and *trans*-zeatin glucoside (tZG) also increased to higher levels at 6 hr after inoculation than in controls, but there were few significant differences at later time points. A greater accumulation of *cis*-zeatin riboside-*O*-glucoside (cZROG) in rust infected plants relative to uninfected controls was observed at most time points over the course of infection, especially at the last time point, 14 d. Levels of ortho-topolin (oT), an aromatic CK, and isopentenyladenine riboside (IPR) and *trans*-zeatin riboside-*O*-glucoside (tZROG) increased significantly after rust infection relative to controls at most time points after inoculation, but increases in isopentenyladenine (IPA) were barely detectible. Taken together, our CK measurements indicate that rust infection modulates the levels of CKs in poplar in a complex time-dependent manner. In general, the various CKs are increased by infection relative to controls, especially at early time points.

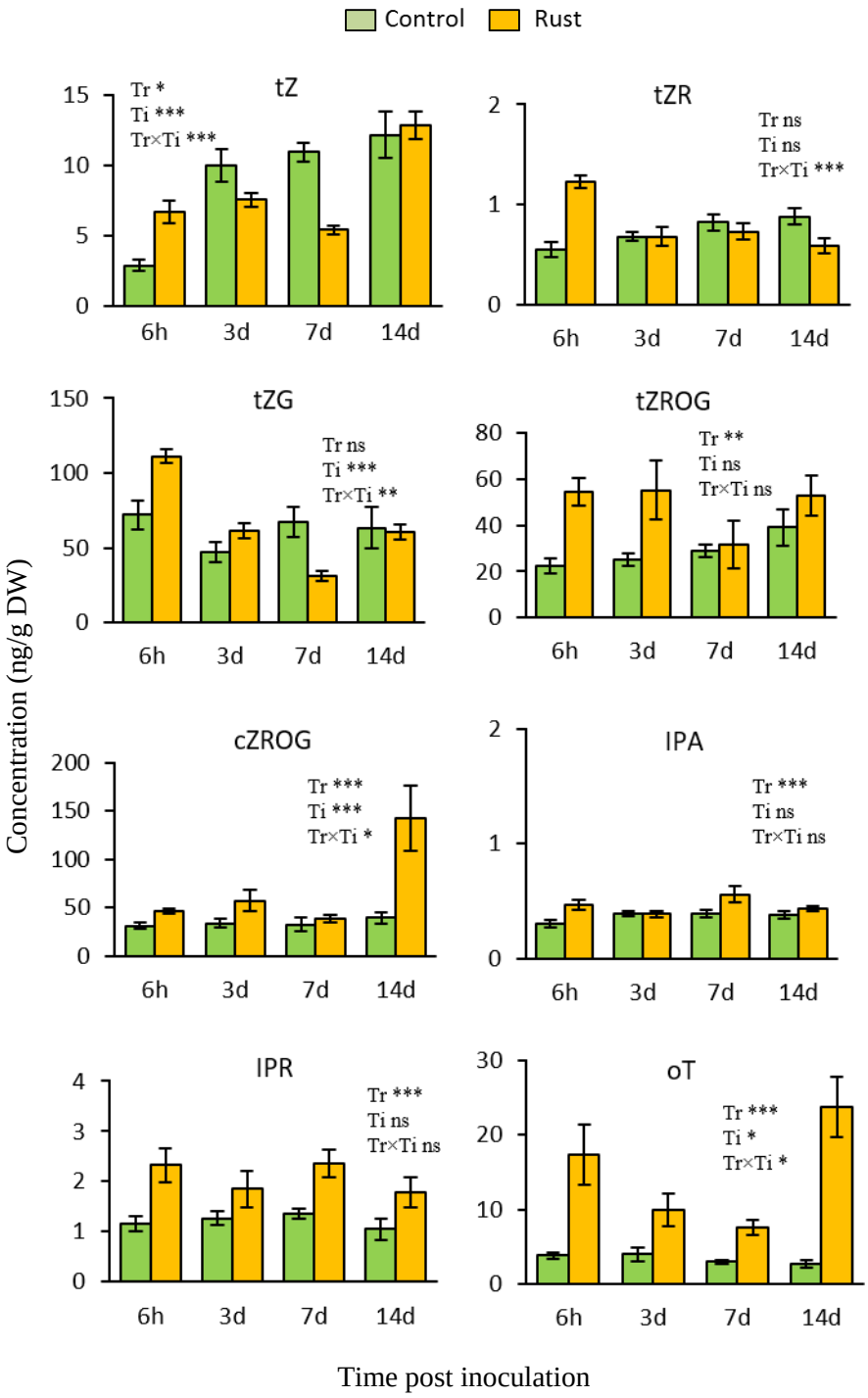

Figure 5: Accumulation of CKs in black poplar leaves with and without rust infection over a two week time course of infection. Leaf samples were collected at different time points post inoculation. CKs were analyzed by LC-MS/MS. Data were analyzed using a two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (Tr-treatment, Ti-time, Tr×Ti- interaction effect, ns-not significant). Data were mean ± SE (n=5). tZ, *trans*-zeatin; tZG, *trans*-zeatin glucoside; cZROG, *cis*-zeatin riboside-*O*-glucoside; IPR, isopentenyladenine riboside; tZR, *trans*-zeatin riboside; tZROG, *trans*-zeatin riboside-*O*-glucoside; IPA, isopentenyladenine; oT, *ortho*-topolin.

3.2. Transcripts of a group of CK biosynthetic genes, the isopentenyltransferases (*IPT*), are downregulated in rust-infected poplar leaves

To investigate the transcriptional dynamics of the genes involved in CK biosynthesis in rust infected poplar leaves, I choose to analyze the expression of isopentenyl transferase (*IPT)* genes. IPTs catalyze the first step in the CK biosynthetic pathway (Kakimoto 2003; Kakimoto 2001). In poplar, nine *IPT* genes have been identified recently (Jaworek *et al*., 2019), of which I analyzed the transcript abundance of a few in rust infected poplar leaves at different time points after inoculation. Transcript levels of all the *IPT* genes, except *IPT*5a, changed significantly (Figure 6) in *M. larici populina*-infected poplar leaves in comparison with control leaves. At 6 h post inoculation, only the *IPT3* gene was up-regulated while the transcript levels of the other genes were not significantly different. All genes except *IPT3* were significantly down-regulated at 3 dpi and 7 dpi. These results suggest that the biosynthesis of CKs was slightly upregulated initially and then downregulated over the time course of rust infection. Thus both our transcript (Figure 6) and metabolite data (Figure 5) indicate that rust infection in poplar trees induces CK biosynthesis at the early period of infection (6h), but down-regulates it during the active colonization of poplar leaves at 3- and 7-days post-inoculation.

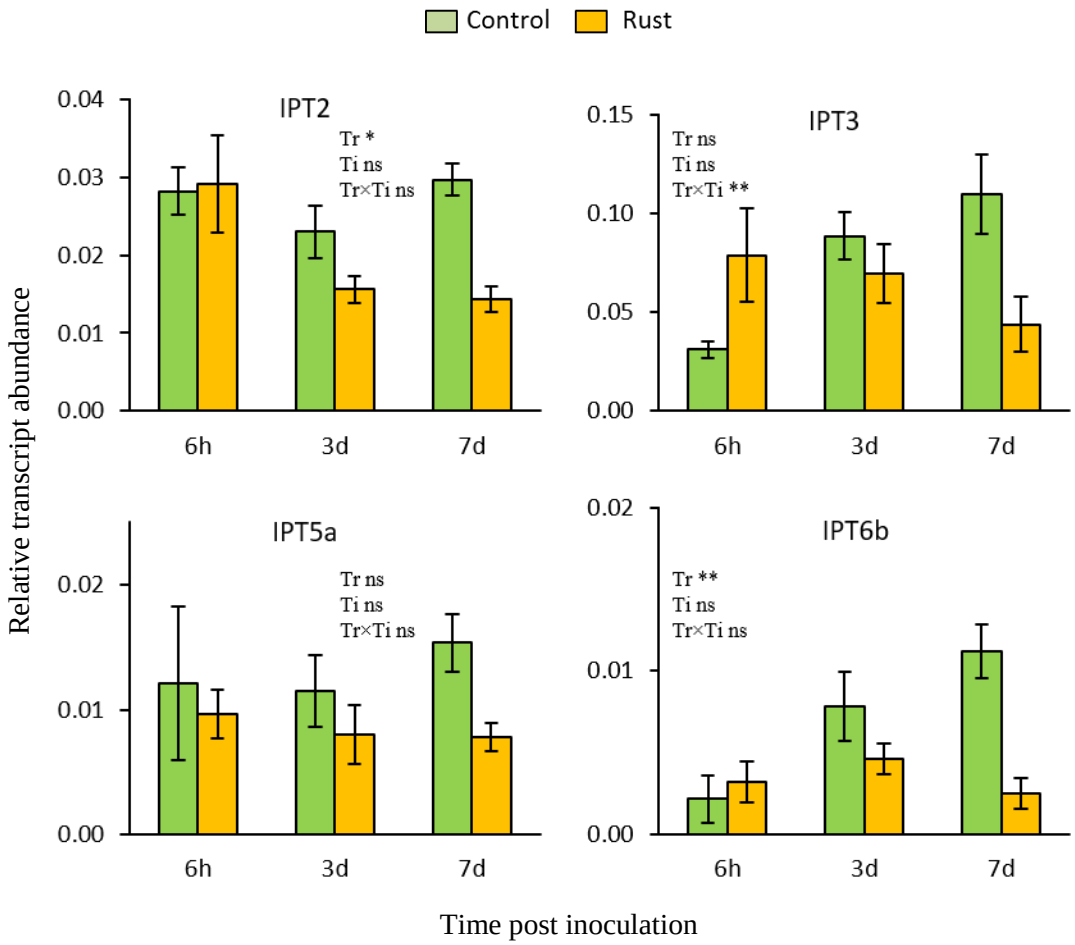

Figure 6: Transcript abundance of the *IPTs*, a group of CK biosynthetic genes, in black poplar leaves with and without rust infection. Transcript levels of each *IPT* gene were normalized to the housekeeping *actin* expression. Data were analyzed using a two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (Tr-treatment, Ti-time, Tr×Ti-interaction effect, ns-not significant). Data were mean ± SE (n=4-5).

3.3 Transcripts of CK metabolism (*CKX*) and signaling genes (*RR*) were down-regulated in rust infected *P. nigra* leaves

The levels of CKs in plants are controlled by degradation as well as by biosynthesis. The CK oxidase/dehydrogenases (*CKX*s) are known to be responsible for the irreversible degradation of CKs (McGaw and Horgan 1983; Sakakibara 2006). To quantify if the degradation pathway of CKs is also changed during rust infection in poplar leaves, I measured the transcript abundance of some selected *CKX* genes using qRT-PCR. The expression of the *CKX*1 gene was significantly lower (Figure 7A) in rust infected poplar leaves compared to the controls over the period of infection, while the expression of *CKX*4 did not change. The transcript

levels of *CKX*6 were significantly induced at 6h after inoculation compared to controls, but downregulated at 3 and 7 dpi.

To measure the level of CK signaling, I determined the expression of response regulators (RR), which are considered as feedback regulators in the CK signaling pathway (Hwang *et al.*, 2012; Muller *et al.*, 2007; To and Kieber 2008). Transcript levels of *RR*7 and *RR*13 decreased significantly in rust-infected poplar leaves over the course of infection (Figure 7B). These results suggest that there is a strong downregulation of the CK signaling pathway at the transcriptional level upon rust infection.

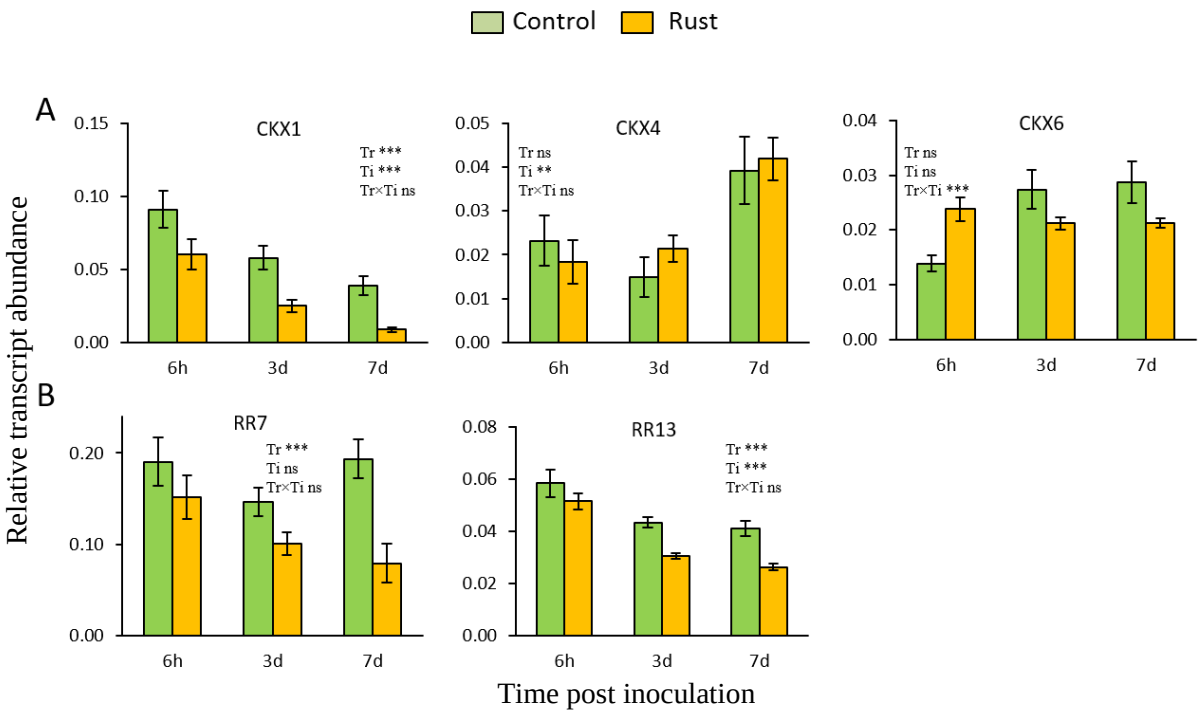

Figure 7: Transcript levels of *CKX* and *RR* genes in black poplar leaves with and without rust infection. A) CK oxidase/dehydrogenases (*CKX*) and B) Response regulator (*RR*) genes. Transcript levels of each *CKX* and *RR* gene were normalized to the housekeeping *actin* expression. Data were analyzed using a two-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) (Tr-treatment, Ti-time, Tr×Ti- interaction effect, ns-not significant). Data were mean ± SE (n=5).

3.4. Exogenous *trans*-zeatin (tZ) is converted to structurally related by inactive forms of CKs in poplar

To determine if exogenous tZ is converted to other forms of CKs in poplar, we sprayed black poplar leaves with two different concentrations of tZ and measured CKs 3 days after spraying. As expected, tZ concentration increased significantly in tZ-treated poplar leaves (Figure 8). Interestingly, the concentrations of tZR, tZG and tZROG increased linearly after

exogenous tZ treatments (Figure 8). In contrast, cZG, cZROG, oT, IPR and IPA did not change significantly after tZ spraying. These data demonstrate that poplar converted exogenous tZ to different inactive forms, which are structurally related to tZ. Amounts of cZROG and cZG were not affected suggesting that there is no possibility of interconversion of tZ to cZ (Vyroubalova *et al.*, 2009; Hosek *et al.*, 2019).

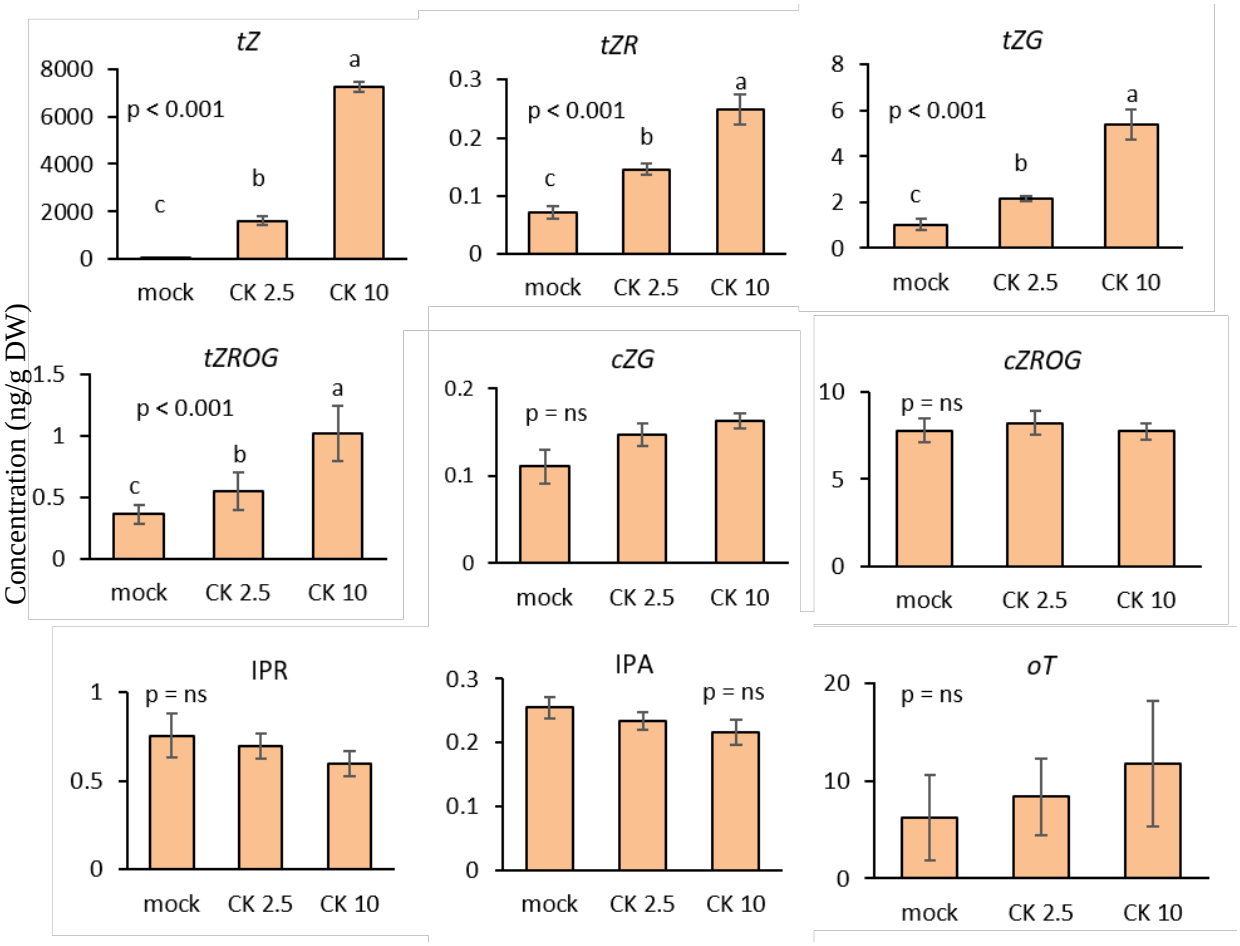

Figure 8: Effect of *trans*-zeatin (tZ) spraying on different forms of CKs in poplar (mock control, CK 2.5 µM and CK 10 µM). Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Data were mean ± SE (n=4). tZ, *trans*-zeatin; tZR, *trans*-zeatin riboside; tZG, *trans*-zeatin glucoside; tZROG, *trans*-zeatin riboside-*O*-glucoside; cZG, *cis*-zeatin glucoside; cZROG, *cis*-zeatin riboside-*O*-glucoside; IPR, isopentenyladenine riboside; IPA, isopentenyladenine; oT, *ortho*-topolin.

3.5. Exogenous cytokinin reduced the accumulation of flavan-3-ols and some phytohormones in *Populus* × *canescens*

In order to examine the effect of exogenous CKs on the accumulation of flavan-3-ols, tZ (1 µM, 2 µM, 5 µM) or 0.2% methanol in water as mock control were sprayed on *P. canescens*. Leaf samples without the mid-rib were harvested at 3 dpi. Flavan-3-ols (catechin,

gallocatechin and PA dimers) accumulated significantly in lower amounts (Figure 9A) at low doses (1 µM and 2 µM) when compared to mock-treated and 5 µM tZ-treated trees (One-way ANOVA, $p < 0.05$). These results indicate that low doses of exogenous CK decreased the levels of flavan-3-ols.

To investigate whether the increased CK levels affect other hormones, I analyzed the level of defense hormones including SA, jasmonates and ABA in CK-treated samples and compared them with mock controls. Interestingly, SA content decreased significantly in CK-treated trees at low doses (1 µM and 2 µM) (One-way ANOVA, $p < 0.01$) compared to mock control-treated trees (Figure 9B). The levels of jasmonic acid glucoside (JAG) decreased significantly in 1 µM and 2 µM CK treated trees in comparison with the mock control ($p < 0.01$, Figure 9B). On the other hand, the levels of salicylic acid glucoside (SAG), JA, OPDA and ABA were not affected by CK treatment. Therefore, our data suggest that exogenous CK treatments decreased the accumulation of flavan-3-ols, which could be due to a negative correlation between the SA and CK signaling pathways.

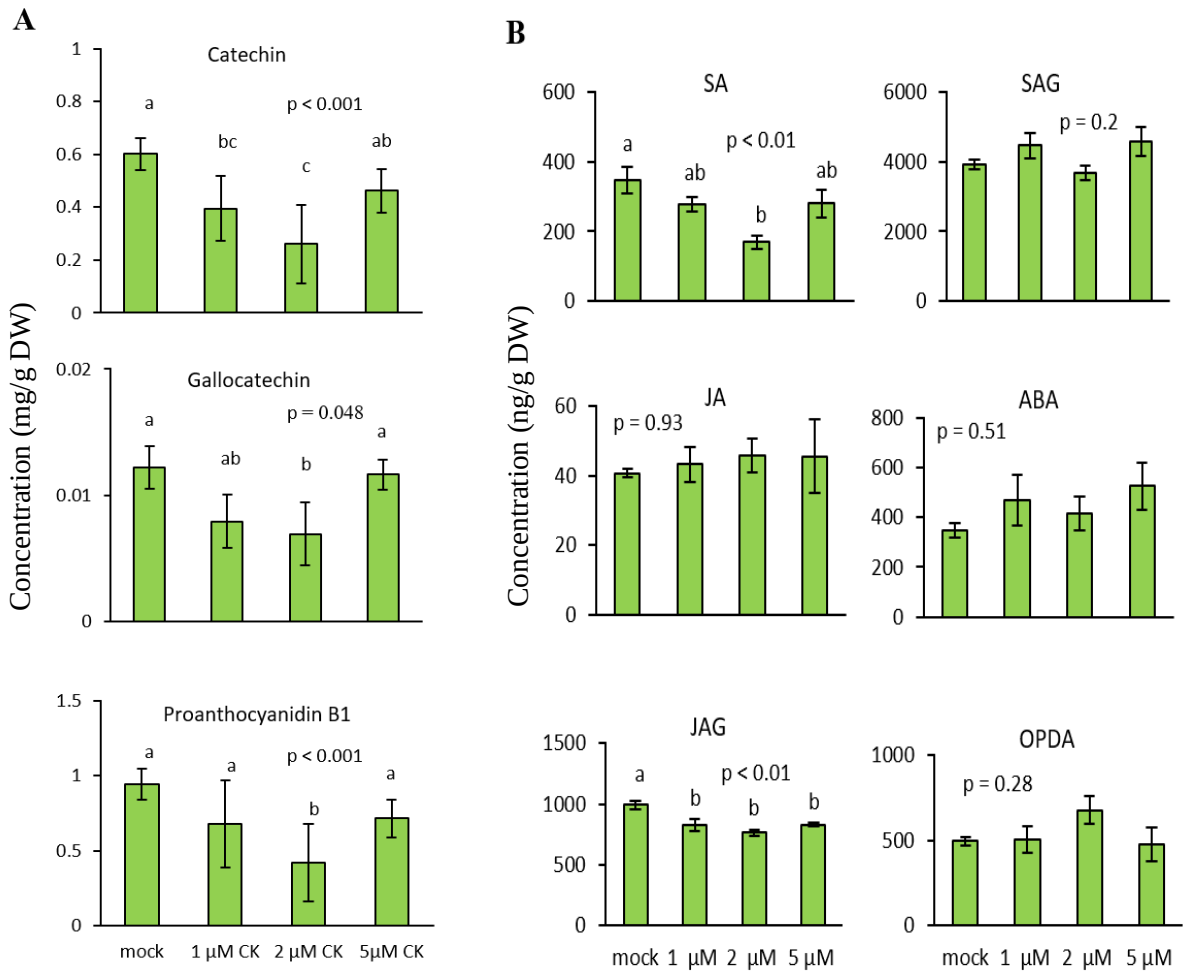

Figure 9: Effect of different doses of CKs on flavan-3-ols accumulation and phytohormones in *P. canescens.* The concentrations of A) flavan-3-ols and B) phytohormones were observed. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Mean groups with different letters are statistically different at 95% confidence. Data were mean ± SE (n=4). (SA, salicylic acid; SAG, salicylic acid glucoside; JA, jasmonic acid; ABA, abscisic acid; JAG, jasmonic acid glucoside; OPDA, oxo-phytodienoicacid).

3.6. Transcripts of flavan-3-ol biosynthetic genes in *Populus* × *canescens* leaves were partially down-regulated by CK treatment

Then we measured the relative transcript abundance of *PnLAR* genes and *PnANR* gene using qRT-PCR. *LAR* and *ANR* genes are responsible for catalyzing the last steps of flavan-3-ol biosynthesis in poplar (Ullah *et al.*, 2017). The expressions of two *LAR* and one *ANR* genes were partially down-regulated after treatment with 1 µM and 2 µM CK, but the differences were not statistically significant. Our transcripts (Figure 10) and the corresponding metabolite data (Figure 9) suggest that exogenous CK treatments decreased flavan-3-ol biosynthesis and accumulation.

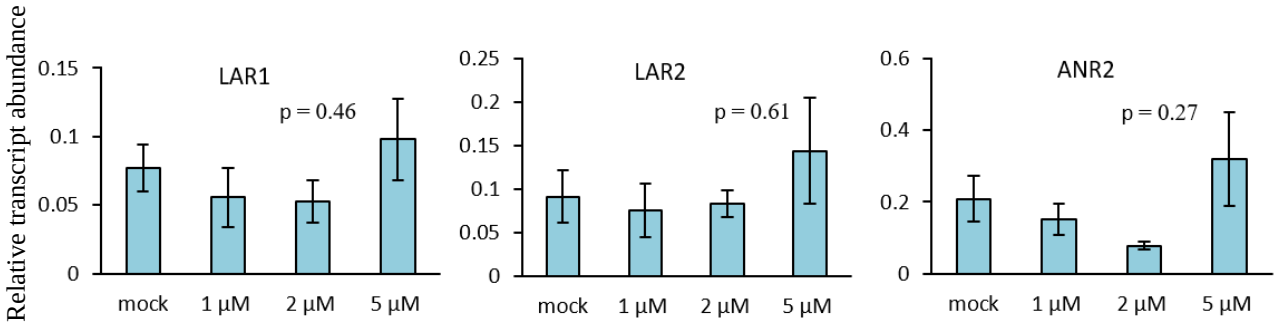

Figure 10: Transcript levels of flavan-3-ol biosynthetic genes in *P. canescens*. Transcript levels of each gene were normalized to the expression of the housekeeping gene *actin*. Data were analyzed using one-way ANOVA. Data were mean ± SE (n=4). Leucoanthocyanidin reductases (*LAR*) and anthocyanidin reductase (*ANR*).

3.7. Transcripts of CK degradation and signaling genes were up-regulated on CK treatment of *Populus* × *canescens*

Since externally applied CK was converted to different forms of CKs *in planta* (Figure 8), we determined whether the transcripts of CK degradation and signaling genes were changed by the exogenous spraying of CK. Expression of three cytokinin oxidase/dehydrogenase genes (*CKX*) and two signaling response regulator (*RR*) genes were analyzed by qRT-PCR. The transcripts of *CKX*1 and *RR*7 increased significantly in CK-treated trees in comparison with mock controls. *CKX*4, *CKX*6 and *RR*13 transcripts were also induced in CK-treated trees compared to mock controls, but the differences were not significant. The above results indicate a strong upregulation of *CKX* and *RR* genes after exogenous CK application.

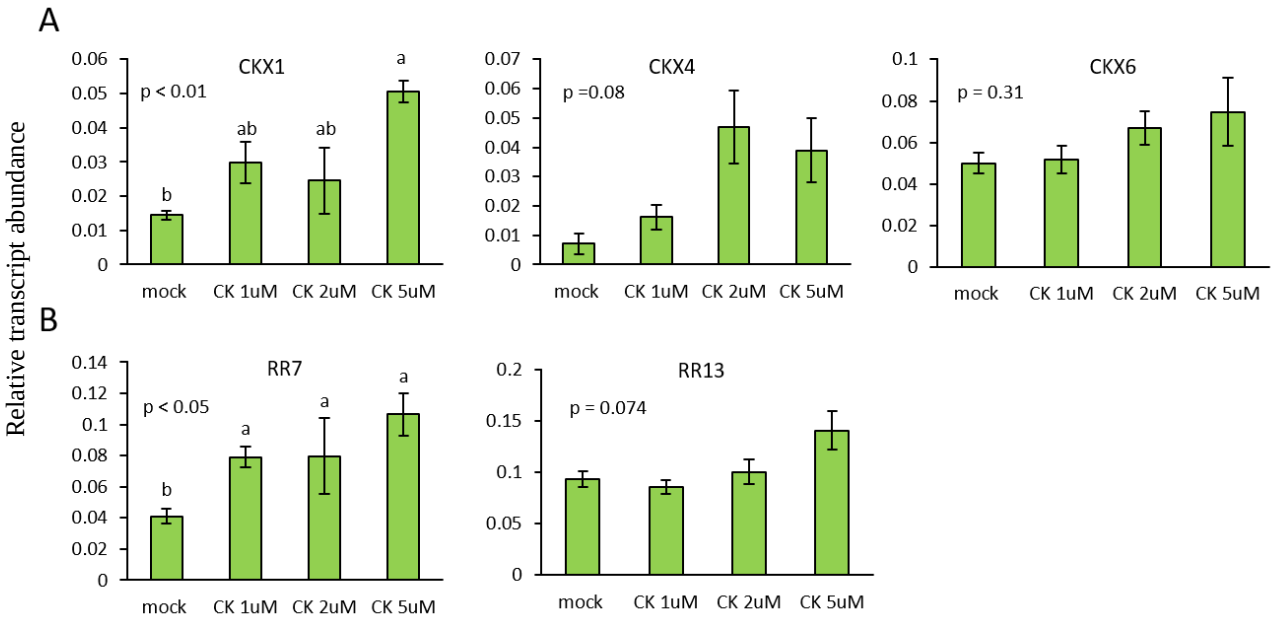

Figure 11: Transcript abundance of *CKX* and *RR* genes in *P. canescens* saplings treated with CK A) CK oxidase/dehydrogenases (*CKX*) degradation genes, B) response regulator (*RR*) genes. Transcript levels of each gene were normalized to the expression of the housekeeping gene *actin*. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Data were mean ± SE (n=4). Different letters mean groups are statistically different at 95% confidence.

3.8. Pre-treatment with CK decreased rust resistance in poplar

To quantify the effect of CK (tZ) pre-treatment on rust infection in black poplar leaves, both treated and un-treated saplings were inoculated with rust fungus 1d after spraying. Samples were harvested at 3 dpi, ground in liquid N_2, RNA extracted and rust colonization measured using qRT-PCR. We measured transcript abundance of the rust *Actin* gene and normalized it to poplar *ubiquitin* (*UBQ*) expression. The colonization of rust fungus in CK pre-treated trees was significantly higher in comparison with the mock-treated trees at 3 dpi (Figure 12). This result suggests that CK treatment enhanced tree susceptibility to rust.

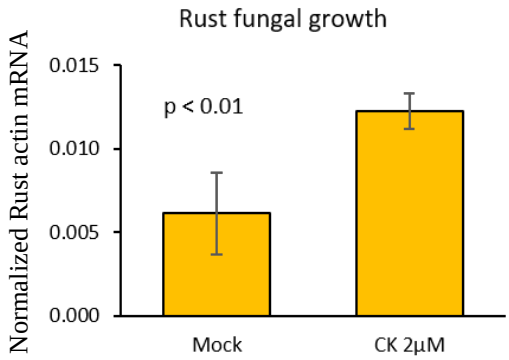

Figure 12: Relative rust colonization in poplar leaves after CK treatment. The mRNA levels of the rust (*M. larici populina*) *Actin* gene measured in samples harvested at 3 dpi were normalized to poplar *UBQ* expression. Data were analyzed using a Student's t-test. Data are presented as the mean ± SE (n=5, two technical replicates per sample).

3.9. Effect of exogenous CK on the accumulation of phytohormones and flavonoids in rust-infected black poplar (*P. nigra*) leaves

In order to determine the effect of rust infection on flavonoids and phytohormones after pretreatment with CK, I measured flavonoids such as naringenin, quercetin and flavan-3-ols from the same samples used for measuring rust infection. The amounts of epicatechin, naringenin and quercetin accumulated in slightly lower amounts than in mock-treated leaves. However, other flavan-3-ols such as catechins, gallocatechin and PAs increased slightly in CK-treated leaves when compared to mock controls (Figure 13A). SA and ABA levels show a decreased trend in CK-treated poplar leaves relative to mock-treated leaves, whereas JAG and OH-JA-Ile levels did not change (Figure 13B).

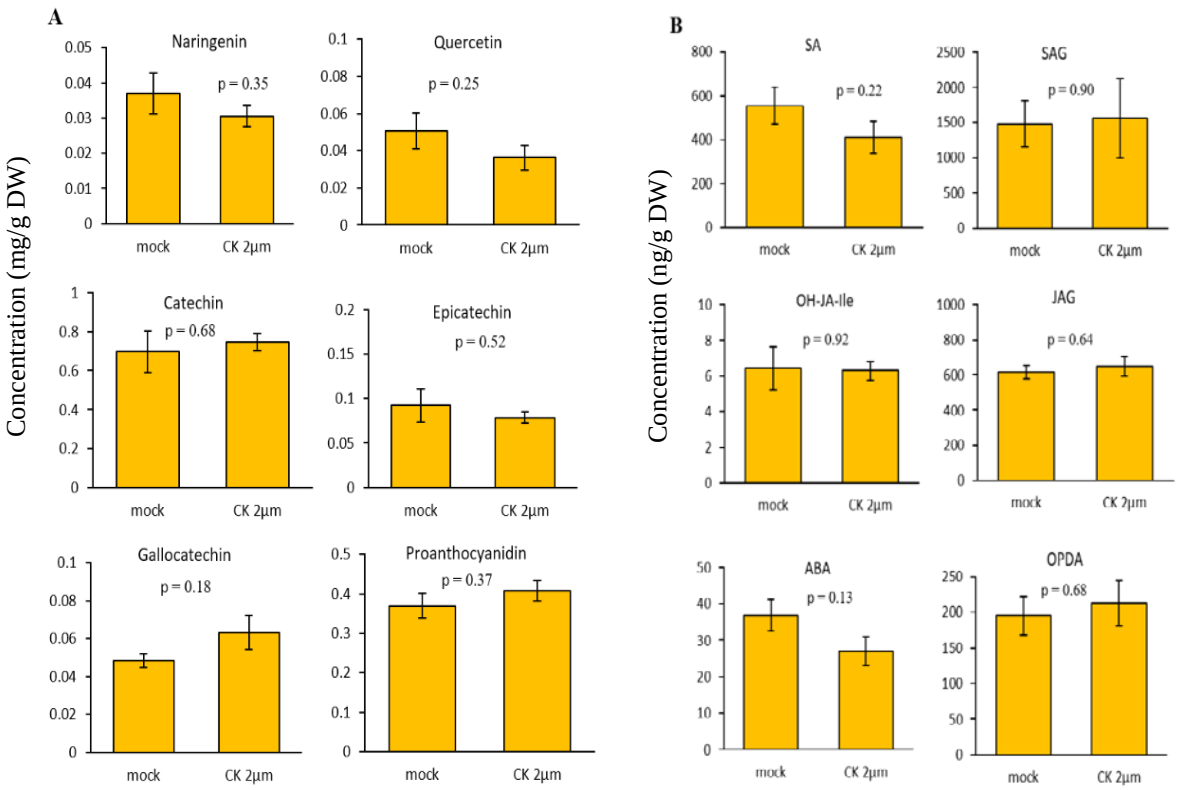

Figure 13: Effect of exogenous CKs on various constituents of rust infected black poplar (*P. nigra*) leaves at 3 dpi. The concentrations of A) flavan-3-ols and other flavonoids B) phytohormones were measured. Data were analyzed using a Student's t-test. Data are presented as the mean ± SE (=5).

3.10 Effect of exogenous CK on transcripts of flavan-3-ol biosynthetic genes in rust-infected black poplar (*P. nigra*) leaves

We also measured the relative transcript abundance of flavan-3-ol biosynthetic genes, two *PnLAR* genes and one *PnANR*, after the same experimental treatment (Figure 14) by using qRT-PCR. The gene expressions of both *LAR* and ANR genes were reduced slightly in CK-treated trees in comparison to mock controls but were not significantly different. The results shown in Figure 13 and14 appear inconsistent. One explanation could be the unusual growth phenotypes of these plants in the greenhouse. It should be noted that plants stopped growing and were exposed to a cold treatment, which induced flashing new leaves. Therefore, it is recommended to repeat this experiment with freshly grown plants.

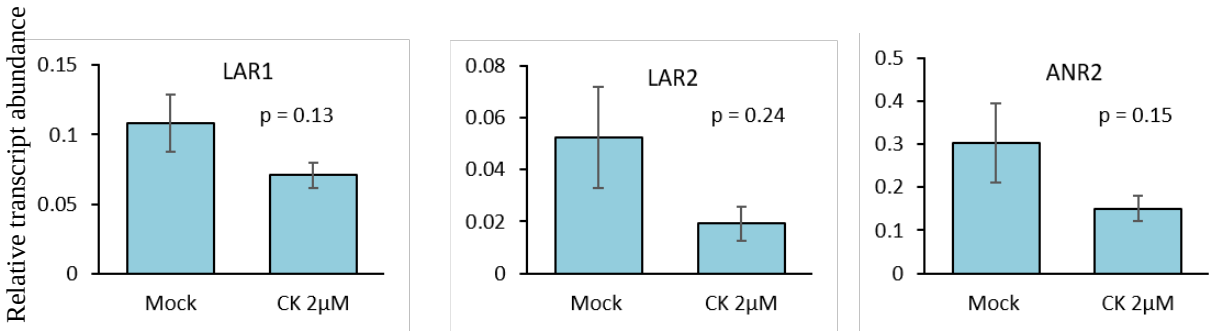

Figure 14: Effect of exogenous CK on transcript levels of flavan-3-ol biosynthetic genes in rust-infected black poplar at 3 dpi. Transcript levels of two leucoanthocyanidin reductase (*LAR*) and one anthocyanidin reductase (*ANR*) gene were measured and normalized to plant ubiquitin expression. Data were analyzed using an independent Student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; not significant, $p > 0.05$). Data are presented as the mean ± SE (=5).

4. DISCUSSION

Cytokinins **(**CKs) are important plant hormones that play a role in defense against pathogens in herbaceous plant species through interacting with the signaling pathways of other hormones (Choi *et al.,* 2010; Großkinsky *et al.,* 2011). However, the role of CKs in tree defense remains unknown so far. To gain an insight into the function of CKs in poplar-rust interaction, we examined the CK contents and transcripts of both CK biosynthesis and signaling genes in black poplar leaves over a course of rust (*M. larici populina*) infection. Then the effects of exogenous CKs on poplar resistance to rust infection and the accumulation of flavonoids and its defense hormones such as SA and jasmonates were investigated. We found that CK treatment decreased resistance to rust infection. While the contents of active CK increased immediately after inoculation, they declined strongly by 7 days post-inoculation. However, the levels of topolin, an aromatic CK, induced throughout the infection period. Most of the genes involved in CK biosynthesis and degradation were also downregulated by rust throughout the experimental period. Previous studies in our group showed a strong induction of SA signaling and in rust-infected poplar leaves compared to controls (Ullah *et al.,* 2017; Ullah *et al.,* 2019b). The downregulation of CK signaling in rust-infected leaves demonstrated in this study suggests a negative association between CKs and flavan-3-ols, which might lead to increased resistance since flavan-3-ols have shown to act in defense against rust. This hypothesis was verified by the exogenous application of an active CK (tZ) to poplar trees, which led to a reduction in flavan-3-ol accumulation and decreased resistance to rust.

4.1 Rust infection modulates the CK signaling pathway in black poplar in a complex time-dependent manner leading to decreased signaling, especially during the later stages of infection

CKs are ubiquitous plant hormones that play a critical role in plant growth and development as well as in defensive responses against biotic stresses (Sakakibara 2006). *Melampsora* species are the most devastating and widespread biotrophic pathogens of poplar trees causing decreased biomass production worldwide (Gerard *et al.,* 2006; Newcombe *et al.,* 2001). Our analysis shows that rust infection modulates the level of CK contents in black poplar leaves in a complex time-dependent manner. We found that the active CKs such as tZ and tZR increased 6 hours post-inoculation and decreased at later stages of infection in rust-infected black poplar trees compared to the controls. On the other hand, other active CKs (IP, IPR),

inactive CKs (tZROG, cZROG, tZG) and an aromatic CK (oT) accumulated in rust-infected trees over the course of infection. Similar results have been shown in the accumulation of CKs in *Plectosphaerella populi* infected poplar stems (Ullah *et al.,* 2019b). In particular, this finding is also similar to the effect of powdery mildew infection in wheat, where there is an increase in CK contents during the sporulation phase, which eventually declines due to the hypersensitive reaction (Serezhkina *et al.,* 2004). Likewise, CK genes were transcriptionally activated in *Nicotiana attenuata* leaves f after wounding and herbivore attack (Schafer *et al.,* 2015).

To look for additional evidence supporting the alteration of CK signaling during poplar-rust interactions, the expression of CK biosynthetic genes was investigated. A major step in the CK biosynthetic pathway is catalyzed by IPTs (Kakimoto 2003; Kakimoto 2001). In *Populus canadensis cv. Robusta,* nine genes encoding IPT were identified recently (Jaworek *et al.,* 2019). In this study, I quantified transcript levels of the *IPT3* gene and found that it was upregulated in rust-infected black poplar leaves compared to controls, but down-regulated compared to uninfected controls at 3 and 7 days post-inoculation. The other tested *IPT* genes were significantly downregulated by rust infection. Previous transcript data on uninfected trees indicated that the *IPT3* gene is highly expressed in mature leaves of poplar (Jaworek *et al.,* 2019). In this study, transcripts of the *IPT3* gene increased in uninfected trees as time progressed but not in rust-inoculated trees suggesting that fungal infection down-regulated CK biosynthesis.

CK signaling may be controlled not only by CK biosynthesis, but also by the rate of CK degradation Thus I investigated the *CKX* genes, whose encoded proteins catalyze the degradation of CKs (Sakakibara 2006; Mcgaw 1983). In the *Populus trichocarpa* genome, eight *CKX* genes have been identified (Immanen *et al.,* 2013). Interestingly, rust infection increased the transcript levels of the *CKX*6 gene, but downregulated expression during the later stages of infection. Also, the expression of the *CKX*1 gene was downregulated throughout the experimental period. Thus, these data indicate that in balance both CK biosynthesis and degradation decline during the later stages of colonization of black poplar leaves by rust fungus. These results are similar to the model suggested for reduction of CK levels in *Arabidopsis thaliana* where the loss of function of *IPT* genes lowered the expression of *CKX* genes (Nishiyama *et al.,* 2011).

To explore the effect of fungal infection on the signaling genes involved in the CK pathway, I measured the expression levels of the *RR* genes, known as feedback regulators that control CK signaling output (Hwang *et al.,* 2012; Muller *et al.,* 2007; To and Kieber 2008). It is interesting that the transcript levels of *RR*7 and *RR*13 were also downregulated by rust infection. A similar pattern of downregulated response regulator action is observed in the literature (Nishiyama *et al.,* 2011) where reduced CK levels suppress the CK signaling pathway. However, the exact mechanism and type of RR involved in our study is still unknown. Together, this study suggests that rust infection down-regulates the CK signaling pathway in poplar based on evidence from biosynthesis, metabolism and signaling.

4.2 Exogenous CKs negatively regulate flavan-3-ol biosynthesis and the SA signaling pathway in *P. canescens*

Flavan-3-ols have been reported to function as effective antifungal compounds in poplar against rust infection (Ullah *et al.,* 2017). Previous work showed that flavan-3-ols including the monomeric catechins and PA oligomers accumulated remarkably in rust-infected leaves compared to corresponding control leaves. Previous experimental data from our group also demonstrated that exogenous CK could suppress the accumulation of flavan-3-ols in *Plectosphaerella populi* infected poplar stems (Ullah *et al.,* 2019b). Here, I investigated the effect of exogenous CK on the accumulation of flavan-3-ols in poplar leaves (*P. canescens*). Our results revealed that low doses of exogenous CK decreased the levels of flavonoids including catechin and PA dimers. I also observed decreased accumulation of SA in poplar (*P. canescens*) trees treated with low doses of CK whereas JA content did not change. Similar patterns were observed in *Plectosphaerella populi* infected poplar stems (Ullah *et al.,* 2019b).

Only a few studies have investigated the interaction of CK and other hormones. For example, CK modulates JA signaling which functions in herbivore-induced defense signaling in *Nicotiana attenuata* (Schafer *et al.,* 2015a; Schafer *et al.,* 2015b). Our work provides some evidence that CKs could negatively regulate the SA signaling pathway. This negative hormonal cross-talk may fine tune the effect of cytokinin on plant immunity. SA is known to play a positive role in regulating flavan-3-ol accumulation in rust infected poplar trees (Ullah *et al.,* 2019b). In contrast with our results, Argueso *et al.* (2012) reported that SA negatively regulates CK signaling in a negative feedback mechanism in Arabidopsis. The effect of CK was dose-dependent, with higher CK concentrations leading to increased defense response against the pathogen whereas low or moderate levels of CK allowed the biotrophic pathogen

to survive (Argueso *et al.,* 2012). However, this generalization does not appear to hold for woody tree species.

The decreased accumulation of flavan-3-ols on CK application provoked us to investigate the transcript abundance of flavan-3-ol biosynthetic genes. The *LAR* and *ANR* genes are involved in the last steps of the flavonoid pathway (Ullah *et al.,* 2017). In our work, both *LAR* and *ANR* genes were partially downregulated at a low dose of CK when compared to control which suggests that flavonoid accumulation reduced their transcriptional levels. Although flavan-3-ol contents decreased quite strongly after CK treatment, we did not find strong down regulation of their biosynthetic genes. One reason could be due to the fact that the down-regulation of biosynthesis happened much earlier. Alternatively, it could also possible that low-molecular-weight flavan-3-ols (catechins and PA dimers) were converted to PA polymers after CK treatment. We conclude that exogenous CKs downregulated the accumulation of flavan-3-ols, which was accompanied by lower levels of SA. Further study is necessary to confirm whether the CK-mediated suppression of flavan-3-ol accumulation is dependent on the down-regulation of SA signaling or not.

CKs play a vital role in regulating cambial development (Nieminen *et al.,* 2008), leaf phyllotaxy (Besnard *et al.,* 2014) and serving as a priming agent in response to biotic stress. For example, CK primed plant defense response against insect herbivores in *Populus* after wounding (Dervinins *et al.,* 2010; Sano *et al.,* 1996). Additionally, CKs are also known to play a role in abiotic stress responses (Nishiyama *et al.,* 2011). Several pieces of evidence suggest that CKs play a role in plant-pathogen interactions. For example, in Arabidopsis, plant-derived CKs have been shown to enhance defense response against the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 Pst via induction of the SA signaling pathway. Interestingly, CK-mediated induction of phytoalexins in tobacco plays an important role in reducing pathogen (*Pseudomonas syringae*) infection independent on SA signaling. The exogenous application of CK leads to changes in susceptibility to pathogens. For example, in *Phaseolus vulgaris*, exogenous application of CK delayed the disease symptoms after infection by white clover mosaic virus (Gális *et al.,* 2004). Conversely, CKs are associated with the development of club root disease symptoms caused by the biotrophic protist *Plasmodiophora brassicae* (Siemens *et al.,* 2006). The study by Pertry *et al.* (2009) revealed that the biotrophic actinomycete *Rhodococcus fascians,* the causal agent for leafy gall disease, secretes CKs, which act through histidine kinases CK receptors in Arabidopsis (AHK2 and AHK3), leading to the rapid development of disease symptoms. All the above

studies suggest that CKs may either have cause an enhanced or reduced resistance towards pathogens and this response may be dependent or independent of the SA signaling pathway.

4.3 Role of CKs in rust infection

In this study, I also investigated the role of exogenous CK in black poplar (*P. nigra*) defense against rust infection. I could show that exogenous CK treatment on poplar leaves resulted in higher rust colonization at 3 days post-inoculation when compared to the trees that were not pre-treated with CKs. Infection on poplar leaves starts with the establishment of infection structures called hyphae and haustoria followed by extensive proliferation of the fungus at 3dpi (Duplessis *et al.*, 2011; Hacquard *et al.*, 2011). Elevated levels of CK content are associated with increased pathogen growth. To verify if the reduced rust colonization in CK-treated trees also correlated with the lower accumulation of flavonoids, we compared flavonoid accumulation in both CK treated and non-treated plants after rust infection. We found that the accumulation of epicatechin and other flavonoids like naringenin and quercetin decreased in rust-infected plants that were pre-treated with CKs. However, other flavan-3-ols such as catechins and PAs failed to show this pattern. Transcript levels of *LAR and ANR2* decreased slightly, which implies that flavan-3-ol biosynthesis was partially down-regulated. These observations imply that exogenous CKs decreased the accumulation of flavonoids, resulting in an enhanced biotrophic fungal growth. Certain plant microbes like *M. oryzae* produce their own CKs, which support fungal growth by increasing plant susceptibility (Chanclud *et al.*, 2016). Various plant pathogens like *A. tumefasciens*, *Pseudomonas savastoni, Pseudomonas solanacearum* and gall-forming pathogens produce CKs or induce CK biosynthesis (Akiyosh *et al.*, 1987, Ashby 2000; Choi *et al.*, 2011). Various fungal and bacterial pathogens produce CKs and an increase in CK content might be associated with the formation of “green islands”, green leaf tissue supporting an area of pathogen growth (Kiraly *et al.*, 1967, Ashby 2000). In the model plant *Arabidopsis*, increased susceptibility to pathogens may also be due to the source-sink regulation, delay of senescence and nutrient acquisition (Argueso *et al.*, 2009). Whether the poplar rust fungus *M. larici-populina* also produces CKs during plant colonization is not known.

In conclusion, upon rust infection, the CK signaling pathway is downregulated in poplar as shown by hormone measurement and transcriptome analysis of the genes involved in CK biosynthesis, metabolism and signaling output. Exogenous treatment with CK (tZ) activated both *CKX* and *RR* genes that are responsible for CK metabolism and signaling, respectively.

The activation of CK signaling leads to a reduced accumulation of flavonoids including flavan-3-ols, which are considered to be chemical defenses against rust infection. The decline in flavan-3-ols is accompanied by a decrease in SA levels in black poplar trees. Therefore, CKs might negatively affect flavan-3-ol accumulation in poplar via their effects on SA signaling. Further work is required to confirm the function of CKs in poplar during poplar-rust interactions. Poplar trees could be genetically modified for enhanced CK biosynthetic capacity by expressing an *IPT* gene, or modified for enhanced CK catabolism by expressing a *CKX* gene. Following stable transformation and regeneration, the engineered poplar trees could be analyzed to check the accumulation of flavonoids and other defense hormones. Finally, how these altered CK-producing trees respond to rust infection could also be tested.

5. SUMMARY

Cytokinins (CKs) are adenine derivative hormones that play a major role in the regulation of plant growth and development as well as defense responses against biotic stresses. CKs affect plant disease resistance in annual plants but their role in perennial tree defense has remained unknown. Poplars (*Populus* spp.) are extremely fast-growing forest tree species widely distributed and known for their great ecological and economic importance. The most devastating and widespread biotrophic pathogens of poplar trees are the rust fungi (*Melampsora* spp.), which can cause decreased biomass production worldwide. Here, I investigated the effect of CKs during poplar-rust interaction. Using analytical and molecular biology tools, I investigated CK accumulation, metabolism, signaling, their potential role in the regulation of flavonoids and possible interactions with other hormones. Black poplar *(P. nigra)* trees that were infected with foliar rust fungus *(M. larici populina)* modulated CK levels over the course of infection. In particular, the active CKs increased quickly after rust inoculation but showed decreased accumulation relative to uninfected controls during the plant colonization phase at 3 dpi, the sporulation phase at 7 dpi, and afterwards in the course of infection (14 dpi). Transcripts of isopentenyltransferases (*IPT*) genes involved in catalyzing the first step reaction in the conversion of adenosine phosphates to CK nucleotides were slightly upregulated initially and then decreased over the course of infection. This study also revealed that the transcripts of CK oxidase/dehydrogenase (*CKX*), an irreversible degrading enzyme as well as the response regulators (*RR*), feedback regulators of CK signaling, were also downregulated upon rust infection. To manipulate the CK pathway, I sprayed poplar leaves with *trans*-zeatin (tZ), an active CK, and measured rust infection, accumulation of flavan-3-ols and other defense hormones. Exogenous CK treatment enhanced poplar susceptibility to rust, decreased the accumulation of flavan-3-ols, and suppressed levels of the phytohormone SA. Here, we found that CKX and RR transcripts were upregulated on exogenous spraying of t*Z*, correlated with higher accumulation of tZ-derived forms of CKs in poplar. Thus, it appears that CKs act to decrease plant resistance to the rust fungus by serving as a negative regulator of flavan-3-ol biosynthesis in poplar, which could be a result of decreased SA signaling. However, this study does not rule out the possibility of SA-independent inhibition of flavan-3-ol accumulation.

6. ZUSAMMENFASSSUNG

Cytokinine (CKs) sind Hormone, die Derivate von Adenin darstellen und eine wichtige Rolle bei der Regulierung von Pflanzenwachstum und -entwicklung sowie bei der Abwehr von biotischem Stress spielen. CKs beeinflussen die Resistenz von einjährigen Pflanzen gegen Pflanzenkrankheiten, aber ihre Rolle bei der Abwehr mehrjähriger Bäume ist bisher unbekannt. Pappeln (*Populus* spp.) sind extrem schnell wachsende Waldbaumarten, die weit verbreitet und für ihre große ökologische und wirtschaftliche Bedeutung bekannt sind. Die verheerendsten und am weitesten verbreiteten biotrophen Pathogene der Pappel sind die Rostpilze (*Melampsora* spp.), die weltweit eine verminderte Biomasseproduktion verursachen können. Hier habe ich die Wirkung von CKs bei der Pappel-Rost-Interaktion untersucht. Mit Hilfe analytischer und molekularbiologischer Methoden untersuchte ich die Akkumulation von CKs, ihren Metabolismus, ihre Signalwirkung, ihre mögliche Rolle bei der Regulierung von Flavonoiden und mögliche Wechselwirkungen mit anderen Hormonen. Exemplare der Schwarzpappel (*P. nigra*), die mit dem Blattrostpilz (*M. larici populina*) infiziert waren, modulierten die CK-Spiegel im Verlauf der Infektion. Insbesondere die aktiven CKs stiegen nach der Rostinokulation schnell an, zeigten jedoch eine geringere Akkumulation im Vergleich zu nicht infizierten Kontrollen während der Besiedlungsphase der Pflanze mit 3 dpi, der Sporenphase mit 7 dpi und danach im Verlauf der Infektion (14 dpi). Die Transkripte der Isopentenyltransferase-Gene (*IPT*), die an der Katalyse der ersten Reaktionsschritte bei der Umwandlung von Adenosinphosphaten in CK-Nukleotide beteiligt sind, waren anfangs leicht hochreguliert und nahmen dann im Verlauf der Infektion ab. Diese Untersuchung zeigte auch, dass die Transkripte von CK-Oxidase/Dehydrogenase (*CKX*), einem irreversibel abbauenden Enzym, sowie die Response-Regulatoren (*RR*), Rückkopplungsregulatoren der CK-Signalisierung, bei einer Rostinfektion ebenfalls herunterreguliert wurden. Um den CK-Signalweg zu manipulieren, besprühte ich Pappelblätter mit *trans*-Zeatin (tZ), einem aktiven CK, und maß die Rostinfektion, die Anhäufung von Flavan-3-olen und anderen Abwehrhormonen. Die exogene CK-Behandlung erhöhte die Anfälligkeit der Pappel für Rost, verringerte die Anhäufung von Flavan-3-olen und supprimierte die Werte des Phytohormons SA. Hier fanden wir heraus, dass die CKX- und RR-Transkripte beim exogenen Sprühen von tZ hochreguliert wurden, was mit einer höheren Akkumulation von tZ-abgeleiteten Formen von CKs in der Pappel korrelierte. Es scheint also, dass CKs die Pflanzenresistenz gegen den Rostpilz verringern, indem sie als

negativer Regulator der Flavan-3-ol-Biosynthese in der Pappel dienen, was ein Ergebnis der verminderten SA-Signaltransduktion sein könnte. Diese Studie schließt jedoch die Möglichkeit einer SA-unabhängigen Hemmung der Flavan-3-ol-Akkumulation nicht aus.

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INDEPENDENT DECLARATION

I hereby declare that I have completed this work independently. This thesis has not been submitted for other examination purposes and has not been published elsewhere. All knowingly used text, ideas, techniques, quotations or content from other authors have been expressly marked as such in accordance with standard referencing practices.

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