Biologie

Dissertationsthema

Studies on the biological activity of chitosans and their mode of action in plant protection

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Don’t criticize what you can’t understand.

Bob Dylan

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Abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
ANOVA	Analysis of variance
cDNA	Complementary DNA
CEBiP	Chitin elicitor binding protein
CERK	Chitin elicitor receptor kinase
CM	Complete medium
CMM	Carboxymethyl cellulose medium
COS	Chitin oligomers
CRISPR	Clustered regularly interspaced short palindromic repeats
Ct	Cycle threshold
CuAc	Copper acetate
D_A	Degree of acetylation
DD_A	Degree of deacetylation
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DPn	Average DP
E	Amplification efficiency
e.g.	*exempli gratia* (for example)
ETI	Effector-triggered immunity
EU	European Union
F_A	Fraction of acetylation
FAO	UN Food and Agricultural Organization
FAOSTAT	FAO statistic data
GlcN	D-glucosamine
GlcNAc	N-acetyl-D-glucosamine
GM	Genetically modified
GO	Gene Ontology
HILIC-ESI-MS	Hydrophilic-interaction liquid chromatography-electrospray ionization mass-spectrometry

HMWC	High molecular weight chitosan
HR	Hypersensitive response
i.a.	*inter alia* (among others)
i.e.	*id est* (this is)
ISR	Induced systemic resistance
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LMWC	Low molecular weight chitosan
LRR	Leucine-rich repeat
LYK	Lysine motif receptor kinase
LYP	Lysine motif containing protein
LysM	Lysine modif
MAPK	Mitogen activiated protein kinase
MIC	Minimum inhibitory concentration
MMWC	Medium molecular weight chitosan
Mn	Number-average molecular weight
mRNA	Messenger RNA
Mw	Weight-average molecular weight
NOX	NADPH-oxidase
NTC	Non-target control
OD	Optical density
OECD	Organization for Economic Co-operation and Development
paCOS	Partially acetylated chitin oligomers
PAL	Phenylalanine ammonia-lyase
PAMP/MAMP	Pathogen or microbe associated molecular pattern
PCA	Principal component analysis
PCR	Polymerase chain reaction
PesCDA	*Pestalotiopsis* sp. chitin deacetylase
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PS	Photosystem

PTI	PAMP-triggered immunity
REST-MCS	Relative expression software tool – multiple condition solver
RK	Receptor kinase
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLU	Relative light units
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SF	Synergy factor
TAL	Tyrosine ammonia-lyase
UN	United Nations

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Abstract

As sessile organisms, plants are constantly exposed to countless stresses. In the course of their evolutionary development, plants have therefore developed elaborate defense mechanisms to defend themselves against all sorts of enemies ranging from tiny unicellular microbes to herbivore mammals. Plant resistance extends from preformed barriers through induced defense reactions to programmed cell death, controlled through various pathogen perception methods and intracellular signaling cascades. Especially microbial pathogens continuously manage to overcome plant resistance factors and cause plant diseases, which latest becomes a global problem if crop plants are affected. Crop protection and pest control have been used for centuries to safeguard crops from devastation and hence secure feeding of the world population. However, the available arable land might not be able to cover the demands of a rapidly increasing world population without considering drastic changes to food distribution, land use and the dimensions of ecological footprints. Due to an overall rising awareness of climate change and general imbalance of food, prosperity and health, a transition to less harmful, environmentally friendly plant protection seems one way to transform conventional agriculture to more sustainability. Chitosan is the deacetylated derivative of chitin, which is occurring naturally as structure molecule in crustaceans, insects and fungi. Displaying a range of antimicrobial and plant-strengthening activities, it is already widely used as fertilizer, fungicide and general plant protection product. Furthermore, being a natural polymer, chitosan is stated as non-toxic, sustainable and biodegradable, which provides many arguments for its usage in agriculture. However, as chitosan is characterized by different parameters, *i.e.* the fraction of acetylation (F_A), the degree of polymerization (DP) and the pattern of acetylation (P_A), a lot of effort still has to be investigated to find the most suitable chitosan for each desired application. In the present work, the antifungal activity of chitosan against the wheat pathogen *Fusarium graminearum* and the resistance-inducing activity of chitosan towards the most important non-grain crop plant *Solanum tuberosum* (potato) was investigated, using a broad range of chitosans differing mainly in their F_A and average DP. It could be shown that low F_A chitosans with small to intermediate DP are best for antifungal activity, whereas intermediate F_A chitosans with intermediate to large DP are most appropriate to elicit plant responses. Chitosan combinations were particularly effective in both cases, suggesting different modes of action that depend on the characteristics of each chitosan. It was finally proposed that combinations of different chitosans have large potential to supersede or at least compete conventional plant protection products due to their high biological activity, accompanied with a high environmental compatibility, and hence contributing to a more sustainable crop protection.

Chapter I

Introduction

1. Introduction

1.1. The global challenge to feed the world

One of the most important, yet not less challenging global tasks, is the feeding of a steadily increasing world population. According to a recent projection by the United Nations (UN), the world's population is expected to rise from 7.6 billion in 2017 to 9.8 until 2050 and eventually reaching 11.2 billion in 2100 (Roser, 2017). Simultaneously, the UN Food and Agricultural Organization (FAO) proposed that the arable land area might further increase, though with a very low rate and eventually might reach its peak in 2050 (FAO, 2012). As people rely on agricultural nutrition, both mentioned projections (Figure 1) inevitably lead to the challenge to optimize the usage of the existing farmland to maintain the food supply for humankind, as the current output of agriculture will quite likely be insufficient to nourish this increasing amount of people.

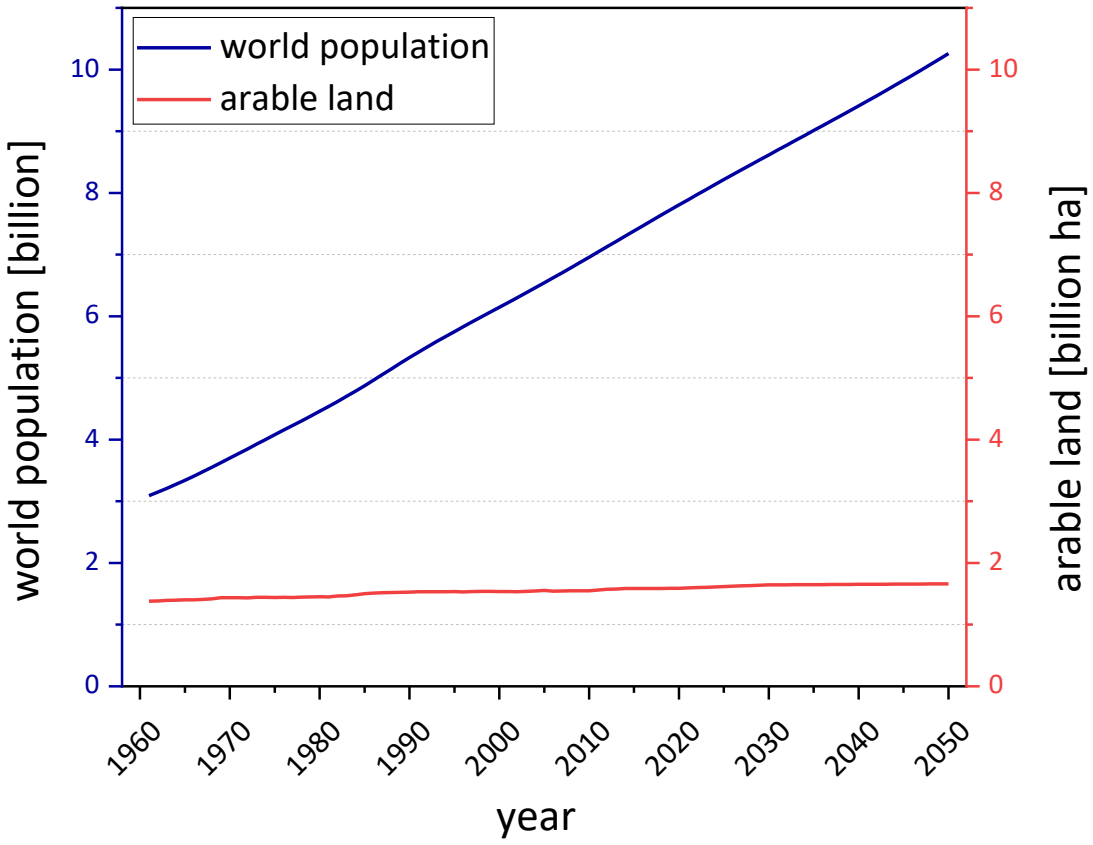

Figure 1: UN projection of the world population growth and FAO projection of the change of arable land size from 1960 to 2050. While according to the UN projection the world population (blue curve) undergoes a drastic increase up to 11 billion of people in 2060, FAO states that the size of arable land (red curve) will almost stay constant between 1.5 and 2 billion hectares.

There are different options imaginable on how to improve utilization of the available farmland. One possibility is to overthink the current usage and reallocate the output and distribution of crop production. By examining the FAO statistic data (FAOSTAT), Cassidy *et al*. concluded that only two thirds (67 %) of globally grown crops are used for direct human consumption while the rest is used for either animal feeding (24 %) and other uses like biofuel production (9 %) (Cassidy et al., 2013). According to FAO, the latter is expected to consume 12 % of coarse grains and vegetable oil as well as 22 % of sugarcane for ethanol production in 2025 (OECD/FAO, 2018). A recent study from the German Biomass Research Center in cooperation with the University of Hamburg suggests to encounter the rising demands of biofuels by halving the meat intake from 200 to 100 g per day and capita, which would allow biofuel production to be increased 7.7 fold (Zech & Schneider, 2019). However, in the long term, biofuel production is required to be shifted completely to non-food sources to end its clash with food security (Abideen et al., 2014). Animal feeding is equally still hugely based on crop plants which hence are unavailable as direct food source for humans (West et al., 2014). Grain-feeding of cattle was mainly introduced in the 1950s because of its ability to improve meat marbling by increasing the intramuscular fat, but changing consumer demands resulted in a decline of grain-fed animals and many producers switched back to grass-feeding (Daley et al., 2010). It is suspected that replacing 50 % of grain-fed to grass-fed animal products could feed additional 2 billion people as this change would make more cereal crops available for food instead for animal feed (Cassidy et al., 2013). On the other hand, grass-fed beef requires a higher land use due to the lower nutrient density and digestibility of grass in comparison to grains (M. Clark & Tilman, 2017). Concerning the usage of crops as animal feed, the amount of land required for meat production tends to be higher than for other food products, especially for beef production which can easily require up to 100 times more land in comparison to cereal crops to produce the same amount of proteins (M. Clark & Tilman, 2017). Thus, there might be no alternative to a general reduction of meat consumption or at least to shifting it from beef to pork and poultry as both latter ones have a much better protein yield to land use ratio (M. Clark & Tilman, 2017). It is out of question that reduction or even renunciation of meat consumption could solve many problems, not only in terms of food security but also animal rights. However, meat consumption was always linked to prosperity and is believed to double by 2050 because of economic growth, especially in developing countries (Bereżnicka & Pawlonka, 2018). Nevertheless, rising knowledge and perception of sustainability, health and livestock farming as well as increasingly represented meat substitutes are believed to have a positive influence on food selection in the future (Happer & Wellesley, 2019; Siegrist & Hartmann, 2019). Apart from rethinking the

allocation of crop production or considering harsh cuts like giving up or substitute meat consumption, optimization of crop production itself is one important and promising option to encounter the rising demand of food and will be covered in a separate chapter (1.3).

1.2. Potato – "a dainty dish even for Spaniards"

By looking at the most important and most grown crops, one cannot avoid noticing that by far most of the agricultural land is used for cereal production. As cereals provide rich sources of proteins, minerals, carbohydrates, fats, oils and vitamins (Sarwar, 2013), it is not surprising that cereals contribute significantly to the nutrition of the world's population. According to FAO, the agricultural land used for cereal production exceeds 700 million hectares while all other crop types take less than 100 million hectares of global land. However, with its high yield and nutritive composition including vitamins, antioxidants and high amounts of starch (Burlingame et al., 2009), the potato (*Solanum tuberosum* L.) does not need to fear comparison. Especially in developing countries, the potato is a crucial element in food security (Scott & Suarez, 2012) and after the top three cereals wheat, rice and maize, potato is the worldwide most important non-grain crop (Zaheer & Akhtar, 2016) with a global production of over 388 million tons in 2018. Its value ultimately becomes clear by looking at FAO's crop yield statistic data in which the potato yield almost reached 50 tons in Western Europe in 2014 while other crops including cereals barely reach 10 tons.

The triumphal march of the potato began as early as in the 15th century. Discovered during a Spanish expedition through Columbia, the conquistador Juan de Castellanos wrote on July 31, 1537 about "truffle" plants with "scanty flowers of a dull purple color and floury roots of good flavor, a gift very acceptable to Indians and a dainty dish even for Spaniards" (Salaman, 2010). In the following decades and centuries, the potato was distributed around Europe. While first being neither accepted as food nor being sufficiently profitable, the potato became an important staple crop in Western Europe in the 17th and 18th century, a process which was sustained heavily by the so called "potato order" by the Prussian king Frederick II, declared on March 24, 1756 (Göse, 2012). The potato's modesty in terms of climate and space was though a double-edged sword. Its already mentioned nutritive composition, packed in high yields, often led to potato dependencies in Western European countries. This monocultural cultivation of few potato varieties that lack genetic diversity consequently facilitated the spread of diseases. The oomycete *Phytophthora infestans* blazed its trail across the Atlantic Ocean in the 1840s and caused over one million deaths in Ireland due to starvation and vitamin C deficiency (Crawford, 1988). This "Great Famine" had great impact on the Irish demography, but also

other countries, as many Irish people left the country to escape this disaster (Fotheringham et al., 2013). Even more important, the famine led to a rethinking of potato cultivation, resulting in the growth of other, more resistant cultivars and - with plant science becoming more developed - identifying novel resistant varieties used for crossing and breeding (Turnin, 2011). Modern genetic engineering approaches include the development of transgenic potato varieties like Fortuna by BASF, designed for reducing fungicide treatments due to the implementation of two wild potato resistance genes (E. van der Vossen et al., 2003; E. A. G. Vossen et al., 2005), but also cisgenic potato varieties (Haverkort et al., 2016). However, genetic engineering often lacks both public acceptance and legal basis (see 1.3), hence crop protection of potato is still highly dependent on the usage of pesticides. In organic farming, copper-based fungicides are used to control late blight (Bangemann et al., 2014) in amounts that can cause environmental harms (Flemming & Trevors, 1989). Nevertheless, although scientists and economists appeal to loosen the restrictions and overcome fears to open up for GM approaches in European agriculture (Dixelius et al., 2012; Gheysen & Custers, 2017), potato agriculture will remain based and dependent on traditional breeding methods for now.

About 5000 potato varieties are believed to exist, however more than half of all varieties only occur at their origin in the Andean mountain region of South America (Zaheer & Akhtar, 2016). Controversial theories describe the origin of today's cultivated potato as well as its initial distribution (Machida-Hirano, 2015). Potato species generally show 12 chromosomes, but form a polyploidy series of diploid (2n = 24), triploid (3n = 36), tetraploid (4n = 48), pentaploid (5n = 60) and hexaploid (6n = 72) variants (Celio et al., 2013). It is believed that originally diploid variants underwent chromosome doubling to achieve tetraploidy, which is the most common polyploid state of commercial potato cultivars today (Iwanaga & Peloquin, 1982). On one hand, the potato can display enhanced biomass and vigor due to its polyploid nature, on the other hand, it suffers inbreeding depression due to selfing of genetically identical individuals (Muthoni et al., 2015). This contrast is a challenge for modern breeding, since heterozygosity both increases the yield and impedes the selection of desirable characteristics after crossing at the same time (Muthoni et al., 2015). The usage of diploid variants can facilitate conventional breeding and genetic studies. With this, the retracing heredity after successful breeding can be overcome (Watanabe, 2015), which eventually culminated in using the homozygous, double-monoploid potato variant DM1-3 516 R44 (DM) to sequence the complete potato genome in 2011 (Xu et al., 2011). According to the sequencing results, the potato genome has a (haploid) length of 844 Mbp with over 39,000 protein-coding genes including 2,642 potato-exclusive ones (Xu et al., 2011). To gain information with more value for field trials and other agricultural

purposes, sequencing of DM was subsequently used to integrate data from RH89-039-16 (RH), a heterozygous, tetraploid potato variant. RH closer resembles commercial potato cultivars which are usually tetraploid (Xu et al., 2011), hence RH data opened new opportunities for investigations of potato genomics and transcriptomics, thus noticeably improving its breeding, cultivation and protection. Also, by analyzing a huge RNA sequencing (RNA-seq) dataset of different DM tissues and various growth conditions, Alicia Massa and her co-workers created a basis for future gene expression studies of different potato varieties (Massa et al., 2011, 2013). It did not take long until first whole transcriptome analyses of potato plants became available, investigating transcriptomic changes of potato plants related to drought stress (N. Zhang et al., 2014; L. Gong et al., 2015; Pieczynski et al., 2018), low temperature stress (Ji et al., 2019) and resistance against a broad range of pathogens including viruses (Goyer et al., 2015), nematodes (Walter et al., 2018), bacteria (Kwenda et al., 2016) and - not surprising - *P. infestans* (Gao et al., 2013; Ali et al., 2014). In the long run, access to the potato genome sequence data has the potential to massively improve breeding programs of potato, as breeding can be more focused on genotypic data and will no longer be limited to selections within F1 populations (Hirsch et al., 2014).

1.3. From classic crop protection to modern crop improvement

Crop protection is as old as agriculture itself. As long as populations changed from a nomadic lifestyle to permanently settled communities, there was need to produce and protect food (Dayan et al., 2009). Modern agriculture still relies on crop rotation, a practice that is known to be used for thousands of years. Crop rotation can increase the harvest yield significantly (Woźniak, 2019), but in contrast, modern fertilizers are able to compensate losses in monoculture nowadays (Nevens & Reheul, 2001). Furthermore, lots of plant species were used in different ways in the history of agriculture to protect crops, *e.g.* as competitor plants or used as extracts, infusions or powders (Secoy & Smith, 1983). Using other organisms for crop protection is a technique that is called biological pest control and contrasts with using solely active biological or chemical agents known as pesticides. Despite of using sulfur compounds as insecticides already around 2,500 BC (Orlob, 1973), the mechanisms of plant protection were not known during ancient and medieval times and were often even imaginarily strengthened via prayer and magic spells. Sophisticated usage and production of plant protection products started not before the late 19^{th} century with the broader usage of Paris green ($Cu(CH_3COO)_2 \cdot 3\ Cu(AsO_2)_2$) and lead arsenate ($PbHAsO_4$) as insecticides (Peryea, 1998)

and the discovery of the efficiency of Bordeaux mixture ($CuSO_4$ + $Ca(OH)_2$) against fungi in vineyards (G. F. Johnson, 1935).

Synthetic pesticides entered the stage in the 1930s to supersede the highly toxic metallic products used so far, despite being originally invented as chemical weapons (Garcia et al., 2001). Probably the most prominent example of a synthetic pesticide today is glyphosate. Glyphosate (N-(phosphonomethyl)glycine) is the worldwide leading herbicide and was introduced in Europe in the 1970s. Finally registered in 1983, glyphosate was questioned ever since first studies revealed hepatic toxicity in rats in the early 2000s (Benedetti et al., 2004). Its impact on European harvests was investigated 2014, indicating huge losses in case of discontinuation (Wynn et al., 2014), but continuously published contradictory studies regarding carcinogenicity or other health concerns still keep the debate about use or harm of glyphosate alive. Despite that, the European Union decided to renew the approval of glyphosate for another five years in December 2017 (https://ec.europa.eu/food/plant/pesticides/glyphosate_en, accessed on 04.06.2019). In a similar way, lothianidin, imidacloprid and thiamethoxam, three important and widely used insecticides, were banned from usage in the European Union in May 2018 https://ec.europa.eu/food/plant/pesticides/approval_active_substances/approval_renewal/neonicotinoids_en, accessed on 04.06.2019) in consequence to studies about their harm on honey bees, butterflies, moths and other wildlife (Wood & Goulson, 2017).

Apart from health concerns, synthetic organic pesticides are susceptible to resistance developments of insects and other pests (Urech et al., 1997). In contrast, copper-based pesticides are attested a low resistance risk (J. M. Clark & Yamaguchi, 2009). Until now, no real copper resistance was ever observed, making heavy metal based pesticides still highly important and reliable candidates for efficient crop protection. Although being successfully used for decades in plant protection, especially in organic farming nowadays, copper pesticides are suspected to build residues in soils, leading to infertile arable land as a consequence of heavy metal toxicity (Wightwick et al., 2013). Not surprisingly, a sustained debate about the usage of copper in agriculture in the European Union led to instructions to reduce the amounts of copper as well as to the directive to eventually find alternatives for copper as active ingredient in plant protection products (La Torre et al., 2018). Nevertheless, with the Commission Implementing Regulation (EU) 2018/1981 the approval of copper compounds as active substances was extended until 2026 (https://eur-lex.europa.eu/eli/reg_impl/2018/1981/oj, accessed on 04.06.2019).

Both biological pest control and pesticide use are dependent on several biotic and abiotic factors like disease pressure (Juroszek & Von Tiedemann, 2011), weather conditions (Vining, 1990) and climate change (Chakraborty & Newton, 2011; Nazir et al., 2018) and often reach their limits when applied solely. Luckily, one can also give the plant a hand by supporting both its growth and disease management by the application of fertilizers. As with plant protection to manage plant diseases, the origin of fertilizers goes far into ancient times, where Egyptian farmers used Nile mud and Babylonian people utilized stable manure to fertilize their crops (Kiiski et al., 2016). Again likewise for disease management, scientific approaches of fertilization started in the 19th century with Justus von Liebig discovering nitrogen, phosphorus and potassium as essential elements for plant growth (Liebig, 1841). Building on this knowledge, the Haber-Bosch and the Oswald processes created a basis for the production of artificial nitrogen fertilizer by enabling industrial production of nitric acid (Patent No. GB190208300, 1903; Patent No. GB190200698, 2015) and ammonia (Haber, 1911). It is believed that around half of today's world population is fed with the help of artificial nitrogen-based fertilizers (Erisman et al., 2008). Additionally, with artificial inorganic fertilizers being easily applicable, they are often the first choice for farmers. However, especially farmers in poorer regions still highly rely on organic fertilization as they are often more affordable due to their natural origin (Mofunanya et al., 2015). Unfortunately, no fertilizing system is free of drawbacks and a potential of environmental damage. Simple over-fertilizing *e.g.* easily leads to water pollution (*i.a.* nitrate leaching) and even the global climate can suffer from the usage of fertilizers via accumulation of nitrous oxide as a strong greenhouse gas in the atmosphere (Shcherbak et al., 2014).

In addition to externally added compounds to support the vigor of plants, resistance breeding is a strong and important technique since many centuries. First sophisticated resistance breeding that goes beyond the simple selection of the most profitable plant individuals in a field is based on hybridization experiments conducted by Gregor Mendel in the 19th century (Mendel, 1865). A worth to mention traditional plant breeding technique is breeding for heterosis. Heterosis or hybrid vigor describes the enhanced quality of an offspring in comparison to its parents. This knowledge was used to create strong hybrid plants displaying yield advantages via crossing of homozygous inbred lines. One of the first hybrid lines with notable yield increases was hybrid corn, developed by William J. Beal (Duvick, 2001). Although the heterosis-effect was known since the Mendel times, the term itself was first defined by George Harrison Shull in the early 20th century (Shull, 1948). The most prominent problem of traditional plant breeding is its immense time expenditure. For example, hybrid vigor is extremely limited to the F1 generation,

hence farmers must repeat crossing of promising parent plants every season to maintain their harvest expectations. Furthermore, resistance breeding is commonly focused on resistances that are known to be dependent on one or at least very few resistance genes (R genes). This facilitates and shortens the post-breeding process of verification of the resistance. The drawback of monogenetic resistance is the so called boom-bust cycle, after which a new resistant plant variety is 'booming' in an area, but its resistance is easily surmountable by a single mutation in the plant pathogen, causing the resistance to 'bust' (Pink & Hand, 2018). Another problem is the unintended introduction of toxins or other undesired features into the target plant since the crossing with its resistant counterpart does not guarantee the introduction of only beneficial genes (National Research Council, 2004). Although traditional resistance breeding methods are used until today, their weak points were a door opener to more modern approaches (Breseghello & Coelho, 2013).

In the 1980s, genetic engineering entered the stage, not only confusing and whirling up all methods of common practice at this time, but also causing big discussions about necessity, risks and ethical concerns until today. Since the first genetically modified (GM) tobacco plants showed antibiotic resistance in 1983 (Fraley et al., 1983), a broad range of techniques and plant species were used to create plants with highly specified features. Most commonly, GM plants are desired to gain higher stress resistance against *e.g.* drought (Pierre et al., 2012), heat (Bita & Gerats, 2013) and salinity (Hu et al., 2006). On the other hand, insertion of certain genes into plants can also support the efficiency of herbicides and insecticides and other plant protection agents. For example, glyphosate-resistant plants significantly enhance both efficiency and application of this herbicide simply by completely withstanding its mode of action (Duke & Powles, 2008). Another approach to genetically engineer resistant plants is using RNA interference (RNAi). Specific RNA fragments complementary to essential pest genes can be introduced to host plants and are shown to successfully suppress damage caused by insects (Gordon & Waterhouse, 2007; Whyard, 2015) and viruses (Galvez et al., 2014). Although the improvement of plant growth and productivity is rarely touched due to being regulated by complex genetic programs, a recent study reports the successful implementation of alternative photorespiratory pathways which lead to an increase of tobacco plant biomass up to 40 % in field trials (South et al., 2019). Not least, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing provides a relatively new and promising way to edit plant genomes to enhance various features of crops (Ran et al., 2013). GM plants are a tough act to follow and are rejected from a broad mass. It might still take a lot of effort to wipe out major concerns about nutrition alterations and toxin formation (Maghari & Ardekani, 2011),

however, future generations might be dependent on biotechnological methods to maintain global food supply. As the European Union follows strict regulations concerning GM plants (Davison, 2010) and Germany used an EU opt-out option (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52015PC0177, accessed on 05.06.2019) to prohibit the use of GM plant as food or feed in 2015, benefits and dangers of GM plants in agriculture will not be further covered here.

Concluding from this, crop protection was always an arms race between pathogens and plants and their farmers and protectors respectively. Increasing world population, decreasing farmland and pathogens developing resistance against common pest control, accompanied by ascending awareness of the importance of sustainability and environmental protection, inevitably leads to the necessity to continue research for novel ways of plant protection and fertilization that are highly efficient, but also environmentally friendly.

1.4. Chitosan – the jack of all trades

Chitosan is a linear polysaccharide, consisting of β-1,4-linked N-acetyl-2-amino-2-deoxy-D-glucose (short N-acetyl-D-glucosamine or GlcNAc) and 2-amino-2-deoxy-D-glucose (short D-glucosamine or GlcN) subunits. Hence, it is a partially (or fully if artificially produced) deacetylated derivative of chitin (poly-β-(1-4)-N-acetyl-D-glucosamine), the most abundant aminosugar biopolymer on earth (Ravi Kumar, 2000). Both chitin (Figure 2) and chitosan (Figure 3) are occurring naturally. While chitin basically acts as a structural polysaccharide in exoskeletons of arthropods and cell walls of fungi and yeasts (Rinaudo, 2006), chitosan was observed to be naturally present in several fungal organisms, for instance supporting cell wall integrity and growth of *Cryptococcus neoformans* (Banks et al., 2005; L. G. Baker et al., 2007) and germination and hyphal growth of *Aspergillus nidulans* (Takaya et al., 1998). Furthermore, deacetylation of surface-exposed chitin in pathogenic fungi was shown to be a protection strategy against plant chitinase activity which would disintegrate the cell wall of potentially intruding fungi (El Gueddari et al., 2002). Before being discovered as a natural compound of living organisms, chitosan was only known as an artificial product, deriving from the treatment of chitin with alkaline solutions. This process was first described by Charles Rouget, a French physiologist who recognized that chitin becomes soluble in organic acids when cooked in concentrated potassium hydroxide (Rouget, 1859). After simply being called “modified chitin” for a few decades, the deacetylated form of chitin was finally named chitosan by the German physiologist and chemist Ernst Hoppe-Seyler (Sturm & Hesse, 2000). Being essential for many living organisms, chitin is a highly abundant source and until today mainly obtained from

crustacean shells as a by- and waste product of the seafood industry (Ravi Kumar, 2000). However, nowadays other chitin extraction sources like fungi or insects are considered as well (Synowiecki & Al-Khateeb, 2003; Ai et al., 2012). After the shell waste is purified from dirt, proteins and minerals, chitosan can be obtained by chemical or enzymatical deacetylation. This deacetylation of chitin leads to free amino groups that can be protonated at pH values below 6 (Rinaudo, 2006), making chitosan the only known naturally occurring cationic biopolymer. Both chitin and chitosan are not defined molecules by their names themselves but have to be characterized via different parameters. The size of one molecule can range from few (oligomers) to several thousand (polymers) monomer units. This feature is described as the degree of polymerization (DP). While chitin contains of only one type of monomeric unit (GlcNAc), chitosan consists of both GlcNAc and GlcN units. The ratio of GlcNAc and GlcN monomers is called degree of deacetylation (DD_A), degree of acetylation (D_A) or fraction of acetylation (F_A). In case of chitin, which is always completely acetylated, the DD_A would be given as 0 %, the as D_A 100 % and the F_A as 1. It has been proposed to set F_A as the standard unit for describing the acetylation degree of chitosan, representing the mole fraction of anhydro-2-acetamido-2-deoxy-D-glucose units (Roberts, 2008). The solubility of chitosan is highly dependent on the scope of its (de)acetylation, with more acetylated units resulting in a more hydrophobic and thus less soluble molecule (Ravi Kumar, 2000). Both DP and F_A determine the molecular weight (Mw) of the molecule. A chitosan-exclusive parameter is the pattern of acetylation (P_A), grouping the chitosan molecules according to alternating, random or block-wise patterns of GlcNAc and GlcN units which depends on the preparation conditions (Weinhold et al., 2009). Hence, strictly speaking, chitin and chitosan are not two, but a family of biomolecules that should be stated as plural terms as chitinS and chitosanS. However, it will subsequently be stuck to the singular forms.

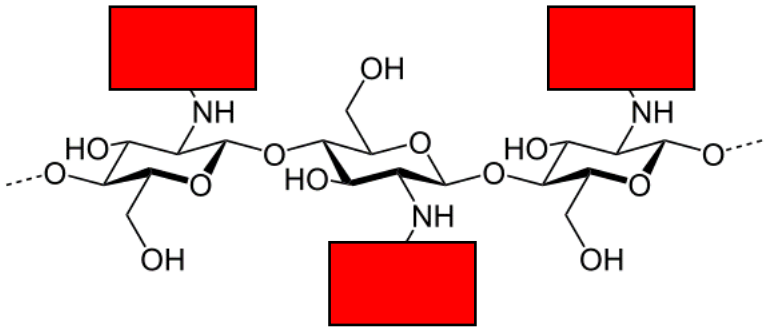

Figure 2: Chemical structure of chitin. Chitin is composed of β-1,4-linked GlcNAc units. The hydrophobic acetyl residues (red) make chitin insoluble in water. Dashed lines indicate the (most common) polymeric nature of the molecule, but short oligomers also exist. The pictured molecule would be characterized as a chitin trimer with DP 3 and F_A 1.

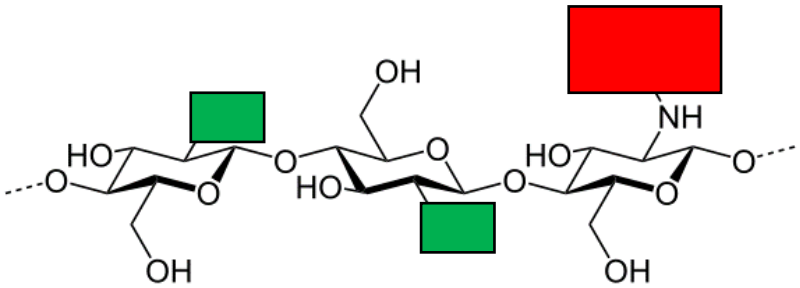

Figure 3: Chemical structure of chitosan. Chitosan is composed of β-1,4-linked GlcNAc and GlcN units. Deacetylated monomers display free amino groups (green) that can be protonated at low pH, making chitosan soluble. Dashed lines indicate the (most common) polymeric nature of the molecule, but short oligomers also exist. The pictured molecule would be characterized as a chitosan trimer with DP 3 and F_A 0,33.

Research on both chitin and chitosan started to increase in the 1930s and 1940s (Annu et al., 2017). Nowadays, chitin is used in food processing for flavor extension and food thickening (Shahidi et al., 1999) and in medicine as bone formation accelerator (Kawai et al., 2009), for sutures (Nakajima et al., 1986) and wound dressings (R. Singh et al., 2008) only to name a few. However, depending on the field of application, the usage of chitin is limited due to its insolubility in water. For instance, fertilizing and antimicrobial effects of a chitin enriched soil (Spiegel et al., 1986) was later considered skeptical, stating that an interaction of plants and pathogens with an insoluble and uncharged molecule is unlikely (Ramírez et al., 2010; R. Sharp, 2013). With its cationic charge and, most importantly, its solubility, the application possibilities of chitosan are broadened and *e.g.* used for films and fibers for various applications (Rinaudo, 2006). The existence of chitosan solutions is also especially useful for agricultural purposes, as solutions can be utilized for seed coating and foliar spray of plant leaves (El Hadrami et al., 2010).

Chitosan is known to be biologically active on a huge variety of plants and against many plant pathogens. The first report of an antimicrobial effect of chitosan celebrates his 40th anniversary this year, describing growth inhibition of a broad range of animal and plant pathogens when exposed to chitosan (Allan & Hadwiger, 1979). This study was confirmed and extended in the following centuries, resulting in today's broad knowledge of antibacterial (No et al., 2002; Benhabiles et al., 2013), antifungal (Muzzarelli et al., 1990; Enio N. Oliveira Junior et al., 2012) and even antiviral (Pospieszny et al., 1991; S. N. Kulikov et al., 2006) and insecticidal (Rabea et al., 2005) activity of chitosan. Large chitosans were observed more effective against bacteria than chitosan oligomers (No et al., 2002). Additionally, the inhibitory effect seems to be depending on the gram type of bacteria (No et al., 2002; Katiyar et al., 2014). DP dependency

with a tendency to an enhanced efficiency of larger chitosan molecules was likewise shown against a broad range of fungi (Enio N. Oliveira Junior et al., 2012). However, more recent investigations tend to suggest a stronger antifungal activity of smaller chitosan molecules, including oligomers (Rahman, Shovan, et al., 2014; Ganan et al., 2019). The actions of chitosan on fungi include alterations in the hyphal morphology (Ghaouth, 1992) and the production and germination of fungal spores (Palma-Guerrero et al., 2008), which again is dependent on the fungal species (Enio Nazaré de Oliveira Junior, 2016). It was even shown that chitosan is actively (*i.e.* ATP-dependently) imported into fungal cells before initiating cell death (Palma-Guerrero et al., 2009). Antiviral activity of chitosan is believed to be based on the inhibition of viral replication or distribution through its host. For example, it was shown that bean plants could withstand bean mild mosaic virus infections when chitosan inhibits viral reproduction and accumulation in inoculated leaves. Furthermore, its distribution to apical leaves was suppressed (S. N. Kulikov et al., 2006). Another study investigated the replication inhibiting effect of chitosan against several viruses, including H7N9 (influenza A), making it a promising candidate as anti-influenza agent (Zheng et al., 2016). However, in general, the antiviral activity of chitosan is not as well-studied and understood as other antimicrobial activities (Chirkov, 2002; Davydova et al., 2011).

Despite plenty of evidence and documentation of the antimicrobial activity, the exact mode of action of chitosan is not yet fully elucidated. The most prominent and commonly stated mechanism is the interaction of the positively charged amino groups of chitosan with negatively charged compounds, *e.g.* phospholipids in the plasma membrane, proteins and different cell wall components (Rabea et al., 2003). It was shown that chitosan is able to disintegrate plasma membranes and cell walls of bacteria and, hence, killing them (Hui Liu et al., 2004). The proposed mechanism was further proven by the fact that more negatively charged cell surfaces showed higher interaction with chitosan, leading to a stronger antibacterial effect (Chung et al., 2004). However, another study stated that the antibacterial activity of chitosan is also highly dependent on its DP, with polymers tending to act bacteriostatic, while oligomers exhibiting bactericidal activity (Benhabiles et al., 2013). A second hypothesis states that chitosan might chelate essential nutrients and metals of microbes and thereby preventing their growth and spread (Li et al., 2008; da Silva Mira et al., 2017). Additionally, a direct interaction of chitosan with nucleic acids in the cells to inhibit gene expression and protein synthesis is assumed (Rabea et al., 2003; Ing et al., 2012). The possible modes of action of chitosan related to antimicrobial activity are shown in Figure 4.

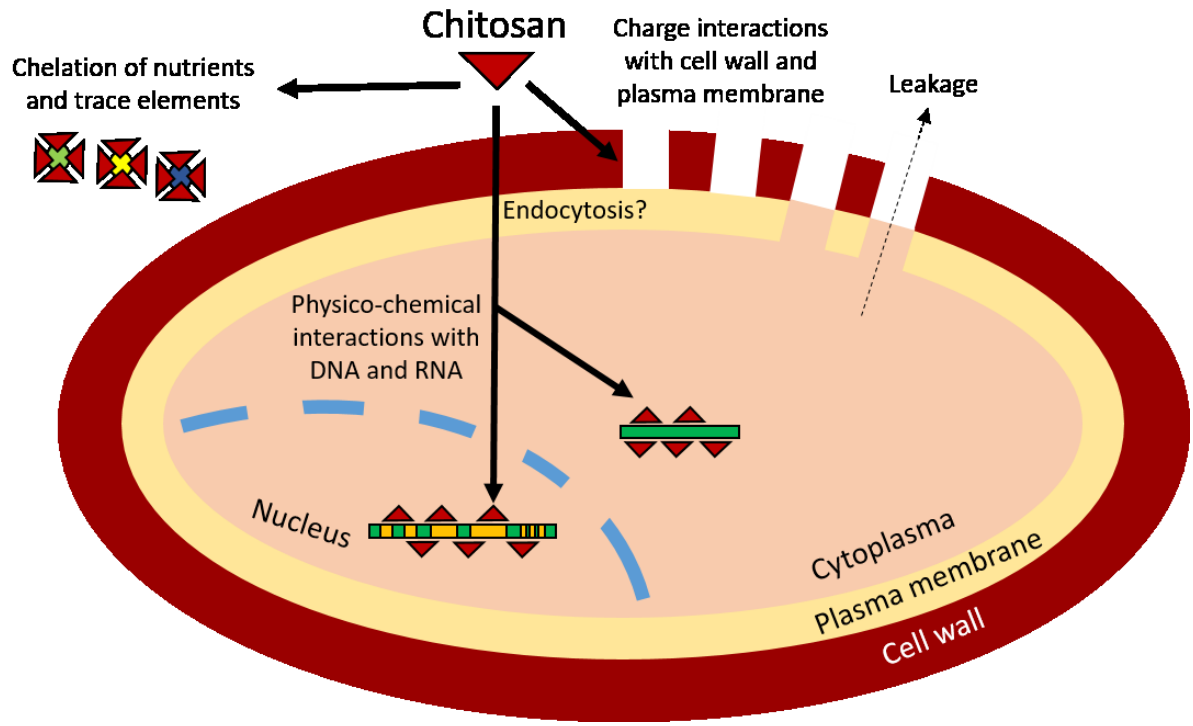

Figure 4: Possible modes of actions of chitosan. The most prominently assumed mechanisms are the interaction of chitosan with negatively charged cell wall or membrane components, the chelation of metals and nutrients and the direct interaction with intracellular nucleic acids.

Independent of which hypothesis is true, it is generally accepted that the F_A is the most crucial parameter of chitosan in terms of its biological activity. Low F_A chitosans are observed to result in higher antimicrobial activity (Omura et al., 2003; Younes et al., 2014), as these molecules display more free amino groups that can be present as charged residues in acidic conditions.

An intermediate to high F_A is in favor of triggering plant activity in response to chitosan treatment (Vander et al., 1998; Nietzel et al., 2018). Therefore, it is proposed that the mode of action of chitosan on plants is less dependent on charge interactions, but rather linked to the perception of chitosan molecules through chitin or chitosan receptors and chitosan binding proteins (Iriti & Faoro, 2009). The Japanese working group of Naoto Shibuya identified a chitin-binding protein (chitin elicitor binding protein, CEBiP) in rice (Ito et al., 1997; H. Kaku et al., 2006; Kouzai et al., 2014) as well as a chitin receptor (chitin elicitor receptor kinase 1, CERK1) in *Arabidopsis* (Miya et al., 2007). Intensive studies on both structures further unraveled the perception mechanics of chitin. Later, it was stated that rice plants require both a chitin binding protein (CEBiP) for perceiving chitin molecules and a receptor kinase (OsCERK1) for signal transduction (heterodimerization), whereas *Arabidopsis* only requires two AtCERK1 molecules for both perception and signaling (homodimerization) (Shinya et al.,

2012). Following studies pursued investigations about chitin perception in plants, reporting that two lysine motif containing proteins LYP4 and LYP6 are important for chitin perception in rice in addition to CEBiP (B. Liu et al., 2012). Concerning *Arabidopsis* on the other hand, a low binding affinity of chitin to AtCERK1 and the discovery of two lysine motif receptor kinases (AtLYK4 and AtLYK5) resulted in the statement that chitin first binds to LYK, which subsequently forms a protein complex with CERK1 to induce plant defense in *Arabidopsis* (Cao et al., 2014). Different models of chitin perception in plants were compared by (Gubaeva et al., 2018), concluding that a 'slipped sandwich' receptor complex is the most likely dimerization mechanism after chitin perception. Independent of the proposed model, chitin binding via lysine motifs (LysM), protein dimerization after chitin perception and signal transduction via kinase domains are in common agreement (Figure 5).

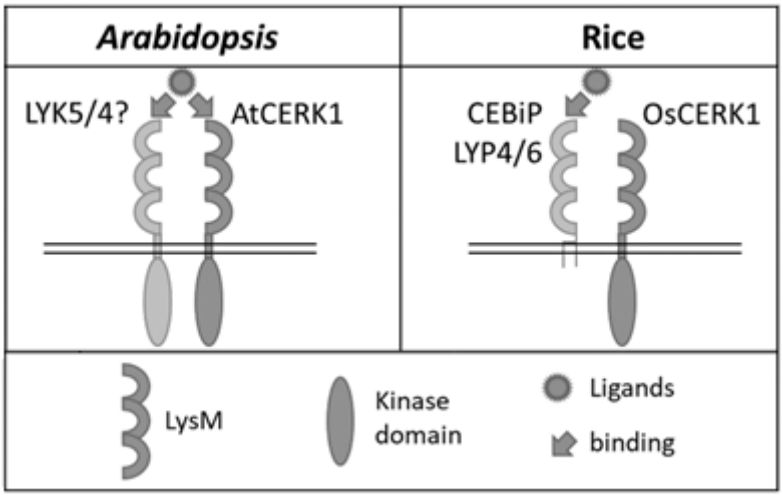

Figure 5: Chitin perception in *Arabidopsis* and rice plants. Chitin perception in *Arabidopsis* requires a lysine motif receptor kinase (LYK4 or LYK5) and a chitin elicitor receptor kinase 1 (AtCERK1). Chitin binding leads to dimerization and subsequent signal transduction via kinase activity of both molecules. In rice plants, chitin binds to either a chitin binding protein (CEBiP) or a lysine motif containing protein (LYP4 or LYP6) which then dimerizes with OsCERK1, providing kinase activity for signal transduction. Modified from (Desaki et al., 2019).

So far, no distinct chitosan receptor could be found. Although older studies assumed that chitosan exclusively elicits plant responses via interactions with negatively charged phospholipids (Kauss et al., 1989), it seems more likely that chitosan to a certain extend is very well perceived by chitin receptors. Concerning the observations that a high F_A results in stronger plant responses (Vander et al., 1998; Cord-Landwehr et al., 2016; Gubaeva et al., 2018; Nietzel et al., 2018), it can be assumed that chitosan molecules are perceived the same way as chitin molecules as long as they display sufficient amounts of grouped GlcNAc units. However, as CERK1-independent chitosan perception is described (Povero et al., 2011), recognition of

chitosan through known chitin receptor molecules seems to be only one possible elicitation mechanism. Physico-chemical interactions of chitosan with cell walls and plasma membranes of plants can still be assumed, as for instance callose synthase was observed to be activated by chitosan (Kohle et al., 1985), which is a Ca^{2+}-dependent process that might occur due to calcium influx as a consequence of altered membrane permeability (Zuppini et al., 2004).

According to (Iriti & Faoro, 2009), chitosan acts as a pathogen or microbe associated molecular pattern (PAMP/MAMP) and is recognized by pattern recognition receptors (PRR) which are usually surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) and act as essential signaling proteins in innate immune responses of plants (Akira et al., 2006; Zipfel, 2008, 2014). This process, which is known as PAMP-triggered immunity (PTI), displays the first layer of induced plant defense and is commonly characterized by synthesis of pathogenesis-related (PR) proteins, release of reactive oxygen species (ROS) and altered phytohormone crosstalk (Jones & Dangl, 2006). Pathogens would now try to outsmart PTI by disguising themselves via secretion of effector molecules, which in turn can be recognized by the plant. This then leads to effector-triggered immunity (ETI), the second layer of induced plant defense (Jones & Dangl, 2006). While ETI is related to a hypersensitive response (HR) of the plant, an ultimate approach to combat pathogen attack via cell death (Wu et al., 2014), chitosan treatment is believed to keep the plant cells on a PTI-level - and thus alive (Lopez-Moya et al., 2019). This matches indications that chitosan might be priming-active, meaning that it can put plants in an alert state to accelerate the execution of defense mechanisms in case of infection (González-Bosch, 2018; Basa et al., 2019). Hence, chitosan treatment of plants is an interesting approach for agricultural purposes and research on chitosan for crop protection has long since developed from a ridiculed footnote to a part of a promising economic branch. In this context, chitosan was shown to elicit a broad range of defense responses in plants (Figure 6). Early events include changes in H^+-ATPase activity and other H^+-mediated processes (Amborabé et al., 2008). This leads to altered proton fluxes which are eventually linked to the induction of various defense responses (Amborabé et al., 2008). One early response is the production of ROS, mostly superoxide ($O_2^{\cdot-}$), mainly via NADPH-oxidase (NOX) mediated electron transfer from NADPH to molecular oxygen (Panday et al., 2015; Jiménez-Quesada et al., 2016). ROS can for example act either as antimicrobial agent to combat pathogens directly or as messenger molecules for activation of subsequent reactions (Bolwell & Wojtaszek, 1997). Release and activity of ROS in response to pathogen infection is widely known as oxidative burst (Wojtaszek, 1997). Further responses to chitosan treatment include the production and release of phytoalexins (Hammerschmidt, 1999) and PR-proteins

(Köhle et al., 1984; Hirano et al., 1990; Vander et al., 1998), activation of lignification by producing lignin precursors and other phenolic compounds (Reddy et al., 1999) and the increase of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities (Khan et al., 2003). Additionally, chitosan was observed to influence phytohormones, *e.g.* by activating jasmonic acid (JA) synthesis (Doares et al., 2006). This suggests that chitosan treatment might lead to induced systemic resistance (ISR), an important JA-mediated resistance mechanism in plants, resulting in preconditioned defense structures that allows systemic resistance against subsequent infections (D. K. Choudhary et al., 2007). More recent approaches using microarray studies revealed that chitosan activity not only triggers resistance by activation of consecutively present proteins, but also through inducing the expression of defense-related genes (Povero et al., 2011).

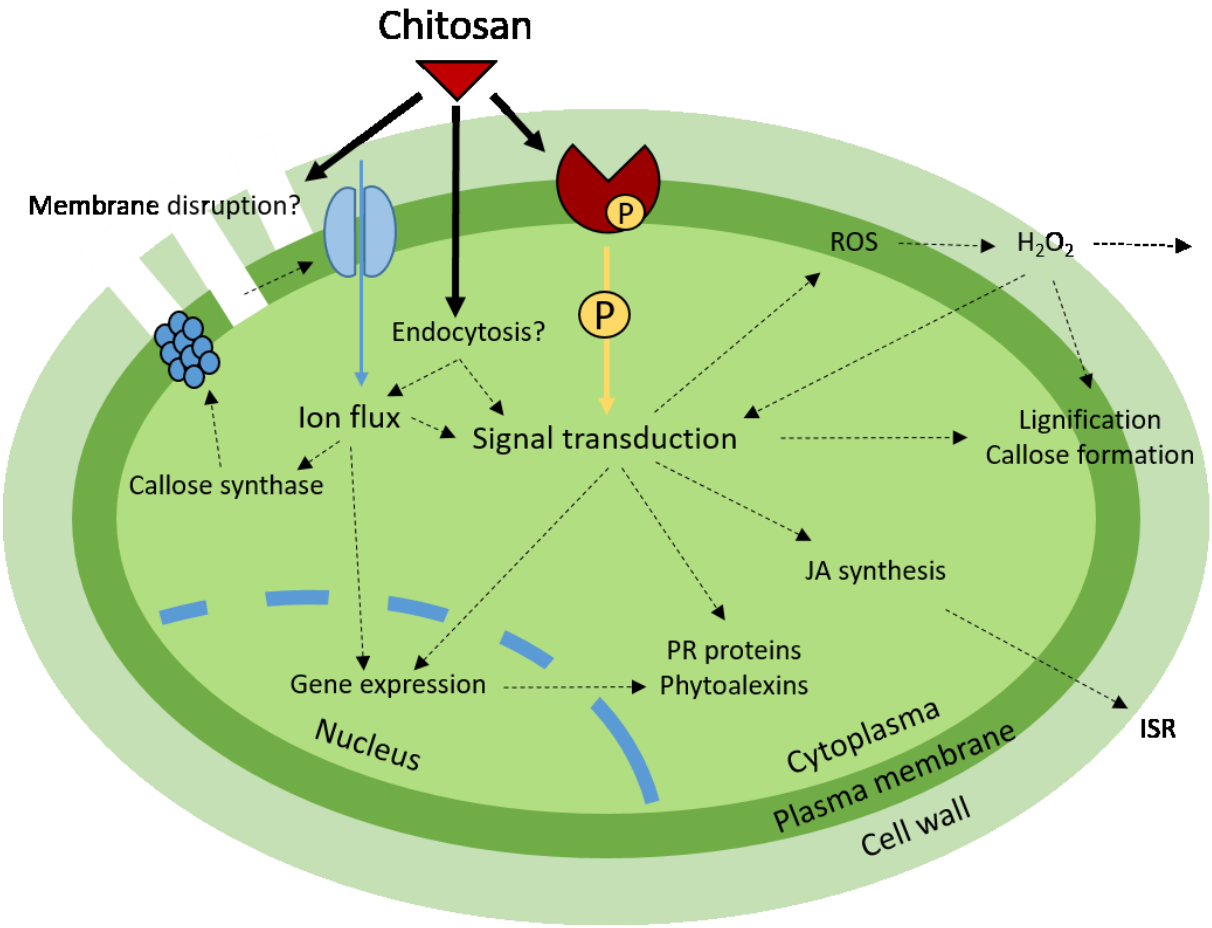

Figure 6: Chitosan as elicitor of plant cell responses. Chitosan might either interact with the plasma membrane, triggering alterations in the ion flux or be perceived by chitin receptors or binding proteins. Chitosan perception leads to kinase activity dependent signal transduction within the plant cell. Both ion and kinase activities can eventually activate a broad range of responses, including the production of ROS, cell wall fortification via callose and lignin and activation of PR proteins and phytoalexins. Furthermore, defense responses as well as other metabolic processes can also be switched on or enhanced via activation of gene expression.

Generally, the efficiency of chitosan on plants seems to base on a dual effect, both attacking pathogens directly via its antimicrobial activity on the one side and inducing resistance in plants on the other side (El Hassni et al., 2004). Furthermore, treating a plant with chitosan influences growth and productivity, not only supporting its vigor and resistance, but also contributing to higher crop yields (Malerba & Cerana, 2016). The latter was furthermore confirmed by recent RNA-seq studies on avocado and strawberry plants, revealing that besides activation of defense-related genes, chitosan treatment leads to a general increase of metabolic activity including activation of photosynthetic processes (Landi et al., 2017; Xoca-Orozco et al., 2017). Worth mentioning, the beneficial activities of chitosan on plants was not only observed using it solely, but also in combination with *e.g.* copper (R. C. Choudhary et al., 2017; Rubina et al., 2017; Rautela et al., 2018) or other conventional fungicides (Rahman, Shovan, et al., 2014), which resulted in enhanced efficiency. This addition of chitosan to a present plant protecting agent usually resulted in less usage metal or fungicide to achieve the same efficacy (Rahman, Shovan, et al., 2014; Rautela et al., 2018). Hence, chitosan might also be used as additive to not only increase the efficacy of commercial products, but through this furthermore enable reduction of chemical usage.

Being a non-toxic, biodegradable and renewable resource (R. Sharp, 2013), chitosan has blossomed out from a barely noticed waste product to a promising plant protection agent. Either as supplement or as standalone product, chitosan has the potential to contribute to the next generation of crop protection without harsh chemicals. This development was especially favored by advances in chitosan research that nowadays allows the reliably reproducible production of certain chitosan molecules of specified length, acetylation degrees and even acetylation patterns (Hamer et al., 2014; Hembach et al., 2017) and not least via approving chitosan hydrochloride as a basic substance for plant protection products in the European Union in 2014 (https://eur-lex.europa.eu/eli/reg_impl/2014/563/oj, accessed on 13.06.19).

1.5. What's it all for?

Knowledge-based plant protection is a global challenge. The demand for arable land space will double within the next decades and the world population will increase further at the same time. Currently, it cannot be foreseen how the world population can be fed in the future, but available projections tend to predict a rather pessimistic development. To defy this dystopian outlook, research on novel plant protection strategies is highly needed. As long as people condemn genetic engineering, these strategies are limited to classic resistance breeding and crop protection through treatment with pesticides and fertilizers. Chitosan is a renewable resource,

biodegradable and non-toxic to humans and animals. Intensive research in the last decades not only led to a broad knowledge about effects and application areas of chitosan, but also to sophisticated procedures for extraction, purification and generation of desired chitosans with defined physico-chemical characteristics. As mentioned, a low F_A results in high antimicrobial activity whereas a high F_A more efficiently triggers plant responses. This means that either finding the most appropriate F_A, displaying both plant and pathogen activity or combining low and high F_A chitosans should lead to a promising plant protection agent. At the same time, both DP and P_A should be considered when screening for the most active chitosan as all parameters of chitosan determine its biological activity in the end. In this study, different chemically produced chitosans are screened for antimicrobial activity against the wheat pathogen *Fusarium graminearum* and for their potential to elicit oxidative bursts in potato plants. As an approach to increase the biological activity, not only single chitosans, but also combinations of different chitosans are tested in both models. These studies regarding antimicrobial and eliciting activity of chitosan in bioassays are furthermore supported by gene expression studies on both *F. graminearum* (real-time PCR) and potato (RNA-seq). In the end, this work should not only broaden the knowledge of chitosan-based plant protection in terms of gaining new information regarding the most appropriate chitosan to apply on plants and against pathogens, but also aims to further enlighten the mode of action of chitosan in both systems (Figure 7).

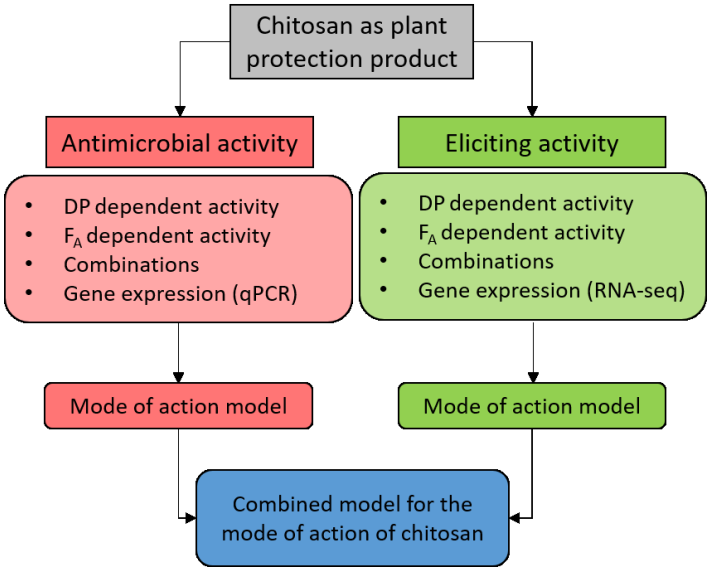

Figure 7: Thesis flowsheet. The suitability of chitosan as a plant protection product was planned to be separately tested against a plant pathogen and on a crop plant. In both cases, the biological activity in dependency of size (DP) and acetylation degree (F_A) was determined before combining different chitosans in order to increase their activity. Bioassays were supported by gene expression studies. The resulting information about the mode of action of chitosan depending on their physico-chemical properties was supposed to be used to create a model of the mechanisms of chitosan when targeting both a pathogen and its host plant.

Chapter II

Material and methods

2. Material and methods

2.1. Chitosan preparation and characterization

Preparation techniques and analysis methods for the used chitosans are described in the following paragraphs. A list summarizing all chitosans including origin and determined physico-chemical properties is given in Table 1.

2.1.1. Chitosan supply sources

The chitosans used in this thesis were all of commercial origin. This guaranteed not only good purity, but also especially sufficient amounts of chitosans, which is crucial for applying it in active amounts on plants. The chitosans were obtained from four different suppliers: Mahtani Chitosan Pvt. Ltd. (Veraval, Gujarat, India), Heppe Medical Chitosan GmbH (Halle, Germany), BioLog Heppe GmbH (Landsberg, Germany) and Bio Base Europe Pilot Plant (Desteldonk, Belgium).

2.1.2. Preparation of chitosan solutions

Chitosan solutions were obtained by dispersing chitosan powder in dH_2O and solubilized with a 5 % molar excess of acetic acid (60 g/mol) relative to the free amino groups in the chitosan used. The overall amount of acetic acid depends on the chitosan mass to be solubilized and was calculated using the formula

$$V_{C_2H_4O_2} = \frac{m_{C_2H_4O_2}}{p_{C_2H_4O_2}} \times \frac{m_{CS} \times \left(1 - \frac{DA}{100}\right) \times \left(1 - \frac{H_2O}{100}\right)}{M_{GlcN} \times \left(1 - \frac{DA}{100}\right) + M_{GlcNac} \times \frac{DA}{100}} \times 1{,}05$$

with $p_{C_2H_4O_2}$ = 1.048 g/mL, M_{GlcN} = 162.16 g/mol, M_{GlcNAc} = 204.09 g/mol and DA = percentage mol of deacetylated residues. As autoclaving leads to de-polymerization of chitosan (No et al., 2003), sterilization of chitosan solutions was limited to sterile filtration through filters with a pore size of 0.22 µm.

2.1.3. Molecular weight analysis

Average DP (DPn) of chitosan was determined using a combined system of high-pressure size exclusion chromatography coupled to refractive index detection and multi-angle laser light scattering analysis (HP-SEC-RID-MALLS) as described in (Schatz et al., 2003).

From this, the dispersity Đ of a chitosan could be calculated using the formula

$$Đ = \frac{Mw}{Mn}$$

with Mw being the weight-average and Mn being the number-average molecular weight.

2.1.4. Fraction of acetylation determination

To determine the F_A of chitosan, proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) was applied according to a method described by (Hirai et al., 1991). This method uses the ratio between the integral of acetylated (*i.e.* methyl) group protons and the integral of GlcN protons with the formula

$$F_A = (\frac{1}{3} \times I_{CH_3})/(\frac{1}{6} \times I_{(H_2-H_6)})$$

with I_{CH_3} being the integral of methyl group protons and $I_{(H_2\text{-}H_6)}$ being the sum of integrals of H_2, H_3, H_4, H_5 and H_6 protons of GlcN (Kasaai, 2010).

2.1.5. Separation of oligomer and polymer fractions of chitosans

Separation of polymer and oligomer fraction from a heterogeneous, highly disperse chitosan (661 in this study, see Table 1) was already conducted by the manufacturer. This process included chitosan precipitation by increasing the pH of the solution to pH 8, followed by centrifugation of the precipitate. The supernatant emerging from centrifugation contained the oligomers, while the polymer fraction remained in the pellet. The oligomer fraction was obtained from the supernatant via lyophilization and re-solubilization in acetic acid. The polymer fraction containing pellet was washed with ammonia and dH_2O and finally also re-solubilized in stochiometric amounts of acetic acid (see 2.1.2).

2.1.6. Enzymatical de-N-acetylation of chitin oligomers

Chitin oligomers (COS) were enzymatically de-N-acetylated to gain partially acetylated chitin oligomers (paCOS). This process was conducted using the chitin deacetylase from *Pestalotiopsis* sp. (PesCDA) (Cord-Landwehr et al., 2016). For de-N-acetylation, a 10 mg/mL COS solution was treated with 99 µL PesCDA in 8.2 mL dH_2O and 12.5 mL NH_4HCO_3 buffer (100 mM, pH 8). COS were incubated at 37 °C for 24 h, followed by enzyme inactivation via heating for 10 min at 80 °C. COS or paCOS respectively were analyzed via hydrophilic-interaction liquid chromatography-electrospray ionization mass-spectrometry

(HILIC-ESI-MS). A detailed description of the enzymatical de-N-acetylation via PesCDA can be found in (Cord-Landwehr et al., 2016).

2.1.7. Chemical re-N-acetylation of chitosans

To prepare a series of chitosans with constant DPn, but varying F_A, fully deacetylated 134 was used for chemical re-N-acetylation. This process was conducted in a hydroalcoholic mixture of propylene glycol and desired amounts of acetic anhydride to gain partial re-N-acetylated chitosans (F_A 0.1, 0.2, 0.3 0.4, 0.5 and 0.6) as described in (Vachoud et al., 1997). The re-N-acetylated chitosans were subsequently neutralized, precipitated with ammonia at pH 8.5, washed with dH_2O and lyophilized to regain chitosan powders. The F_A of the chitosans was determined as previously described.

Table 1: Physico-chemical properties of all chitosans applied in this thesis.

Chitosan	Supplier	F_A	DPn	Đ
651	Mahtani Chitosan Pvt. Ltd.	0.2	450	5
661 [1]	Mahtani Chitosan Pvt. Ltd.	0.2	343 [2]	5.5
661 oligomer fraction	Mahtani Chitosan Pvt. Ltd.	0.2	2-17 [3]	n.d.
661 polymer fraction	Mahtani Chitosan Pvt. Ltd.	0.2	450	5
134 [4]	Mahtani Chitosan Pvt. Ltd.	0.0	1300	2
75/5	Heppe Medical Chitosan GmbH	0.14	170	2.2
75/20	Heppe Medical Chitosan GmbH	0.14	499	1.5
75/100	Heppe Medical Chitosan GmbH	0.15	1081	1.4
90/1000	Heppe Medical Chitosan GmbH	0.14	1671	1.3
90/3000	Heppe Medical Chitosan GmbH	0.14	2087	1.3
CPS 4	BioLog Heppe GmbH	0.10	2-12	n.d.
COS [5]	Bio Base Europe Pilot Plant	~ 1 [5]	4-5	n.d.
paCOS [6]	Bio Base Europe Pilot Plant	~ 0.5 [6]	4-5	n.d.

[1] 661 is derived from 651 via chemical hydrolysis, resulting in a highly disperse chitosan.

[2] 661 consists of around 75 % (w/v) of a polymeric fraction and 25 % (w/v) a mixture of paCOS.

[3] The detection limit of used mass spectrometry is reached with a DP of 17.

[4] 134 was used as starting material for a F_A series of chitosans, ranging from F_A 0.1 to F_A 0.6.

[5] Produced by *E. coli* M61655 pet226 p14 RBSm GRHz. Main components: A4 (10 %), A5 (80 %), A4D1 (10 %).

[6] Produced by enzymatical de-N-acetylation. Main components: A3D2, A3D1, A2D3.

n.d.: not determined.

2.2. Antifungal activity of chitosan

Antifungal activity of different chitosans was investigated using a microtiter plate based growth study of chitosan exposed *F. graminearum* spores. The following chapter describes cultivation of *F. graminearum* as well as execution and analysis of the assay.

2.2.1. Cultivation and induction of conidia production of *Fusarium graminearum*

Mycelium of *F. graminearum* strain DSM 4528 was cultivated and proliferated in petri dishes containing complete medium (CM) (Pontecorvo et al., 1953) agar (Table 2). The plates were incubated in darkness, either at 4 °C for storage or at 26 °C for vegetative growth induction. For conidia induction, precultured mycelium in CM was transferred to fresh CM medium containing carboxymethyl cellulose (CMM) (Cappellini & Peterson, 2007) (Table 3). After 8 days of shaking with 120 rpm at 26 °C in darkness, conidia were harvested via filtering the liquid culture through mesh or cotton. CMM was removed from conidia via centrifugation and re-suspending in dH_2O.

Table 2: Complete medium for *F. graminearum* cultivation. This recipe provides liquid culture medium for *F. graminearum* cultures in flasks. For agar plates, 1.5 % (w/v) agar-agar was added. pH was adjusted to 5.8 with KOH and the medium was autoclaved before usage.

Component	Concentration
Yeast extract	0.1 % (w/v)
Casein hydrolysate	0.1 % (w/v)
Sucrose	1 % (w/v)
Tryptone	0.2 % (w/v)
Salt stock solution [1]	5 % (v/v)
Vitamin stock solution [2]	0.1 % (v/v)
Trace elements stock solution [3]	0.2 % (v/v)

[1] 10.4 g/L KCl, 10.4 g/L $MgSO_4 \cdot 7\ H_2O$, 30.4 g/L KH_2PO_4

[2] 0.5 g/L biotin, 16 g/L 4-aminobenzoic acid, 20 g/L pyridoxine hydrochloride, 50 g/L nicotinic acid

[3] 1 g/L $FeSO_4 \cdot 7\ H_2O$, 0.15 g/L $CuSO_4 \cdot 5\ H_2O$, 1.61 g/L $ZnSO_4 \cdot 7\ H_2O$, 0.1 g/L $MnSO_4 \cdot H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$

Table 3: Carboxymethyl cellulose medium for *F. graminearum* conidia induction. Autoclaved before usage.

Component	Concentration
Carboxymethyl cellulose	1.5 % (w/v)
NH_4NO_3	0.1 % (w/v)
KH_2PO_4	0.1 % (w/v)
Yeast extract	0.1 % (w/v)
$MgSO_4 \cdot 7\ H_2O$	0.05 % (w/v)

2.2.2. Antifungal activity

The antifungal activity of chitosan was measured in a 96 well microtiter plate according to (E. N. Oliveira et al., 2008). Accordingly, 10 µL of a spore suspension (7 x 10^3 conidia per mL) or dH_2O (blanks) were added to 150 µL CM, supplemented with 40 µL of a solution of single or combined chitosans. The plates were incubated under agitation as described above and fungal growth was recorded by UV/Vis spectrophotometric determination of the optical density at 600 nm (OD_{600}) every 24 h for a total of 96 h. In case a dose-dependent antifungal activity of different chitosans was compared, the minimum inhibitory concentration to inhibit 50 % of fungal growth (MIC_{50}) was determined and used as a benchmark. MIC_{50} was given as the chitosan concentration, which resulted in an at least halved OD_{600} value in comparison to the negative control (dH_2O) value.

2.3. Eliciting activity of chitosan

Eliciting activity of chitosan was determined via oxidative burst measurements (*i.e.* H_2O_2 production/release with potato leaf discs using a 96 well microtiter plate chemiluminescence assay (Albert et al., 2016), which allowed multiple screening of different chitosans in varying concentrations. The following paragraphs describe cultivation of potato plants, the preparation of leaf discs and elicitors as well as the measurement of the oxidative burst itself.

2.3.1. Cultivation of potato plants

Potato plants were cultivated in an environmental chamber with constant temperatures under long-day conditions (16/8 h photoperiod, 24/18 °C). Light was produced via fluorescent tubes (Sylvania Cool White F48T12/CW/VHO, 115 W) from Osram Sylvania Inc. (Danvers, Massachusetts, USA). For proliferation, germinated potato tubers were planted into pots and covered with propagation substrate (Einheitserde- und Humuswerke Gebr. Patzer

GmbH & Co.KG; Sinntal, Germany). The tubers and plants were irrigated manually upon need. To avoid insect infestations through *e.g.* white or fruit flies, the chamber was equipped with blue and yellow sticky traps if necessary. *S. tuberosum* cv. Sárpo Mira, a highly late blight resistant cultivar (Rietman et al., 2012; Tomczyńska et al., 2014), was used to assay the eliciting activity of chitosans, as it was observed to react very sensitively in the conducted assay.

2.3.2. Preparation of leaf discs

Leaf discs were prepared from fully-grown, mature potato leaves that were detached from a plant. Using a cork borer (Ø 5 mm), leaf discs were cut by gently pressing the sharp tool on the lamina part of the leaf (both veins and midrib were excluded in cases of strong expressions). Each disc was subsequently transferred to a well of a 96 well microtiter plate containing 100 µL of dH_2O. The plates were covered with aluminum foil and incubated at room temperature over night to allow de-stressing of the freshly cut leaf discs.

2.3.3. Oxidative burst assay

After removing the water from overnight incubation, the leaf discs were exposed to 200 µL of a 1:1 mixture of the putative elicitor (solution of single or combined chitosans) and 0.5 mM of the luminol derivative L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione) (Nishinaka et al., 1993) in 10 mM MOPS/KOH buffer (pH 7.4). H_2O_2 was quantified by a special microplate reader, which measures the light emission caused by the reaction of H_2O_2 and L-012 (Albert & Fürst, 2017). In this reaction, L-012 is oxidized, forming the unstable dianion 3-aminophthalate. 3-aminophthalate gains stability by changing electrons from excited state to ground state which is emitting energy as a photon (C. J. Baker, 1995). As one photon is released per H_2O_2 molecule, the detected chemiluminescence is directly proportional to chitosan-triggered H_2O_2 release from the leaf discs. Chemiluminescence was given as relative light units (RLU) and was continuously measured for 5000 ms per well every 8 min over a total time of 120 min. This time course measurement not only resulted in a burst curve, revealing the intensity of the oxidative burst response over time, but also allowed to determine the maximum RLU value (RLU_{max}) produced by each chitosan. RLU_{max} was subsequently used for analysis and comparison of the eliciting potential of different chitosans. Oxidative burst assays generally suffer from high standard deviations due to plant age, temperature, humidity and other barely controllable conditions. Therefore, the RLU_{max} values of all biological replicates were used to create boxplots, which allows covering all measured single values including deviation and median value.

2.4. Test for synergistic activity

Combinations of different chitosans were tested for both eliciting and antifungal activity to determine whether chitosan combinations are able to show enhanced biological activity in comparison to the usage of a single chitosan. By definition, a mixture of biological active components shows synergism if the activity of a mixture is greater than the sum of the activities of the individual components. In contrast, activity of a mixture that equals the sum of the individual components of the mixture is termed additive, an activity that is less than the sum antagonistic (Kosman & Cohen, 1996; Chou, 2006). However, an activity is only defined as synergistic if both individual components show activities by their own. If the activity of one component is increased by another, completely inactive component, it should be stated as enhancement or potentiation (Kosman & Cohen, 1996; Chou, 2006). From several methods of analyzing the biological activity of mixtures, the most prominent ones are by using formulas described by either W. S. Abbott (Abbott, 1987) or F. M. Wadley (Wadley, 1945). While Abbott's formula is applied when independent modes of actions of the components of a mixture are assumed, Wadley's formula is used in case both components show the same mode of action and one compound could substitute the other in constant proportions (Cohen & Levy, 1990). Since different modes of actions for different chitosans can be assumed (see 1.4), Abbott's formula was applied in this thesis using the formula

$$C_{exp} = A + B - (\frac{A \times B}{100})$$

with C_{exp} being the expected activity (in this case either inhibition of fungal growth or elicitation of an oxidative burst) calculated from the activities from the individual components A and B. If the ratio (also known as synergy factor SF) of the experimentally observed activity (C_{obs}) and C_{exp} is greater than 1, the mixture shows synergistic activity, whereas a ratio close to 1 indicates an additive activity (Cohen & Levy, 1990).

2.5. Statistics

Experimental data concerning the antifungal and eliciting activity of chitosan was statistically analyzed via analysis of variance (ANOVA) test (Fisher, 1921) and post hoc analysis via Tukey test (Benjamini & Braun, 2002) using OriginPro 2019b (OriginLab Corporation, Northampton, Massachusetts, USA) or GraphPad PRISM (GraphPad Software, Inc., San Diego, California, USA).

2.6. Gene expression studies

The following chapter describes all preparation and analysis steps required for gene expression studies in both SYBR green based real-time PCR and RNA-seq experiments. Real-time PCR experiments were conducted self-directed, whereas RNA-seq was performed by GeneVia Technologies (Tampere, Finland).

2.6.1. Plant treatment

For investigations regarding the gene expression of chitosan-treated potato leaves, mature leaves were detached from a potato plant (see 2.3.1 for cultivation description) one day prior to treatment. Detached leaves were incubated overnight in the potato plant growth chamber on petri dishes containing water agar (0.5 % agar) to maintain leaf humidity and to support de-stressing from detachment (as described for the oxidative burst assay in 2.3). For treatment, undersides of the leaves were sprayed either with a 651 solution (500 µg/mL) or with dH_2O (as control treatment) until small droplets formed on the leaf surface. After spraying, the leaves were incubated in the potato growth chamber again until being frozen in liquid nitrogen at desired time points. Time points ranged from few hours to several days, but were later narrowed to the first 5 h after treatment as chitosan-induced gene expression was expected in this time range.

2.6.2. Pathogen treatment

Liquid cultures of *F. graminearum* (strain DSM 4528) were prepared by adding mycelium-covered agar pieces to CM. These cultures were pre-grown for 3 days under agitation at 26 °C in darkness before treatment. For treatment, 661 (final chitosan concentration 150 µg/mL, final pH 5.3), pH-adjusted CM (pH 3.0 and pH 6.5) or dH_2O (as control treatment) was added to the *F. graminearum* precultures. 3 h and 24 h after chitosan treatment, the mycelium was harvested by separating it from CM via vacuum filtration and frozen in liquid nitrogen. Frozen samples were subsequently lyophilized to remove any remaining culture medium.

2.6.3. RNA extraction

To isolate RNA from either frozen leaf tissue or freeze-dried *F. graminearum* mycelium, samples were grinded using mortar and pestle under liquid nitrogen. RNA was isolated from the resulting powder using the column-based innuPREP RNA Mini Kit from Analytik Jena (Jena, Germany). Concentration and purity of isolated RNA was determined via Nanodrop 2000

photometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For storage, RNA samples were kept at -80 °C.

2.6.4. cDNA synthesis

First strand cDNAs were synthesized from 500 ng of total RNA using PrimeScript RT Master Mix from Takara Bio Inc. (Kusatsu, Shiga, Japan) by enzyme-driven reverse transcription at 37 °C for 15 min, followed by enzyme inactivation at 85 °C for 5 s. The cDNA samples were kept at 4 °C for storage.

2.6.5. Primer design

Primer design was conducted using NCBI Primer Blast (Ye et al., 2012), using either the potato genome sequence data (http://solanaceae.plantbiology.msu.edu/cgi-bin/gbrowse/potato, accessed on 16.06.19) (Xu et al., 2011; Sharma et al., 2013; Hardigan et al., 2016) or the genome sequence data of *Fusarium graminearum* strain PH-1 (https://fungi.ensembl.org/Fusarium_graminearum/Info/Index, accessed on 16.06.19) (Trail et al., 2003; Cuomo et al., 2007; Ma et al., 2010; King et al., 2015). Primers were designed to have a melting temperature (T_m) of 60 °C, a length of 20 base pairs (bp) and lead to amplicon lengths of ideally 100 to 300 bp, but exceptions were accepted in case the primer design demanded to further increase the amplicon length. To exclude self- and cross dimerization of the designed primers, the web tool Multiple Primer Analyzer (https://www.thermofisher.com/de/de/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html, assessed on 16.06.19) from Thermo Fisher Scientific (Waltham, Massachusetts, USA) was used, based on a method described in (Breslauer et al., 1986). Housekeeping genes of elongation factor 1-α (ef1α) and 18S rRNA were used as reference genes for potato leaf gene expression studies (Nicot et al., 2005; Goyer et al., 2015), whereas a gene coding for actin was used as reference for *F. graminearum* gene expression studies.

To test all primers for correct amplification, conventional PCR was conducted with a random cDNA sample of the according organism to verify single fragment amplification and fitting amplicon sizes. Tables 4, 5 and 6 lists all primers including their sequences, targets, amplicon sizes and efficiencies E (see next paragraph).

Table 4: List of primers used for real-time PCR studies on *F. graminearum*.

Annotation	Gene	Reference	Primer sequence	Amplicon	E	R^2
Actin gene [1]	actin	(X. Liu et al., 2013)	fwd ATCCACGTCACCACTTTCAA rev TGCTTGGAGATCCACATTTG	309 bp	3	0.97
Stress-activated protein kinase OS-2	os2	(Sella et al., 2014)	fwd TATTGACTTCGCGCCCTCTC rev TCATCTGGGGGTCCTGGATT	192 bp	2.19	0.97
Velvet protein 1	ve1	(Lan et al., 2014)	fwd GTCACGAGAGGAAACCGGAG rev TGAGCCAACGTACCTTTCATACC	101 bp	1.78	0.99
Trichothecene transcription regulator	tri6	(Merhej et al., 2010; Nasmith et al., 2011)	fwd TGAAAGCGGACGGGACTTTA rev AGCTGACTGAGGGCATTCTG	466 bp	3.19	0.92

[1] Reference gene

Continuation of Table 4.

Annotation	Gene	Reference	Primer sequence	Amplicon	E	R^2
Sterol C-24 reductase	erg4	(X. Liu et al., 2013)	fwd CGAGGTTTGGTTCCTCGTCA rev AGCATGAAGCCCCACTTCTC	114 bp	2.05	0.97
C-24 sterol methyl transferase	erg6	(Dou et al., 2019)	fwd GATTGCGCCGAAAGGAACAA rev TTCAGGCTTCTTCCCAACCA	124 bp	2.49	0.99
Cytochrome P450 sterol 14α-demethylase	erg11	(Dou et al., 2019; Fan et al., 2013)	fwd CCAAGGCAATGGCTGAGATA rev TGTTCGAATGCCCCCTTTT	248 bp	1.74	0.97
Aurofusarin polyketide synthase	pks12	(X. Liu et al., 2010)	fwd TGGTGTAGATGCTGTTCGTGT rev TGAACTTTTCGAGGACGGAT	292 bp	0.94	2.13
Aurofusarin synthesis gene	aurO	(X. Liu et al., 2010)	fwd TCGGCACATCAGTATCTCCAA rev CAATACTATCGCCTGTCGCTT	275 bp	1.83	0.99

Table 5: List of primers used for pre-RNA-Seq real-time PCR studies on potato leaves.

Annotation	Gene	ID	Primer sequence	Amplicon	E	R^2
Elongation factor 1-alpha[1,2]	ef1-α	PGSC0003DMG400023270	fwd TGAGGCAAACTGTTGCTGTC rev TGGAAACACCAGCATCACAC	126 bp	2,03	0,97
S. tuberosum gene for 18S rRNA[1]	18s	GenBank: X67238.1	fwd TGATAACTCGACGGATCGCA rev TGGATGTGGTAGCCGTTTCT	166 bp	1,88	0,99
Conserved gene of unknown function [3]	wrky	PGSC0003DMG400009434	fwd ACCACCGCGATAAAACCAGT rev CGTTGGACAAGTGTGACATCC	135 bp	1.80	0.85 [4]
ABC transporter family protein	abc	PGSC0003DMG400014879	fwd TCATCACCCCGACAGTATGAC rev CAGGACTTGGACCTGCCTT	134 bp	1.83	0.91
Respiratory burst oxidase homolog C	rbohc	PGSC0003DMG400014168	fwd GGAGTGTTCTACTGTGGGGC rev ACTGTCCGTGGCAGTTTTGA	168 bp	2.12	0.99

[1] Reference genes

[2] Sequence adapted from (Llorente et al., 2010)

[3] VQ-motif containing protein, interacting with wrky transcription factors as stated in (Goyer et al., 2015)

[4] This R^2 value was low, however considered as still acceptable.

Table 6: List of primers used for post-RNA-Seq real-time PCR studies on potato leaves.

Annotation	Gene	ID	Primer sequence	Amplicon	E	R^2
Elongation factor 1-alpha[1,2]	ef1-α	PGSC0003DMG400023270	fwd TGAGGCAAACTGTTGCTGTC rev TGGAAACACCAGCATCACAC	126 bp	2,03	0,97
S. tuberosum gene for 18S rRNA[1]	18s	GenBank: X67238.1	fwd TGATAACTCGACGGATCGCA rev TGGATGTGGTAGCCGTTTCT	166 bp	1,88	0,99
Photosystem Q(B) protein	psbA	PGSC0003DMG400004211	fwd TAGAGAGACGCGAAAGCGAA rev TCTACTGGAGGAGCAGCAATG	163 bp	1,72	0,99
Photosystem II CP47 chlorophyll apoprotein	psbB	PGSC0003DMT400096732	fwd TCGAAGAGTTAGTGCTGGGC rev TGTCCTAACCATCCAACCGC	174 bp	2,08	0,99
Photosystem II D2 protein	psbD	PGSC0003DMG400017258	fwd GTAGGGGGTTGGTTCACAGG rev CTTGTGCTTCAGGACCCCAT	163 bp	2,13	0,99
Apocytochrome f	petA	PGSC0003DMG400002905	fwd CCACAAGCGGTACTTCCTGA rev AATACGATCGGGAGGGGCTA	162 bp	2,05	0,98
NAD(P)H-quinone oxidoreductase chain,	NDH	PGSC0003DMG401011339	fwd AAACAATACGAGCCGCCAGA rev TGTATTCGGCCTCGGAAACG	133 bp	2,03	0,99

[1] Reference genes

[2] Sequence adapted from (Llorente et al., 2010)

2.6.6. Primer efficiencies

Being a highly sensitive method for gene expression quantification, the outcome of real time PCR experiments strongly depends on the efficiency of each primer pair. Ideally, the target sequence molecules should double in each PCR cycle, corresponding to a 100 % primer efficiency. However, non-optimal primer design or reaction conditions can lead to less efficient amplification. Hence, determination of the efficiency of primers is a crucial step in case of comparing the expression of multiple genes of interest and was set as essential information by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009). To measure the primer efficiency, a standard curve was generated, using a high-quality cDNA template (as determined via Nanodrop) in several factor 10 dilution steps (1:10 to 1:1000000). The standard curve was generated by plotting the log of the dilution factor of the cDNA against the cycle threshold (Ct) value obtained during amplification. An R^2 value > 0.9 was considered as sufficient fitting of the experimental data to the regression line. The primer or amplification efficiency E is eventually calculated from the standard curve slope by the formula

$$E = 10^{-1/slope}$$

with E = 2 meaning a 2-fold increase in the number of cDNA copies and thus being the ideal efficiency of 100 %. Hence, the ideal slope of a standard curve is given as

$$2 = 10^{-1/slope} \Leftrightarrow Slope = -3.32$$

2.6.7. Real-time PCR

Real-time PCR was done using a CFX96 Touch Real-Time PCR Detection System by Bio-Rad Laboratories, Inc. (Hercules, California, USA) with an initial denaturation at 95 °C for 3 min, followed by 44 cycles of 95 °C for 3 s and 60 °C for 20 s. To verify single fragment amplification and exclude genomic DNA contamination (M. et al., 2006) subsequent melting curve analysis was performed with increasing temperatures from 58 to 95 °C whereby the temperature was increased by 0.5 °C every 5 s. The samples contained 2.5 µL of 1:50 diluted cDNA or dH_2O (as non-target control (NTC)), 2.5 µL of a 1:250 diluted mix of one primer pair and 5 µL of KAPA SYBR FAST qPCR Master Mix from Sigma-Aldrich (St. Louis, Missouri, USA). NTCs were not used for analysis, but for verification that no unspecific dye binding occurred due to primer dimerization, which would cause false-positive fluorescence signals.

Real-time PCR data was analyzed regarding relative gene expression ratio normalized to the chosen reference genes using the formula

$$ratio = \frac{(E_{target})^{\Delta Ct_{target}(control-sample)}}{(E_{ref})^{\Delta Ct_{ref}(control-sample)}}$$

with E_{target} being the primer efficiency of the gene of interest, E_{ref} the primer efficiency of the reference gene(s) and ΔCt the Ct deviation of control minus sample of either the gene of interest (target) or the reference genes (ref) (Pfaffl, 2001). REST-MCS (relative expression software tool – multiple condition solver) was used for direct multiple comparison of different time points, treatments and genes of interest (Pfaffl, 2002).

2.6.8. RNA-seq

For RNA-seq, plant leaves were prepared as described for real-time PCR experiments and treated with the same chitosan (651) and in the same concentration (500 µg/mL). Likewise, RNA isolation was conducted. Time points for RNA-seq were set to 2 h and 5 h after treatment as using these time points resulted in interesting and promising gene expression changes in preliminary real-time PCR studies. Using two treatments (chitosan and dH_2O as control) and two time points in biological replicates resulted in a total of 12 RNA-seq samples, which from now are referred to as H_2O_2h, H_2O_5h, CS_2h and CS_5h.

All following steps were carried out by GeneVia Technologies as mentioned before. A Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA) was used to analyze the quality of the RNA prior to sequencing.

2.6.8.1. Sequencing

The Illumina TruSeq mRNA protocol (https://support.illumina.com/downloads/truseq_stranded_mrna_sample_preparation_guide_15031047.html, accessed on 16.06.19) was used to prepare the sequencing library. For sequencing, an Illumina HiSeq 3000 (Illumina, San Diego, California, USA) was used. All 12 samples were sequenced on one HiSeq lane, resulting in 21 to 26 million reads of 50 bp per sample.

2.6.8.2. Raw data analysis and quality control

For raw sequencing output analysis, the potato reference genome and its annotation file was obtained from (https://plants.ensembl.org/Solanum_tuberosum/Info/Index, accessed on 18.06.19). For quality control, FastQC (Frenkel, 2009) and TrimGalore! (Krueger, 2018) were ran on the RNA-seq reads.

2.6.8.3. Read alignments and read counts

STAR aligner 2.5.2 (Dobin et al., 2013) was applied on the RNA-seq reads to align them to the potato reference genome and to obtain gene-level read counts. The read counts were subsequently normalized via regularized log transformation using the DESeq2 R package (Love et al., 2014), transforming the count data to the $\log_2$ scale. The samples were furthermore inspected visually using principal component analysis (PCA) and Pearson's correlation heatmap. For following analysis, technical replicates were combined by averaging their gene counts.

2.6.8.4. Differentially expressed genes

DESeq2 R package was furthermore used to normalize the data and to analyze it with regards to differentially expressed genes (DEG). DEG were determined via comparing the sample groups H_2O_2h and CS_2h as well as H_2O_5h and CS_5h. DEGs were visualized using bar diagrams as well as by creating heatmaps and volcano plots using pheatmap (Kolde, 2019) and ggplot2 (Wickham, 2011) respectively. As huge datasets and multiple comparisons increase the chance of false statistical analysis (S. Y. Chen et al., 2017), p-values had to be corrected for multiple testing according to the Benjamini-Hochberg adjustment procedure (Hochberg, 1995). Only genes with adjusted p-values lower than 0.05 and absolute $\log_2$ fold changes higher than 1 were considered as significantly differentially expressed.

2.6.8.5. Enrichment analysis

DEG from the different comparisons were subjected to enrichment analysis considering associations to Gene Ontology (GO) biological process terms. This analysis determined whether certain DEG could be classified to any GO terms and hence be further assigned to a defined biological function.

Enrichment analysis was conducted using the potato transcript and corresponding protein IDs from http://rsat.eead.csic.es/plants/data/genomes/Solanum_tuberosum.DM.v4.03.PGSC/genome/peptidic_sequences.fasta (accessed on 18.06.19) and assigning it to Ensembl protein

IDs and their corresponding GO terms. The R GO database (Meurk et al., 2013) was used to associate GO terms with their corresponding descriptions. All potato genes with GO annotation were used as a background set for the enrichment analysis. As mentioned for DEG analysis, the p-values were accordingly adjusted (Hochberg, 1995). Only GO terms with adjusted p-values below 0.05 and with at least 2 included potato genes were considered as significantly enriched. GO term comparisons were eventually visualized using –log10(p-value), commonly known as significance score.

2.6.8.6. Kyoto Encyclopedia of Genes and Genomes pathway annotation

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map of the for the photosynthetic light reaction in potato (Ogata et al., 1999) was used to allocate certain genes to their functions in the pathway map. The usage of the KEGG pathway was officially granted by Kanehisa Laboratories (Ogata et al., 1999; Kanehisa et al., 2017, 2019).

Chapter III

Results

3. Results

3.1. Antifungal activity of chitosan against *Fusarium graminearum*

3.1.1. Antifungal activity of COS and paCOS

Fully acetylated chitin oligosaccharides (DP 4-5) did not display strong antifungal activities, especially in comparison to copper acetate (CuAc) as positive control. However, significantly less OD_{600} was observed from a concentration of 60 µg/mL of COS (Figure 8). Nevertheless, OD values (and thus fungal growth) never reached values below 0.9, indicating only very slight antifungal activity. Furthermore, the antifungal activity did not increase with rising concentrations of up to 1500 µg/mL (tested, but not shown).

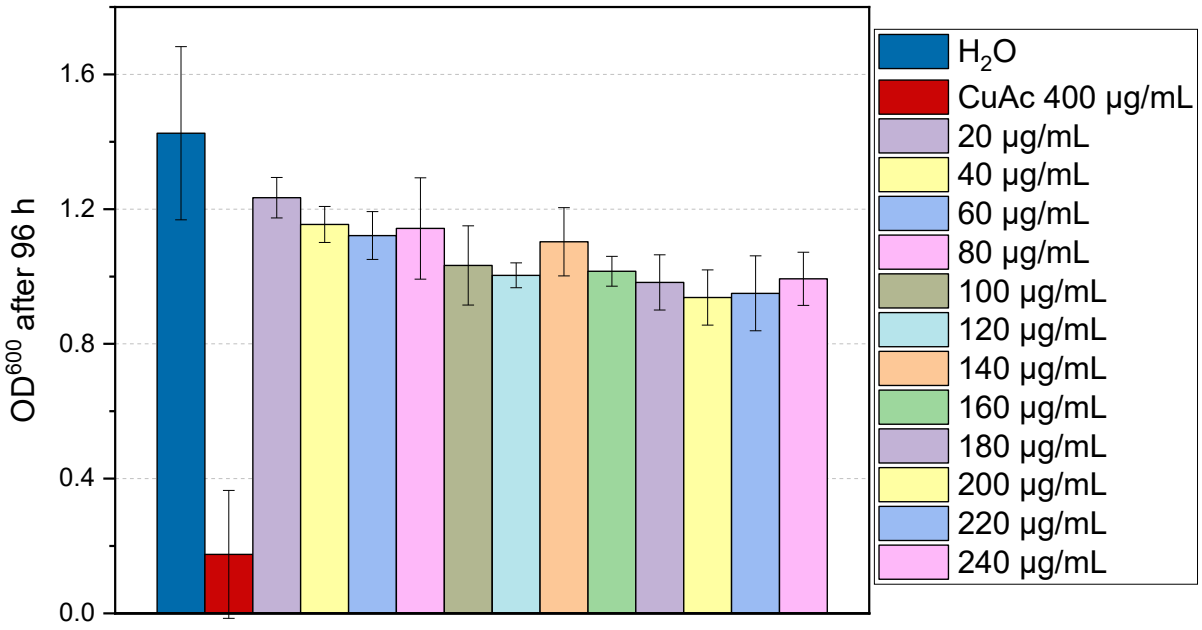

Figure 8: Antifungal activity of COS against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control, copper acetate (CuAc) as positive control. A significant antifungal activity was calculated at concentrations starting from 60 µg/mL according to ANOVA and post hoc Tukey test. N = 3, n = 18.

In contrast, partially acetylated COS (DP 4-5) derived from enzymatical de-acetylation did not show any antifungal activity. paCOS rather seemed to display a growth promoting effect with increasing paCOS concentrations resulting in higher OD values and thus more fungal biomass. Starting from concentrations of 250 µg/mL, fungal growth was significantly increased compared to H_2O control treatment (Figure 9).

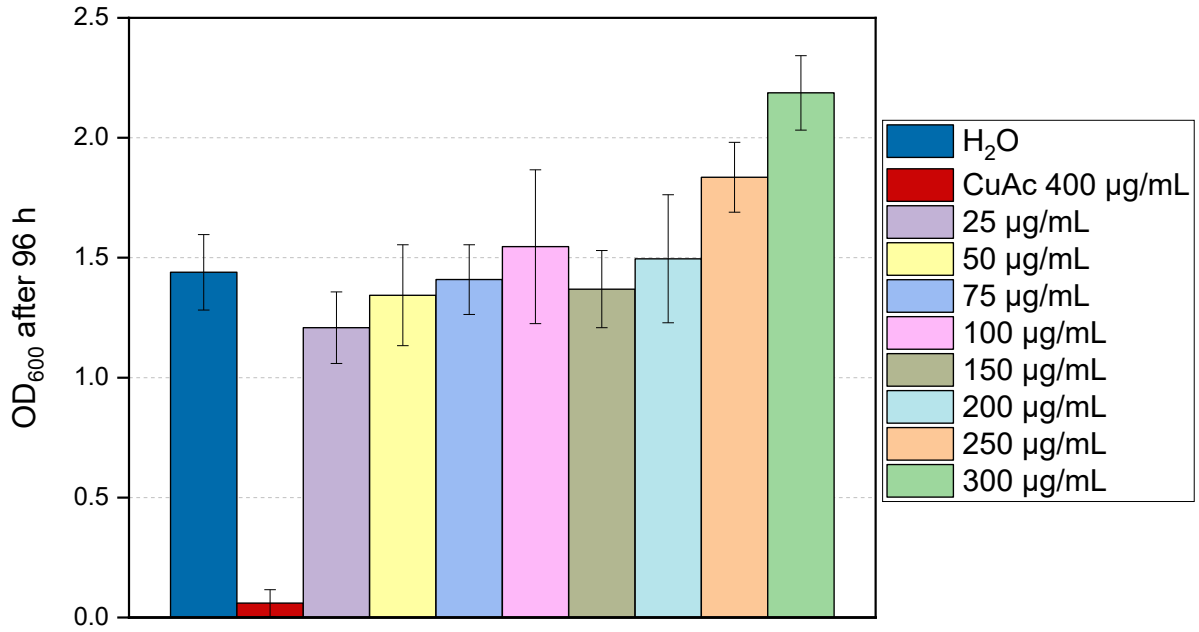

Figure 9: Antifungal activity of paCOS against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control, copper acetate (CuAc) as positive control. Significantly increased OD values in comparison to the negative control were calculated for concentrations starting from 250 µg/mL according to ANOVA and post hoc Tukey test. N = 1, n = 6.

These results lead to the assumption that antifungal activity of chitosan requires a certain minimum DP that was not reached by the tested COS and paCOS.

3.1.2. F_A-dependent antifungal activity

The dependency of the antifungal activity of chitosan on the F_A was tested using a chitosan series with F_A between 0.0 and 0.5, measured at a constant concentration of 30 µg/mL. In this experimental series, no significant F_A-dependent antifungal activity could be measured (Figure 10). Despite observing a slight trend of higher acetylated fractions displaying lower antifungal activity - when treating F_A 0.1 results as outliers -, high deviations between the fractions prevent any clear statements.

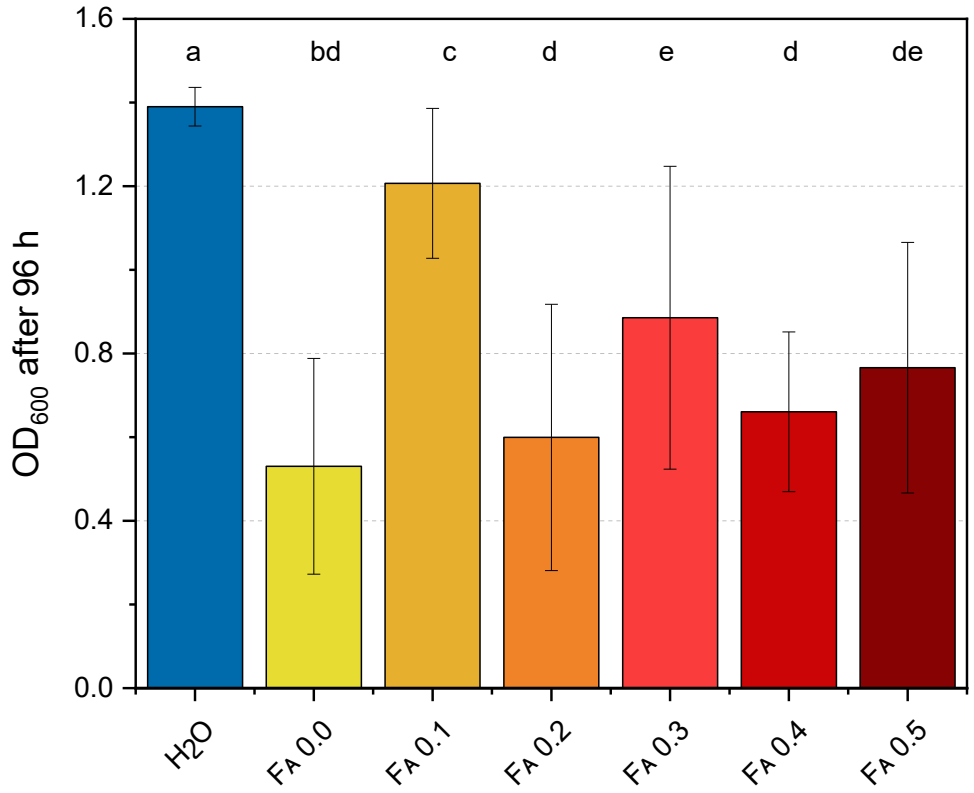

Figure 10: F_A-dependent antifungal activity of 134 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control. The 134 fractions were measured at a constant concentration of 30 µg/mL. N = 4, n = 24.

As the F_A-dependent antifungal activity was only measured at one concentration and with a chitosan of one (high) DPn, the question whether the antifungal activity of chitosan is dependent on the F_A could not be answered with this experimental series.

3.1.3. DP-dependent antifungal activity

Subsequent experiments focused on low F_A chitosans between 0.14 and 0.2, but with varying DPn. In a first experimental series, 651 was used as a commercially available chitosan with an intermediate DPn of 450 and an F_A of 0.2 (Figure 11). 651 displayed antifungal activity with a MIC_{50} of around 60 µg/mL.

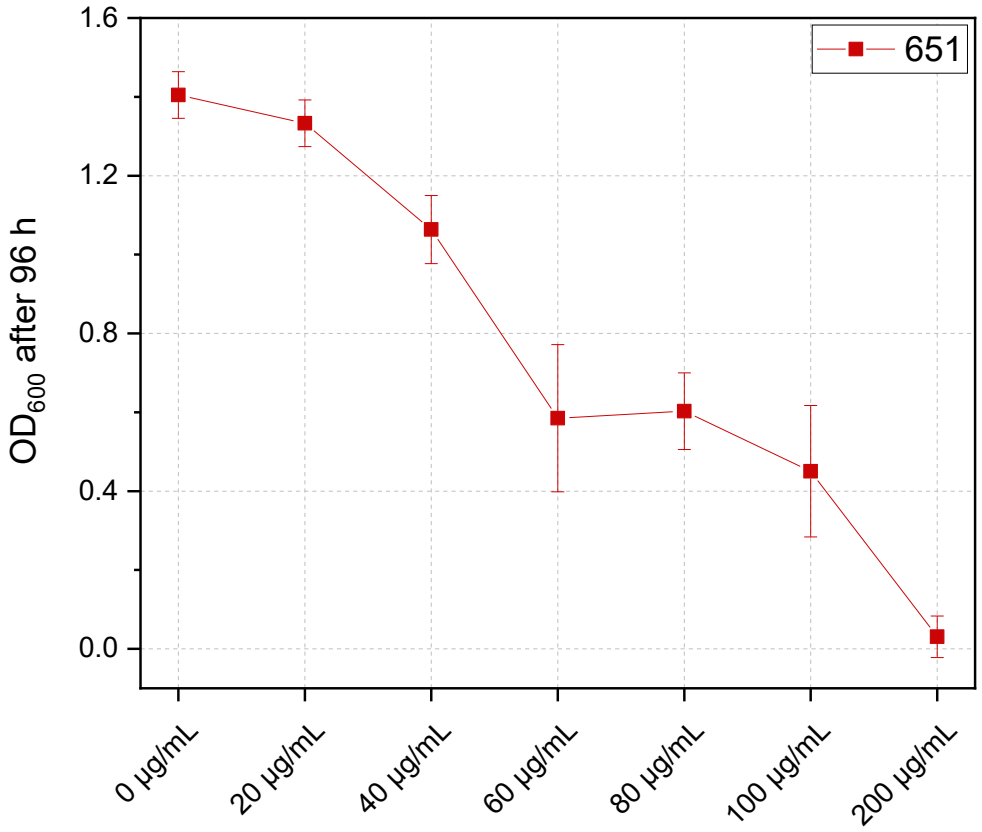

Figure 11: Antifungal activity of 651 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. A concentration of 0 µg/mL corresponds to H_2O supplementation as negative control. The MIC_{50} of 651 was observed at around 60 µg/mL. N = 4, n = 24.

In a second experimental series, 661 was tested for antifungal activity (Figure 12). 661 is the hydrolysis product of 651, containing of a fraction of small oligomers and a fraction of polymer molecules. 661 displayed a stronger antifungal activity with a MIC_{50} of around 30 µg/mL. Furthermore, separated oligomer and polymer fractions of 661 were screened for antifungal activity and compared with unseparated 661. The oligomer fraction 661 was comparably active to unseparated 661 with the same MIC_{50} of around 30 µg/mL, whereas the polymer fraction was observed to display lower antifungal activity with a MIC_{50} of around 50 µg/mL. Especially in intermediate concentrations, the antifungal activity of the polymer fraction was determined significantly lower compared to its oligomeric counterpart and the unseparated 661.

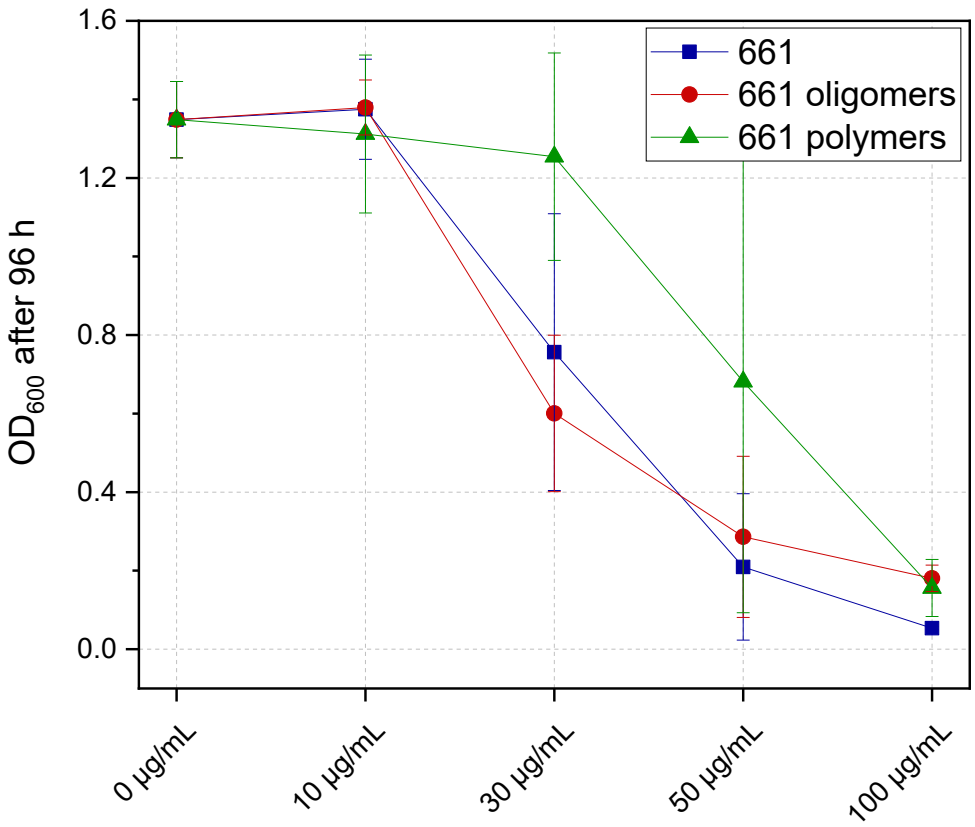

Figure 12: Antifungal activity of 661 (unseparated and separated oligomer and polymer fractions) against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. A concentration of 0 µg/mL corresponds to H_2O supplementation as negative control. Antifungal activity of the polymer fraction is significantly lower at 30 and 50 µg/mL according to ANOVA and post hoc Tukey test. The MIC_{50} of unseparated 661 and the 661 oligomer fraction was observed at 30 µg/mL, whereas the MIC_{50} of the 661 polymer fraction was observed at around 50 µg/mL. Unseparated 661: N = 6, n = 36. Separated 661 fractions: N = 8, n = 48.

In a third experimental series, three additional chitosan polymers were tested with a slightly lower F_A compared to 651 and 661 and a broader DPn range from 170 to 1081 (Figure 13). The DP-dependent antifungal activity already observed for 661 with smaller chitosans showing higher efficiency could be confirmed in this series. Particularly 75/100 showed visibly less antifungal activity in intermediate concentrations, however, no significance could be determined in this series. This chitosan showed a MIC_{50} of around 20 µg/mL whereas both 75/20 and 75/5 showed a slightly lower MIC_{50} of approximately 15 µg/mL.

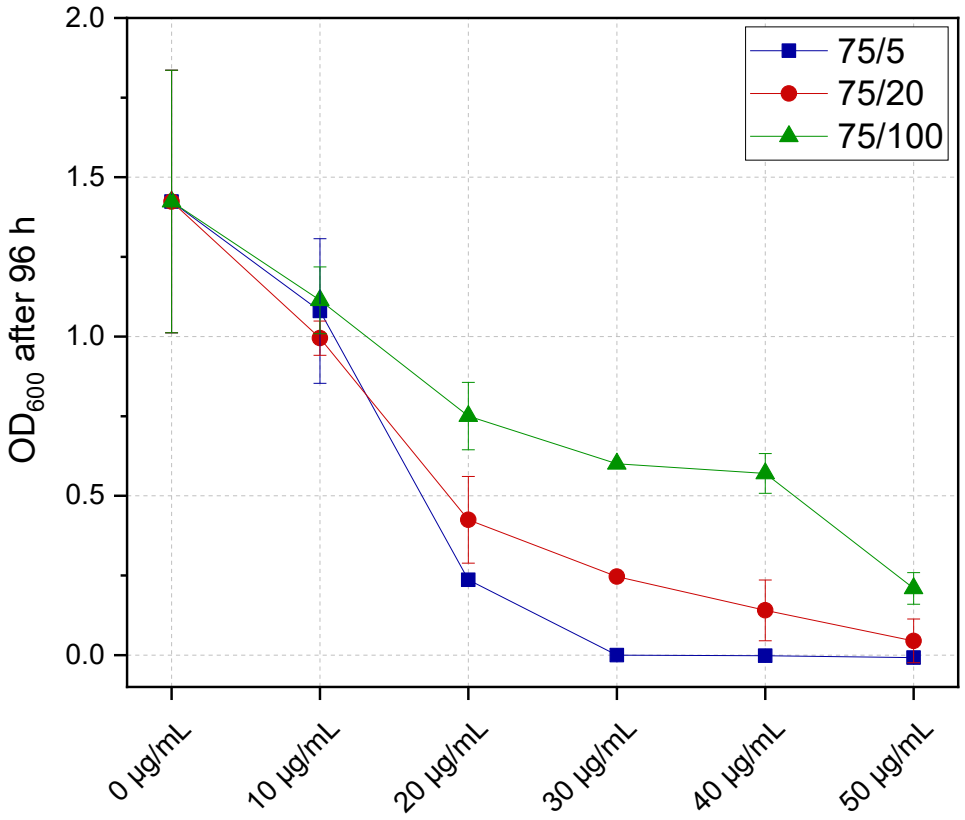

Figure 13: Antifungal activity of 75/5, 75/20 and 75/100 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. A concentration of 0 µg/mL corresponds to H_2O supplementation as negative control. MIC_{50} was determined as 15 µg/mL for 75/5 and 75/20 and as 20 µg/mL for 75/100, respectively. Despite of visible differences in antifungal activity in intermediate concentrations, no significance could be determined via ANOVA and post hoc Tukey test. N = 2, n = 12-24.

In summary, a DP-dependent antifungal activity of chitosans with a nearly constant F_A could be observed with smaller molecules showing higher efficiency. The MIC_{50} values of all chitosans screened for antifungal activity in a concentration range are summarized in Table 7.

Table 7: MIC_{50} values for chitosans screened for antifungal activity in a concentration range. Chitosans are sorted concerning their MIC_{50} from low to high.

Chitosan	F_A	DPn	Đ	MIC_{50}
75/5	0.14	170	2.2	15 µg/mL
75/20	0.14	499	1.5	15 µg/mL
75/100	0.15	1081	1.4	20 µg/mL
661 oligomer fraction	0.2	2-17	n.d.	30 µg/mL
661	0.2	343	5.5	30 µg/mL
661 polymer fraction	0.2	450	5	50 µg/mL
651	0.2	450	5	60 µg/mL

3.1.4. Antifungal activity of chitosan combinations

Synergistic activity of combinations of fungicide are described since decades and likewise synergy between fungicides and chitosans was observed. In this chapter, different chitosans were combined and tested whether this approach could increase the antifungal activity beyond the sum of activity of the individual chitosans.

In a first experimental series, 75/5, 75/20 and 75/100 were combined in an intermediate concentration of 30 µg/mL which resulted in different antifungal activity depending on the DPn as shown in the previous chapter (see Figure 13). It was observed that combinations of the three chitosans among each other lead to a complete inhibition of fungal growth. While the combinations of 75/5 with either 75/20 or 75/100 was determined as additivity, as 75/5 alone already resulted in total growth inhibition at 30 µg/mL, the combination of 75/20 and 75/100 was determined to have synergistic antifungal activity (Figure 14).

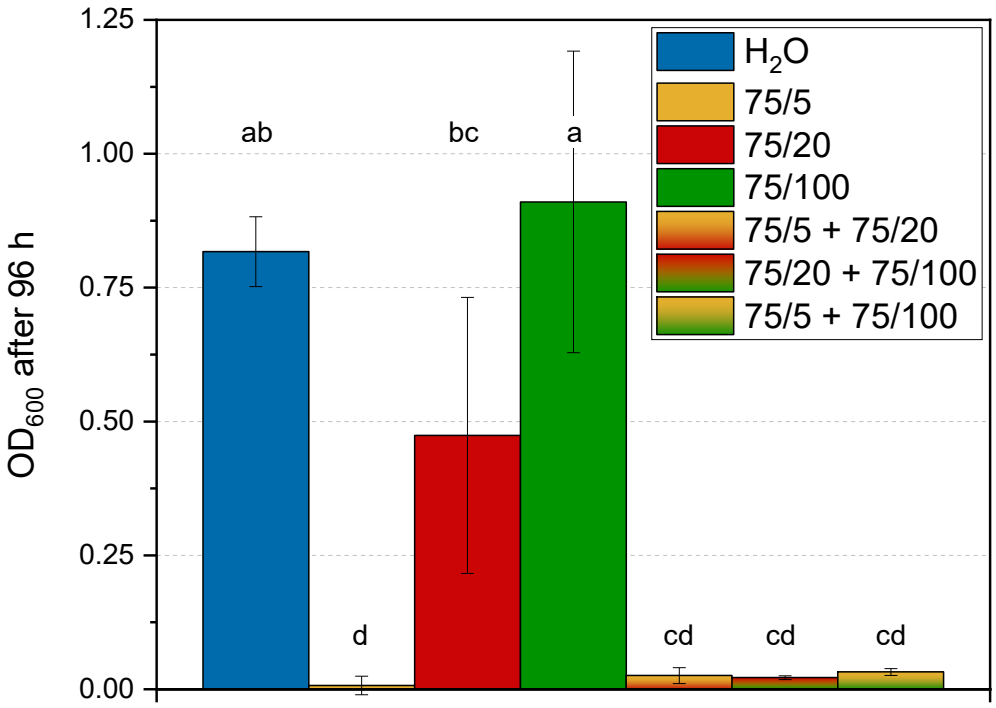

Figure 14: Antifungal activity of combinations of 75/5, 75/20 and 75/100 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control. Individual chitosans and chitosan combinations were tested at a constant concentration of 30 µg/mL. Combinations of 75/100 with 75/5 and 75/20 were determined significantly different to solely used 75/100 as calculated via ANOVA and post hoc Tukey test. The combination of 75/20 and 75/100 displayed high synergistic activity as determined via Abbott's formula. N = 3, n = 18-36.

These first experiments showed that combinations of chitosan polymers can successfully enhance their antifungal potential in a synergistic manner. The calculations concerning synergism according to Abbott's formula (Abbott, 1987) are listed in Table 8.

Table 8: Calculations for synergistic activity of 75-series chitosan combinations. The OD_{600} for H_2O treated *F. graminearum* conidia after 96 h was used as reference to determine the relative growth inhibition values for chitosan. This growth inhibition is the observed antifungal activity C_{obs}. The expected antifungal activity C_{exp} was calculated according to (Abbott, 1987). A synergistic factor SF close to 1 resembles additive activity whereas a SF greater than 1 resembles synergism.

	OD_{600}	**% inhibition (C_{obs})**	**C_{exp}**	**SF (C_{obs}/C_{exp})**
H_2O	0.817	0		
75/5	0.007	99.12		
75/20	0.474	41.99		
75/100	0.91	-11.36		
75/5 + 75/20	0.025	96.90	99.49	0.97
75/20 + 75/100	0.022	97.37	35.41	2.75
75/5 + 75/100	0.032	96.05	99.02	0.97

It was subsequently tested whether combinations of a small polymer with oligomers can lead to the same effect. For this, 75/5 was combined with the commercially available chitosan oligomer (paCOS) mixture CPS 4. Therefore, an inactive concentration of CPS 4 (30 µg/mL) was combined with a range of inactive concentrations of 75/5 (between 1 and 10 µg/mL). The individual antifungal activity of both chitosans are shown in Figure 15.

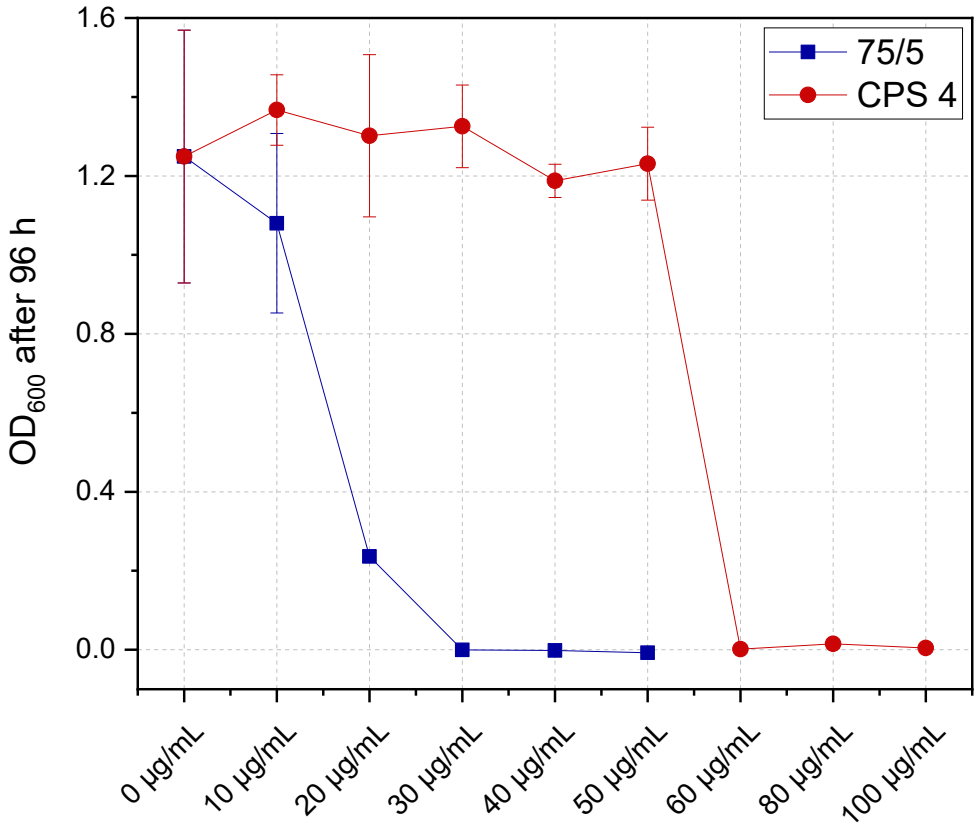

Figure 15: Antifungal activity of 75/5 and CPS 4 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. A concentration of 0 µg/mL corresponds to H_2O supplementation as negative control. MIC_{50} is around 15 µg/mL for 75/5 and around 55 µg/mL for CPS 4. 75/5: N = 2, n = 12-24. CPS 4: N = 1, n = 12.

Combining 30 µg/mL of CPS 4 with increasing concentrations of 75/5 did not lead to a gradual increase in antifungal activity. The activity slightly increased with 1 µg/mL and 2.5 µg/mL of 75/5, whereas concentrations of 5 µg/mL or 10 µg/mL of 75/5 resulted in a sudden and complete drop of fungal growth (Figure 16). All combinations of CPS 4 and 75/5 were determined as synergistic in comparison to the individual chitosans.

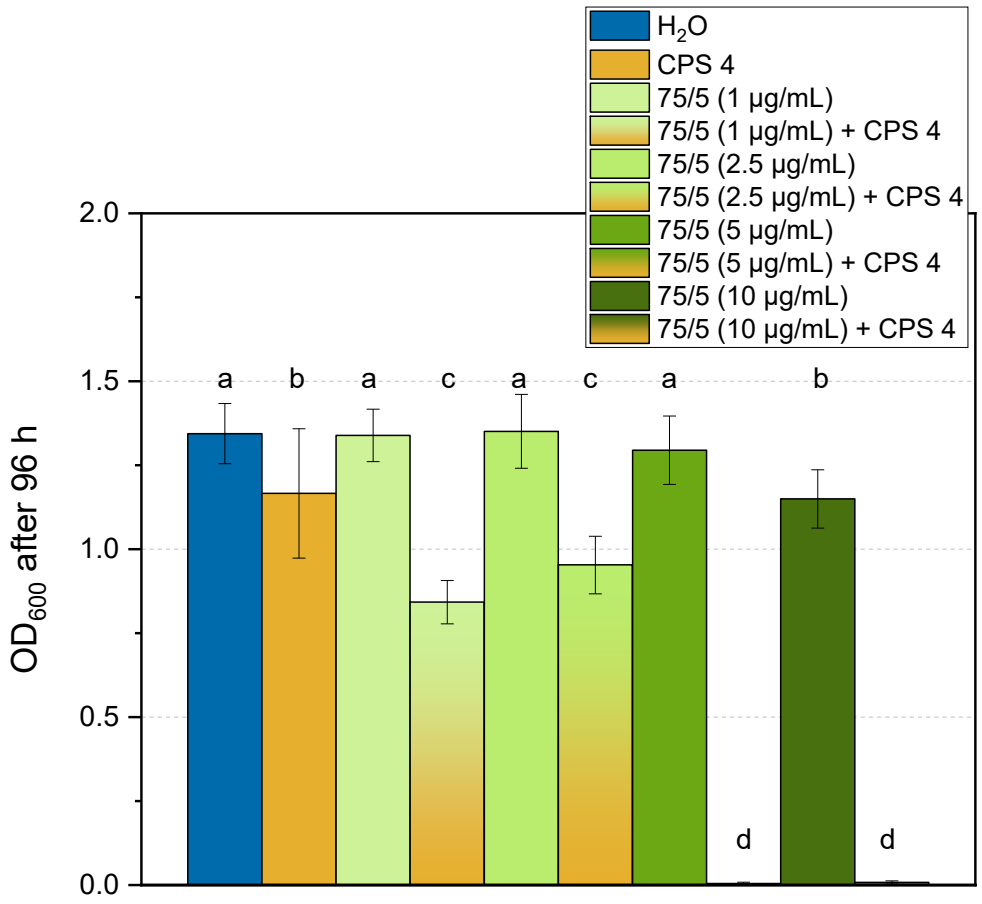

Figure 16: Antifungal activity of combinations of 75/5 and CPS 4 against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control. A constant concentration of 30 µg/mL of CPS 4 was combined with a range of low concentrations (between 1 and 10 µg/mL) of 75/5. Both chitosans had no to low antifungal activity at these concentrations when applied solely, whereas all combinations were determined to show synergism according to Abbott's formula and showed significantly higher antifungal activity compared to the individual chitosans as calculated using ANOVA and post hoc Tukey test. N = 4, n = 6-42.

With these experiments, it could be shown that synergistic activity of two different chitosans can also be achieved by mixing a polymer with an oligomer. Synergism calculations concerning the data seen above are listed in Table 9.

Table 9: Calculations for synergistic activity of chitosan combinations of CPS 4 and 75/5. The OD_{600} for H_2O treated *F. graminearum* conidia after 96 h was used as reference to determine the relative growth inhibition values for chitosan. This growth inhibition is the observed antifungal activity C_{obs}. The expected antifungal activity C_{exp} was calculated according to (Abbott, 1987). A synergistic factor SF close to 1 resembles additive activity whereas a SF greater than 1 resembles synergism.

	OD_{600}	**% inhibition (C_{obs})**	**C_{exp}**	**SF (C_{obs}/C_{exp})**
H_2O	1.344	0		
CPS 4	1.166	13.24		
75/5 1 µg/mL	1.339	0.37		
75/5 2.5 µg/mL	1.351	-0.51		
75/5 5 µg/mL	1.295	3.66		
75/5 10 µg/mL	1.149	14.45		
75/5 1 µg/mL + CPS 4	0.842	37.34	13.56	2.75
75/5 2.5 µg/mL + CPS 4	0.953	29.1	12.79	2.27
75/5 5 µg/mL + CPS 4	0.004	99.72	16.41	6.07
75/5 10 µg/mL + CPS 4	0.008	99.44	25.77	3.86

To gain knowledge about which fraction of a chitosan is the more important, hence the more active component, 661 and its separated fractions were used once again. This time, the ratio of polymer and oligomer fraction was altered while keeping the concentration constant at 30 µg/mL. The ratio of polymer and oligomer fraction was either shifted to an excess of polymer fraction or to an excess of oligomer fraction. The different ratios were compared to the 661 starting product and the sole separated fractions (Figure 17).

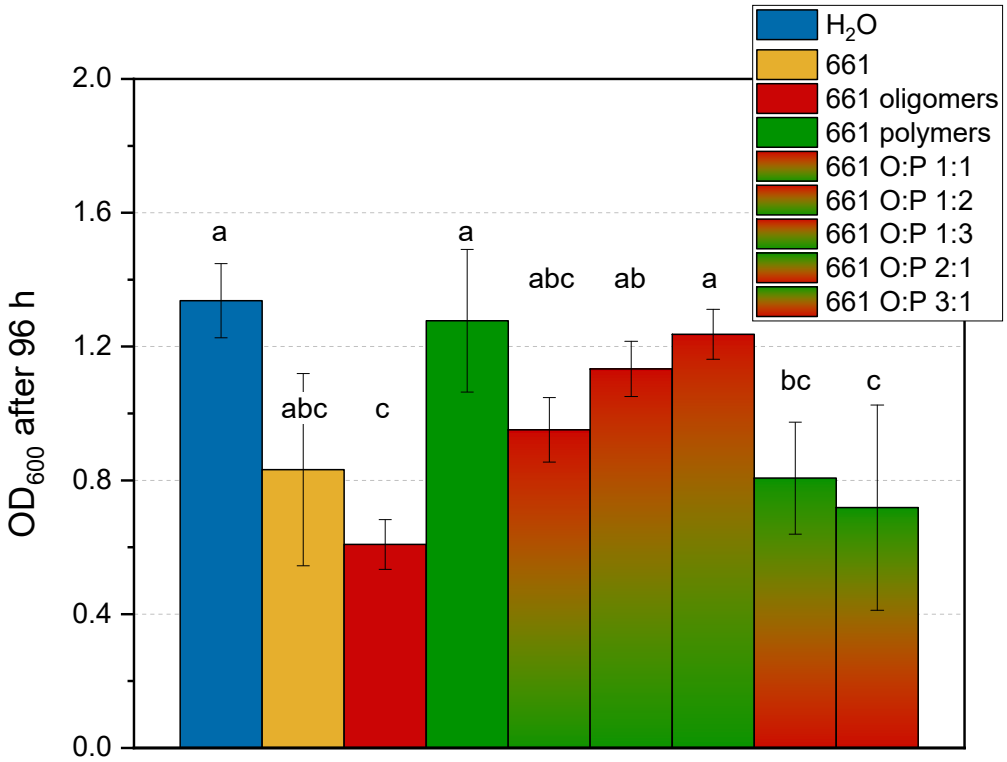

Figure 17: Antifungal activity of different ratios of 661 polymer and oligomer fraction against *F. graminearum*. Y-axis plots the OD at 600 nm with higher values indicating more conidia germination and, hence, fungal growth. H_2O supplement to CM growth medium was used as negative control. All chitosans were tested at a constant concentration of 30 µg/mL. Different ratios resulted either in an excess of polymers (P) or oligomers (O). A shift to an excess of polymers resulted in significantly less antifungal activity, whereas a shift to an excess of oligomers resulted in significantly higher antifungal activity, respectively, determined via ANOVA and post hoc Tukey Test. N = 3, n = 18.

While the antifungal activity of an oligomer/polymer ratio of 1:1 roughly corresponds to the antifungal activity of unaltered 661, it could be clearly observed that increasing the proportion of polymers decrease the antifungal activity, whereas increasing the proportion of oligomers increase the antifungal activity. Ratios in which one fraction was three times in excess roughly showed the antifungal activity induced by the excess fraction alone. These results showed that oligomers seem to be the driving force of the antifungal activity of chitosans.

To conclude, chitosan combinations are indeed capable of synergistically increasing the antifungal activity in comparison to the usage of the according individual chitosans. It was furthermore shown that chitosan oligomers generally seem to have a higher potential to display antifungal activity than chitosan polymers. Hence, defined mixtures of certain biologically active chitosans have huge potentials as antifungal agents without displaying the drawbacks of conventional, chemical-based fungicides.

3.2. Eliciting activity of chitosan

Eliciting activity of different chitosans was analyzed by triggering an oxidative burst in potato leaf discs. The oxidative burst was quantified via chemiluminescence-induced H_2O_2 production by the leaf discs in response to chitosan treatment. As part of PTI, an oxidative burst is a fast response of a plant to an elicitor and is characterized by a vast release of ROS over a short period of time (exemplarily shown in Figure 18). For evaluation and especially comparison purposes, the peaks of the burst responses (RLU_{max}) triggered by different chitosans were used as benchmark values.

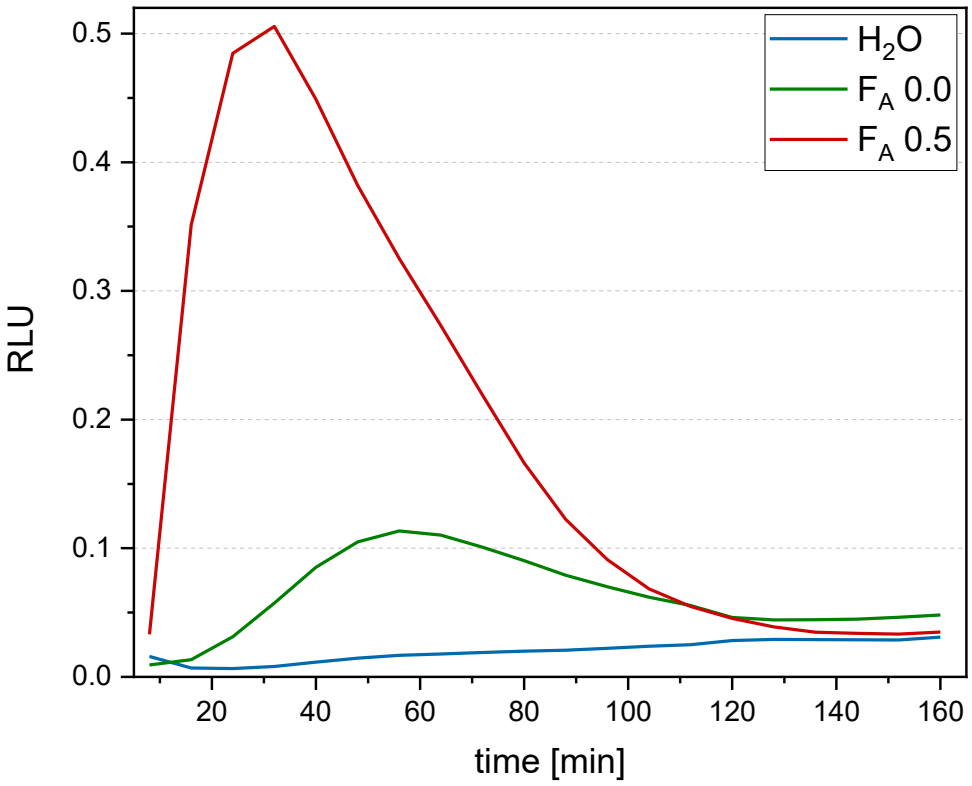

Figure 18: Oxidative burst curves. The diagram shows the oxidative burst response in RLU (Y-axis) over time (X-axis), using the example of two chitosans with an F_A of either 0.0 (pure chitosan) or 0.5 (based on 134). H_2O is used as negative control as it does not trigger an oxidative burst. Standard deviations are not shown.

3.2.1 Eliciting activity of COS and paCOS

No significant eliciting activity could be observed with COS in neither low (10 µg/mL) or high (100 µg/mL) concentration. Likewise, CPS 4 paCOS did not display eliciting activity in low concentration. Only a high concentration (100 µg/mL) of paCOS resulted in oxidative burst activity in the leaf discs (Figure 19).

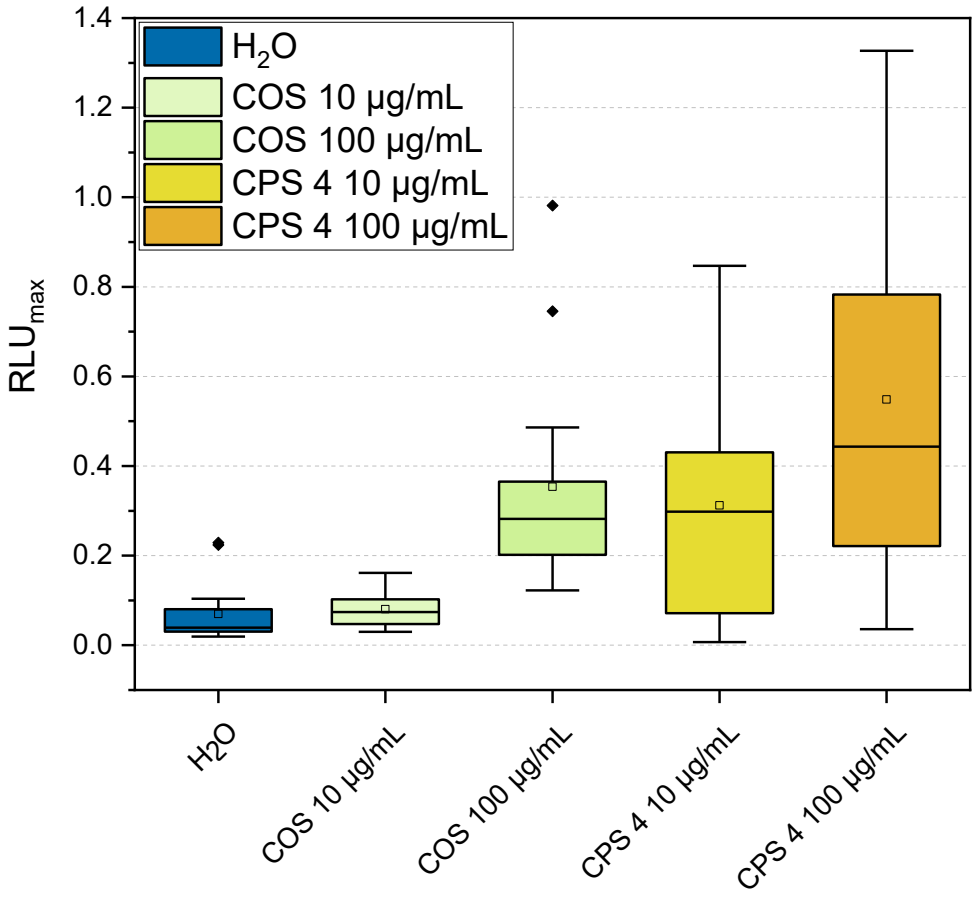

Figure 19: Eliciting activity of COS and paCOS (CPS 4) on potato leaf discs. Y-axis shows the maximum RLU measured for the tested chitosans. H_2O was used as negative control. Eliciting activity of COS in both concentrations and CPS 4 paCOS in low concentration did not significantly differ from H_2O as determined via ANOVA and post hoc Tukey test. COS: N = 2, n = 16. CPS 4: N = 3, n = 20.

Nevertheless, comparability of COS and paCOS is limited in this case, as CPS 4 paCOS instead of COS-derived paCOS were tested, hence, both chitosans had a difference in DPn and especially in the range of differently sized oligomers.

3.2.2 F_A-dependent eliciting activity

The eliciting activity of a chitosan polymer (100 µg/mL) was observed to be highly F_A-dependent, with higher F_A leading to stronger oxidative bursts. However, the highest oxidative burst responses were measured with an F_A between 0.4 and 0.5, while an F_A of 0.6 showed decreasing eliciting activity (Figure 20). This led to the conclusion that the eliciting activity is not fully proportionally dependent on the acetylation degree, but is furthermore influenced by other physico-chemical properties of chitosan.

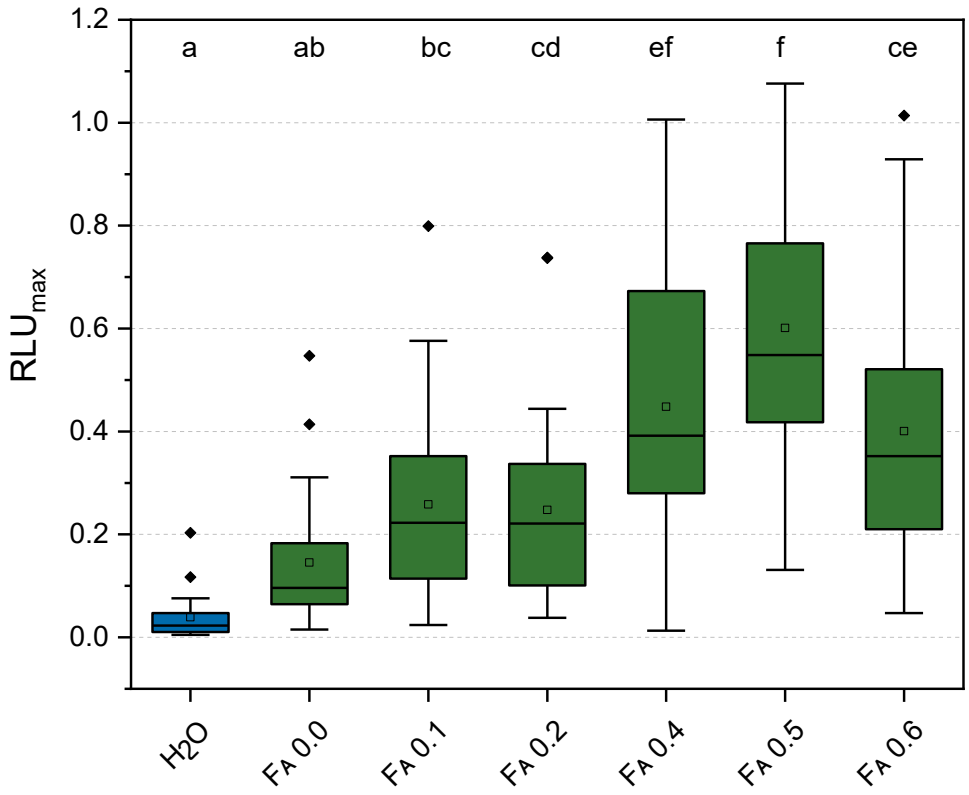

Figure 20: F_A-dependent eliciting activity of 134 on potato leaf discs. Y-axis shows the maximum RLU measured for the tested chitosans. H_2O was used as negative control. Chitosans were tested at a constant concentration of 100 µg/mL. Eliciting activity of F_A 0.4 and F_A 0.5 was significantly higher than for lower and higher F_A, but not among each other (determined via ANOVA and post hoc Tukey test). N = 5, n = 36-40.

3.2.3 DP-dependent eliciting activity

Analogously to the antifungal activity, the influence of DPn on the eliciting activity of chitosan was investigated, again using a constant F_A of between 0.1 and 0.2. As oxidative burst assays suffer from high deviation due to natural variation of plants and barely controllable abiotic conditions, comparing the maximum eliciting activity of biological replicates or even different experimental series is not feasible. Instead, it was focused on the eliciting-active concentration range of a chitosan for comparison purposes. The active concentration range was determined via ANOVA and post hoc Tukey test for significant differences of an active chitosan concentration to the corresponding H_2O negative control.

The eliciting activity of the commercial paCOS CPS 4 is shown in Figure 21. CPS 4 was observed to be active within a broad concentration range from 10 to 750 µg/mL.

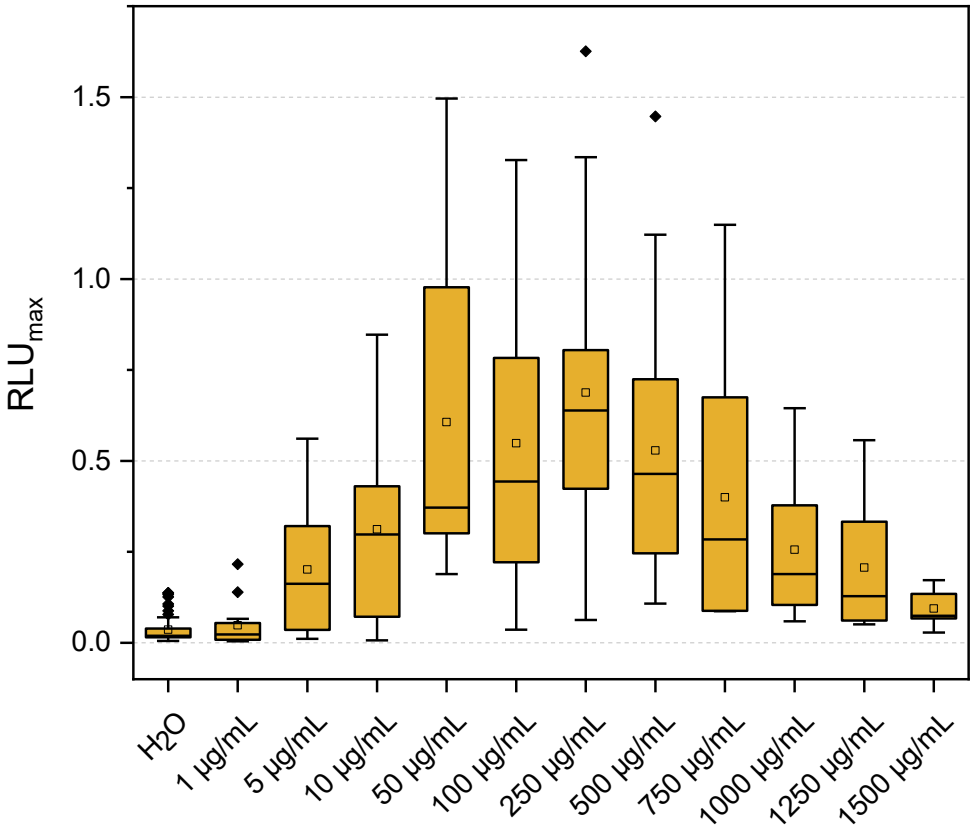

Figure 21: Eliciting activity of CPS 4 on potato leaf discs. Y-axis shows the maximum RLU measured for each chitosan concentration. H_2O was used as negative control. The eliciting-active concentration range was determined to reach from 10 to 750 µg/mL according to ANOVA and post hoc Tukey test. N = 3, n = 24-56.

Unseparated 661 and its oligomer and polymer fractions were furthermore tested in a second experimental series (Figure 22). The 661 oligomer fraction displayed the broadest range of eliciting-active concentrations (between 10 and 200 µg/mL), whereas the eliciting activity of unseparated 661 was already rapidly decreased at a concentration of 200 µg/mL, which did not significantly differ from the H_2O control. The 661 polymer fraction did not show eliciting activity at concentrations of 100 µg/mL or higher. Furthermore, its eliciting activity seemed nearly limited to a narrow range of low concentrations. In addition, the eliciting activity of the 661 oligomer fraction seemed slightly enhanced in comparison to the eliciting activity of unseparated 661, however, this increase was not significant according to ANOVA and post hoc Tukey test.

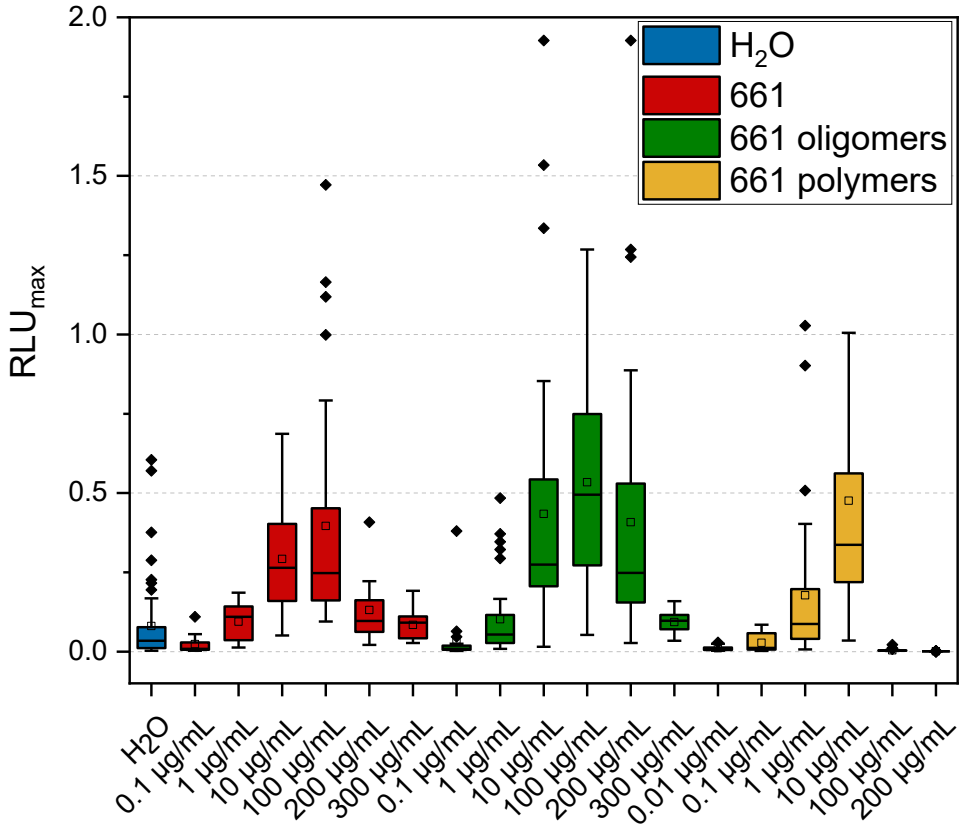

Figure 22: Eliciting activity of 661 (unseparated and separated oligomer and polymer fractions) on potato leaf discs. Y-axis shows the maximum RLU measured for each chitosan and each chitosan concentration. H_2O was used as negative control. The eliciting-active concentration ranges of 661 and the 661 oligomer fraction was observed to be between 10 and 100 µg/mL. The 661 polymer fraction only showed eliciting activity at 10 µg/mL as determined via ANOVA and post hoc Tukey test. N = 7, n = 16-41.

As the group of the next larger chitosans used in this study, the eliciting activity of 75/5, 75/20 and 75/100 were analyzed as shown as in Figure 23. This series confirmed the results of the eliciting of 661 concerning the broadness of the eliciting-active concentration range. Accordingly, 75/5 and 75/20 as the smaller chitosans of this series displayed the broadest eliciting-active concentration range (between 1 and 100 µg/mL), whereas the active concentration range of 75/100, the largest chitosan in the series, was already observed to be narrower, displaying decreased eliciting activity in higher concentrations of 100 µg/mL and beyond. However, according to ANOVA and post hoc Tukey test, 100 µg/mL of 75/100 still resulted in significantly higher eliciting activity in comparison to the H_2O negative control.

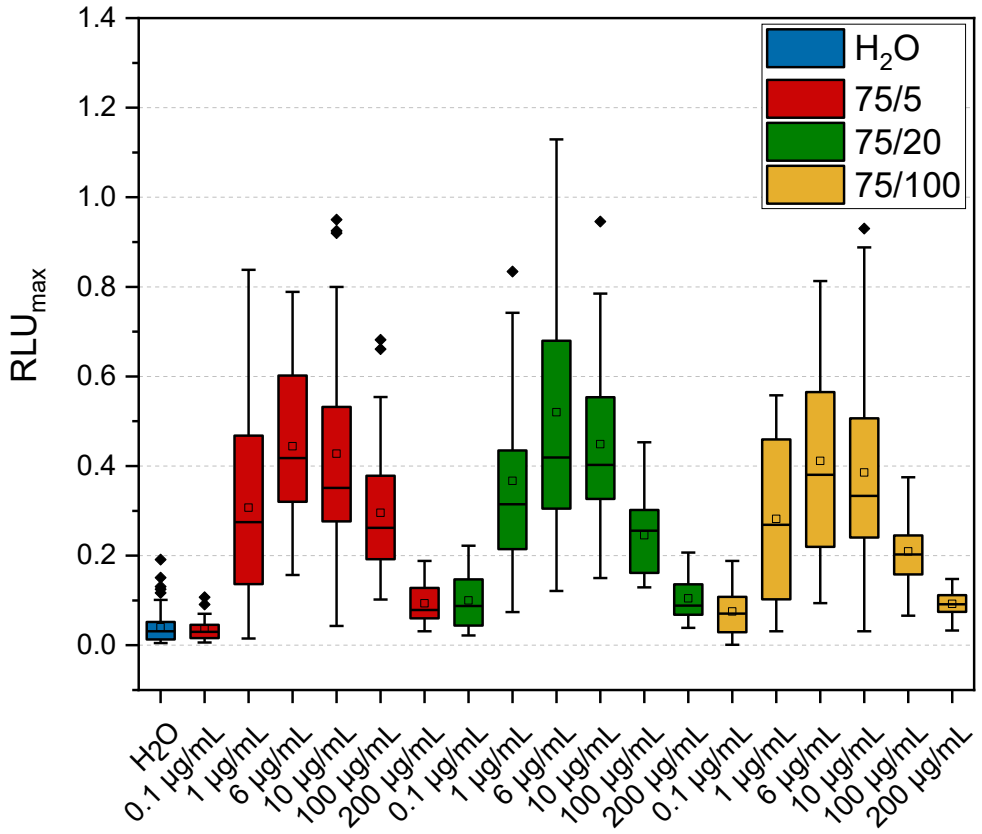

Figure 23: Eliciting activity of 75/5, 75/20 and 75/100 on potato leaf discs. Y-axis shows the maximum RLU measured for each chitosan and each chitosan concentration. H_2O was used as negative control. The range of eliciting-active concentrations was determined to be between 1 and 100 µg/mL according to ANOVA and post hoc Tukey test. N = 13, n = 16-60.

To further investigate the influence of the DPn on both the maximum eliciting-active concentration and the corresponding concentration range, two large chitosan polymers (90/1000 and 90/3000) were included in the oxidative burst analyses (Figure 24). The concentration range in which the chitosans show eliciting activity was even more narrowed in comparison to the previously analyzed chitosans. 90/1000 was observed to be active at concentrations between 0,5 and 10 µg/mL, whereas the concentration range of 90/3000 was once more narrowed (1 µg - 10 µg/mL.

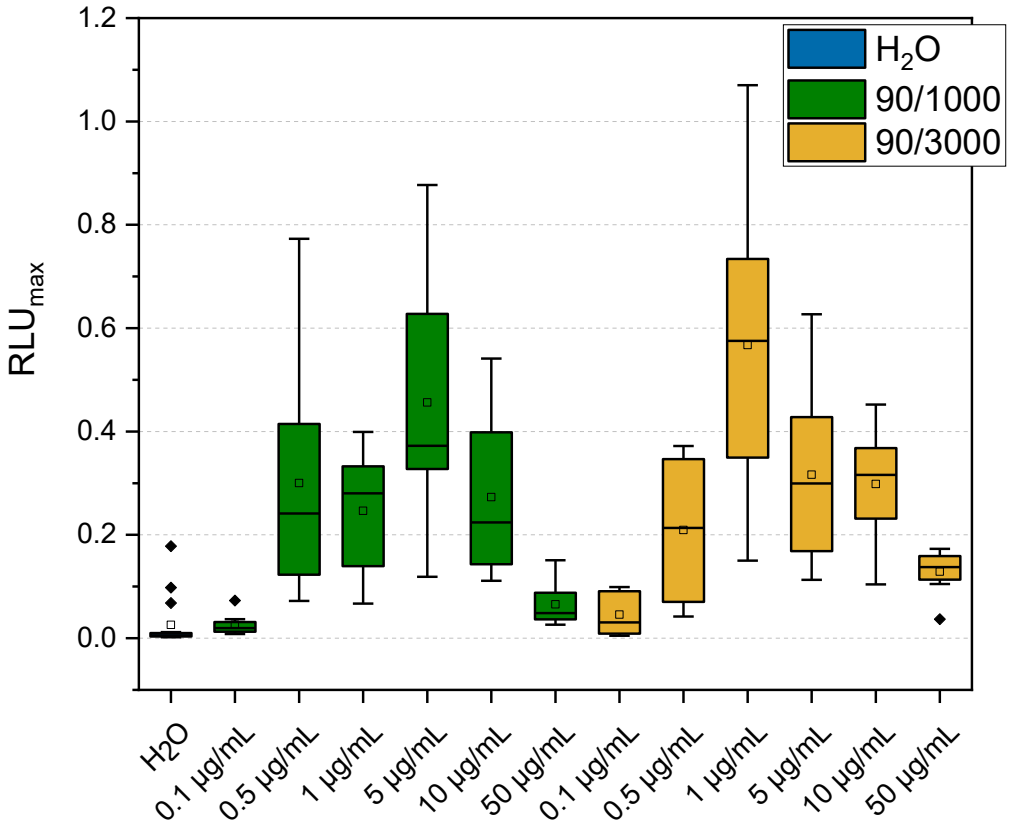

Figure 24: Eliciting activity of 90/1000 and 90/3000 on potato leaf discs. Y-axis shows the maximum RLU measured for each chitosan and each chitosan concentration. H_2O was used as negative control. The eliciting-active concentrations ranged from 0,5 to 10 µg/mL for 90/1000 and from 1 to 10 µg/mL for 90/3000, respectively, as determined via ANOVA and post hoc Tukey test. N = 2, n = 8-16.

In conclusion, the DP of a chitosan highly influences its eliciting activity. It could be shown that the concentration range in which a chitosan is biologically active in terms of oxidative burst responses is broader for smaller molecules than for larger molecules. Furthermore, small molecules can be used in higher concentrations, whereas large molecules more likely become inactive in high concentrations. On the other hand, large chitosan molecules showed eliciting activity already in lower concentrations compared to small chitosans. These observations are summarized in Table 10.

Table 10: Concentration range of eliciting activity. The minimum and maximum concentrations, determining the range of each chitosan in terms of triggering an oxidative burst in potato leafs was determined via ANOVA and post hoc Tukey test regarding significant differences in eliciting activity compared to the H_2O negative control. Chitosans are sorted concerning their active concentration ranges from broad to narrow.

Chitosan	F_A	DPn	Đ	Concentration range
CPS 4	0.1	2-12	n.d.	10 - 750 µg/mL
661 oligomer fraction	0.2	2-17	n.d.	10 - 200 µg/mL
75/5	0.14	170	2.2	1 - 100 µg/mL
75/20	0.14	499	1.5	1 - 100 µg/mL
75/100	0.15	1081	1.4	1 - 100 µg/mL
661	0.2	343	5.5	10 - 100 µg/mL
90/1000	0.14	1671	1.3	0,5 - 10 µg/mL
90/3000	0.14	2087	1.3	1 - 10 µg/mL
661 polymer fraction	0.2	450	5	5 - 10 µg/mL [1]

[1] 5 µg/mL of the 661 polymer fraction was observed to have eliciting activity significantly different compared to H_2O treatment. The result is not included in the corresponding figure.

3.2.4 Eliciting activity of chitosan combinations

To investigate possible synergistic activity of a chitosan combination concerning its potential to trigger an oxidative burst in potato leafs, the small polymer chitosan 75/5 was combined with the large polymer chitosans 90/1000 and 90/3000 respectively. 75/5 was used in a concentration of 1 µg/mL which was the lowest concentration found to be eliciting-active (Figure 23). On the other hand, 90/1000 and 90/3000 were used in a low concentration of 0.1 µg/mL, which was observed to be almost biologically inactive (Figure 24). As expected, 1 µg/mL of 75/5 triggered oxidative bursts, while 0.1 µg/mL of neither 90/1000 nor 90/3000 resulted in eliciting activity that significantly differed from the H_2O negative control. However, combination of 75/5 with either 90/1000 or 90/3000 resulted in visibly higher oxidative burst responses on potato leaf discs (Figure 25). The eliciting activity triggered by the combination of 75/5 and 90/1000 was significantly higher than its individual components according to ANOVA and post hoc Tukey test and displayed synergistic activity as determined via Abbott's formula (see Table 11).

Although the combination of 75/5 and 90/3000 was not determined to be significantly enhanced in comparison to the individual components, synergism was indeed confirmed (see Table 11).

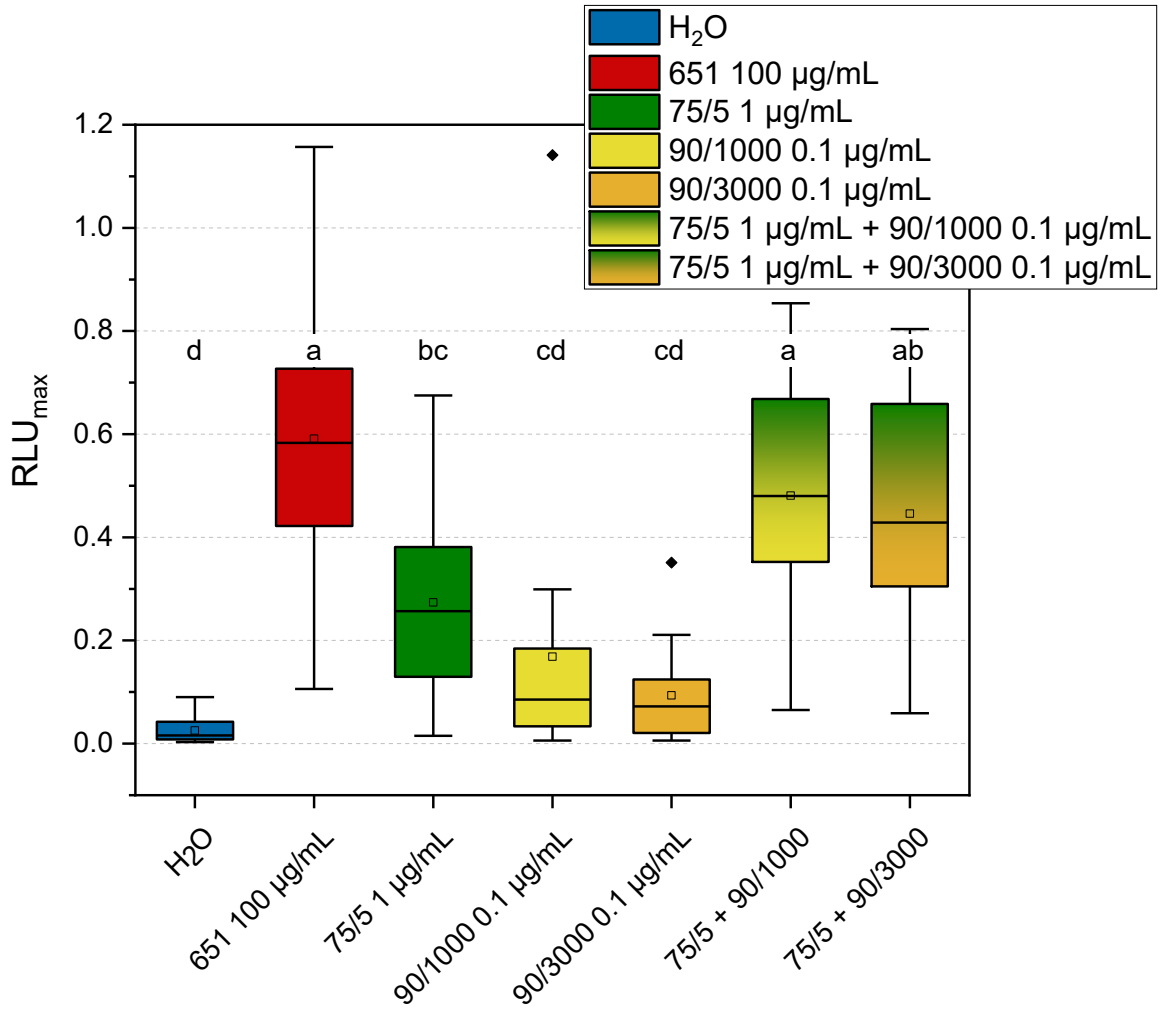

Figure 25: Eliciting activity of combinations of 75/5 with either 90/1000 or 90/3000. Y-axis shows the maximum RLU measured for each chitosan and each chitosan concentration. H_2O was used as negative control, 651 as positive control. Both combinations lead to visibly higher eliciting activity. The combination of 75/5 with 90/1000 was determined to be significantly increased compared to its individual components according to ANOVA and post hoc Tukey test. Both combinations show synergistic activity as calculated via Abbott's formula. N = 6, n = 16-48.

Table 11: Calculations for synergistic activity of 75/5 and 90/1000 or 90/3000 respectively. The RLU_{max} median value for 651-triggered oxidative bursts was used as reference to determine the relative eliciting activities. This eliciting activity is the observed activity C_{obs}. The expected eliciting activity C_{exp} was calculated according to (Abbott, 1987). A synergistic factor SF close to 1 resembles additive activity whereas a SF greater than 1 resembles synergism.

	RLU_{max}	**% elicitation (C_{obs})**	**C_{exp}**	**SF (C_{obs}/C_{exp})**
651 100 µg/mL	0.583	100		
75/5 1 µg/mL	0.257	44.08		
90/1000 0.1 µg/mL	0.086	14.67		
90/3000 0.1 µg/mL	0.072	12.35		
75/5 + 90/1000	0.48	82.33	52.28	1.57
75/5 + 90/3000	0.429	73.50	50.99	1.44

Similar to what was investigated concerning the driving force of antifungal activity, it was furthermore analyzed whether a similar trend could be observed concerning the eliciting activity of chitosan. As shown in Figure 26, shifting the 661 composition towards an excess of oligomers slightly enhanced the eliciting activity until a ratio of 1:5 of unseparated 661 to 661 oligomer fraction. With ratios of 1:4 and 1:5, the eliciting activity was significantly enhanced in comparison to the activity of unseparated 661 as calculated via ANOVA and post hoc Tukey test. A ratio of 1:6 however resulted in a sudden drop of eliciting activity to the activity of the 661 oligomer fraction alone. On the other hand, a shift towards an excess of 661 polymers led to a very low eliciting activity already at a 1:2 ratio (comparable to the H_2O negative control according to ANOVA and post hoc Tukey test). The higher the excess of polymers, the less the eliciting activity.

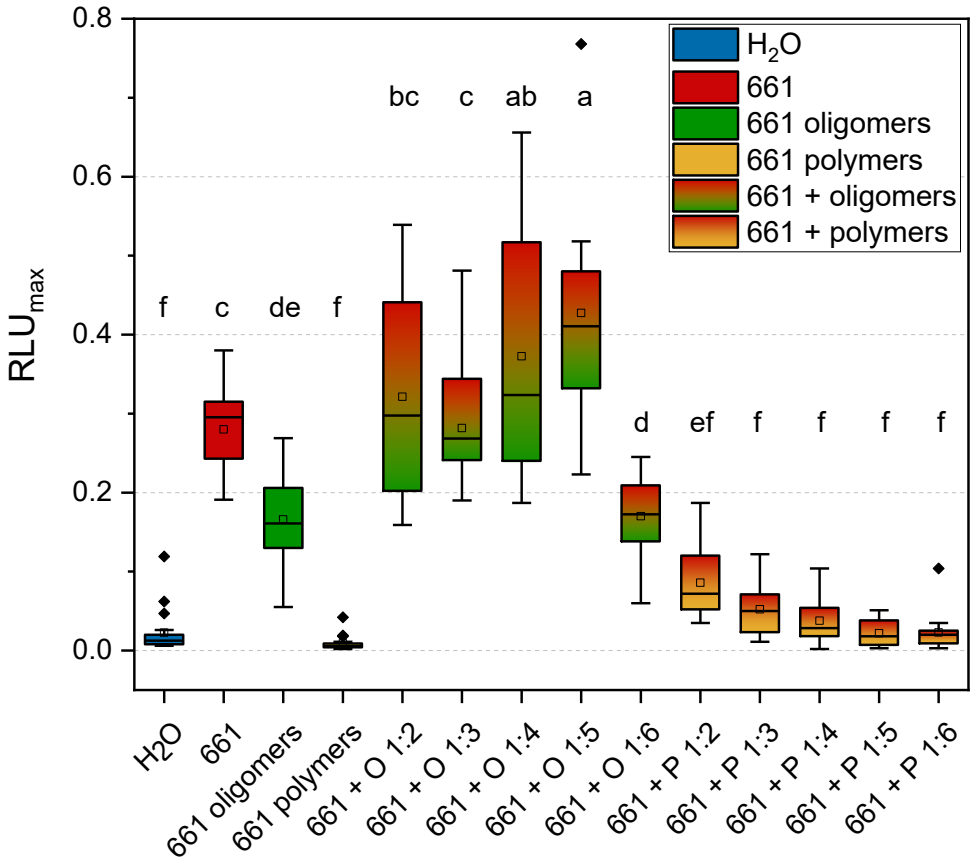

Figure 26: Eliciting activity of different ratios of unseparated 661 to either 661 oligomers or 661 polymers. Y-axis shows the maximum RLU measured for each chitosan and each chitosan concentration. H_2O was used as negative control. All samples were measured at a constant concentration of 100 µg/mL. Generally, an excess of 661 oligomers increases the eliciting activity (up to a ratio of 1:5), an excess of 661 polymers decreases the eliciting activity. Significant increases were determined with 1:4 and 1:5 ratios of unseparated 661 to 661 oligomers, significant decreases were determined with all ratios with polymer excess. N = 3, n = 18.

It was shown that chitosan combinations have the potential to act synergistically, enhancing the eliciting potential to an activity that cannot be achieved by the individual chitosans. As shown with 90/1000 and 90/3000, even almost inactive chitosans or chitosan concentrations, respectively, can contribute crucially to the eliciting activity. As shown Figure 26 though, not only finding the appropriate chitosans, but furthermore selecting the most suitable ratio of each individual component seems to be a critical parameter of a biologically active chitosan mixture.

3.3. Chitosan-induced gene expression

In both organisms used in this study, exposition to chitosan resulted in gene expression alterations. Whereas it was focused on stress-related gene expression concerning *F. graminearum*, a whole transcriptome analysis was conducted with potato leaves, giving a deeper insight into the gene expression profile.

3.3.1. Gene expression profile of *F. graminearum* in response to chitosan

Investigations about chitosan-triggered gene expression changes in *F. graminearum* were roughly categorized into two gene groups. The first gene group contained genes related to pathogenesis, including pathogenicity and virulence factor genes as well as genes involved in mycotoxin biosynthesis (Figure 27). The second group contained 3 genes from the ergosterol biosynthesis pathway, which is a prominent target for fungicides, as ergosterols are essential components of fungal cell walls (Figure 28).

In response to 661, the gene expression of all pathogenesis-related genes was downregulated 3 h after treatment (hours past treatment, hpt), especially ve1 with a $\log_2$ fold change of almost -5. This downregulation attenuated 24 hpt with no gene expression exceeding the threshold of a fold change of ± 1 - excluding tri6 due to a very high standard deviation. This downregulation pattern corresponded with the gene expression change of *F. graminearum* exposed to a pH of 6.5, which caused even stronger downregulation of all genes at both time points. Gene expression of pH 3.0 exposed *F. graminearum* remained elusive due to high standard deviations 3 hpt, however, a strong downregulation of tri6, pkw12 and aurO, *i.e.* mycotoxin-related genes, could be observed 24 hpt. Generally, the observed gene expression profile suffered from high standard deviations, resulting either from average values taken from biological replicates (in case of os2) or from the fact that the experiment was only conducted once (in case of ve1, tri6, pks12 and aurO). Overall, 661 treatment of *F. graminearum* resulted in least gene expression changes of the observed pathogenesis-related genes 24 hpt, whereas both pH 3 and pH 6.5 resulted in downregulation of these genes.

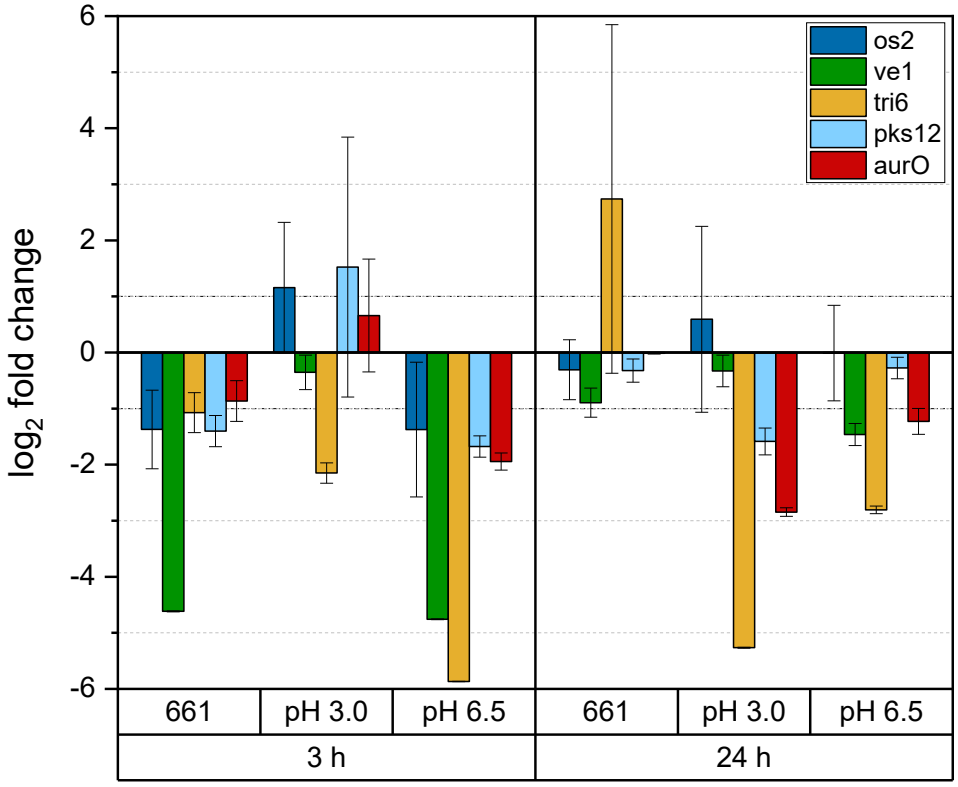

Figure 27: Real-time PCR results of *F. graminearum* exposed to 661 or pH-adjusted CM (pathogenesis-related genes). Gene expression was determined relative to gene expression of water treated *F. graminearum*. The relative gene expression is given in the log_2 fold change, plotted on the Y-axis. Lines at log_2 fold changes of -1 and 1 indicate the threshold of relevant differential gene expression levels. N = 1-3, n = 3-9.

The expression profile of genes related to ergosterol biosynthesis was altered equally, independent from exposure of *F. graminearum* to 661 or pH-adjusted CM. 3 hpt, gene expression of erg4 was slightly upregulated (only $log_2 > 1$ for pH 3), whereas gene expression of erg6 and erg11 was downregulated ($log_2 > 1$ for 661 and pH 6.5). The same expression pattern could be observed 24 hpt, however, upregulation of erg4 was stronger ($log_2 > 2$ for all treatments), whereas downregulation of erg6 and erg11 was weaker in comparison to 3 hpt. As for the first gene group containing pathogenesis-related genes, the results suffered from high standard deviations, preventing any clear statements concerning expression changes of ergosterol-related genes in response to chitosan treatment.

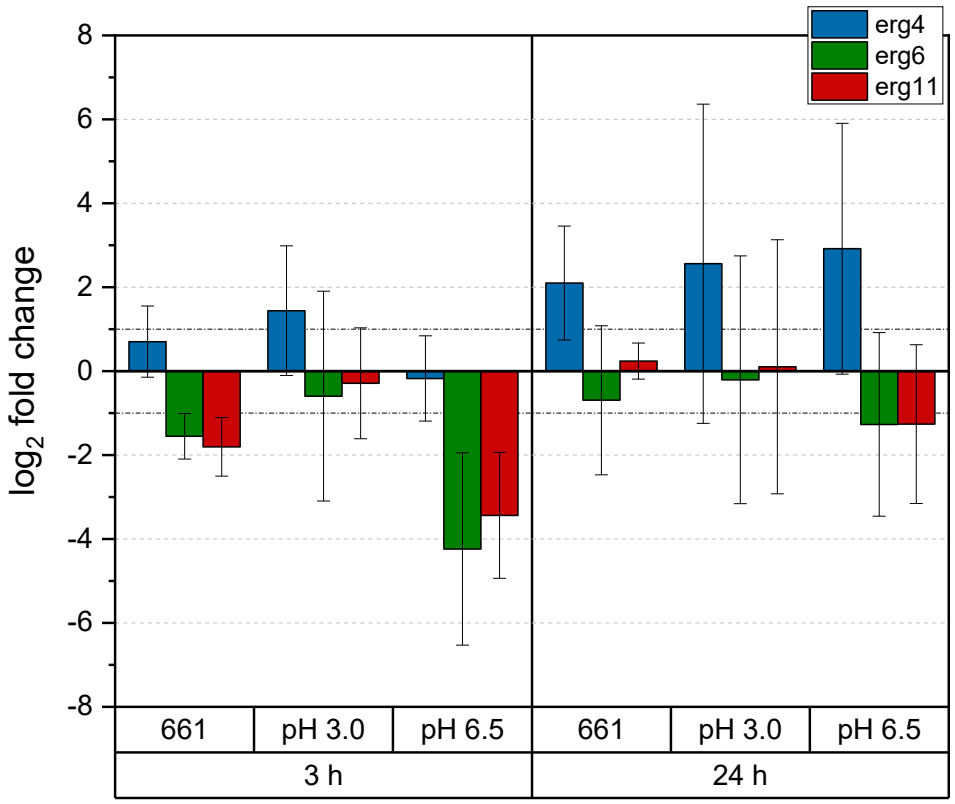

Figure 28: Real-time PCR results of *F. graminearum* exposed to 661 or pH-adjusted CM (ergosterol biosynthesis related genes). Gene expression was determined relative to gene expression of water treated *F. graminearum*. The relative gene expression is given in the log_2 fold change, plotted on the Y-axis. Lines at log_2 fold changes of -1 and 1 indicate the threshold of relevant differential gene expression levels. N = 2-3, n = 6-9.

As already mentioned, mean log_2 fold changes (in case more than one biological replicate was available) were shown in the above depicted figures. This was done to improve clarity of the figures, albeit contributed to high standard deviations which hampered the possibility to give clear statements. To proof that the achieved results still show significance, tables 12 and 13 show the two-way-ANOVA calculations, comparing the statistically significance of all genes in terms of time point, treatment and interaction of time point and treatment. ANOVA analysis could show that the gene expression changes were significantly different in most cases, indicating that *F. graminearum* indeed responded to exposure to 661 or pH-adjusted CM in different ways. This was concerned as a first hint of the ability of chitosan treatment to change the gene expression profile of *F. graminearum* towards less toxicity or even less pathogenicity in general.

Table 12: Two-way-ANOVA analysis of virulence-related genes of *F. graminearum*. ANOVA was conducted with GraphPad PRISM (GraphPad Software, Inc., San Diego, California, USA). For os2, analysis results of all replicates are shown. Significance levels are indicated with asterisks. * = p < 0.05, ** = p < 0.05, *** = p < 0.005, **** = p < 0.0005. n.s. = not significant.

Gene	time	p-value	treatment	p-value	interaction	p-value
os2	****	< 0.0001	**	0.012	****	< 0.0001
	****	< 0.0001	**	0.0012	**	0.0022
	****	< 0.0001	n.s.	0.1204	****	< 0.0001
ve1	n.s.	0.2088	***	0.0010	***	0.0008
tri6	***	0.0004	***	0.0001	*	0.252
pks12	****	< 0.0001	***	0.0001	****	< 0.0001
aurO	****	< 0.0001	****	< 0.0001	****	< 0.0001

Table 13: Two-way-ANOVA analysis of ergosterol-related genes of *F. graminearum*. ANOVA was conducted with GraphPad PRISM (GraphPad Software, Inc., San Diego, California, USA). Analysis results of all replicates are shown. Significance levels are indicated with asterisks. * = p < 0.05, ** = p < 0.05, *** = p < 0.005, **** = p < 0.0005. n.s. = not significant. n.s. = not significant.

Gene	time	p-value	treatment	p-value	interaction	p-value
erg4	****	< 0.0001	**	0.0043	**	0.0059
	****	< 0.0001	****	< 0.0001	*	0.0331
	****	< 0.0001	**	0.0022	****	< 0.0001
erg6	****	< 0.0001	**	0.0025	***	0.0001
	**	0.0030	n.s.	0.1362	*	0.0108
erg11	****	< 0.0001	****	< 0.0001	****	< 0.0001
	**	0.0087	n.s.	0.5638	*	0.0492

Nevertheless, the results concerning the *F. graminearum* gene expression changes in response to chitosan treatment are based on comparably few experiments, especially on few biological replicates and should thus be treated with caution.

3.3.2. Transcriptome analysis of chitosan-treated potato leaves

Whole transcriptome sequencing of potato leaves was based on preliminary real-time PCR studies to identify appropriate sequencing time points after chitosan treatment. Preliminary investigations with three defense-related genes resulted in noticeably upregulation of 2 out of 3 tested genes 2 hpt (Figure 29). This upregulation was considered as significant and hence decided to be used as transcriptome sequencing time point. 5 hpt was decided as a second transcriptome sequencing time point to detect putative downstream genes of 2 hpt upregulated genes.

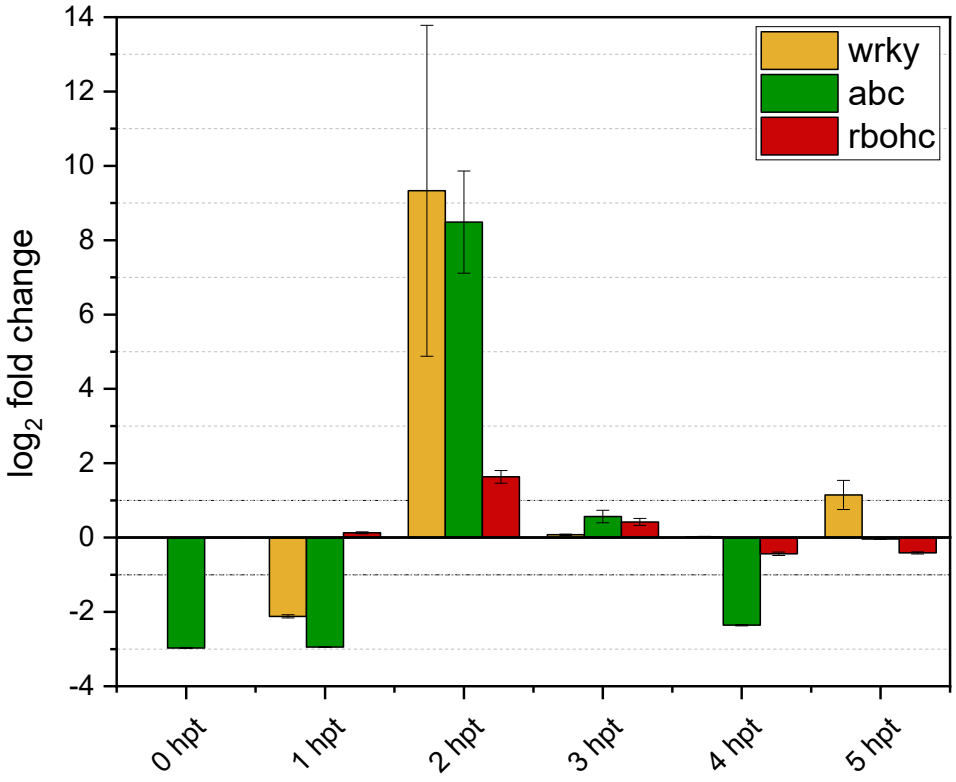

Figure 29: Pre-RNA-seq real-time PCR of chitosan treated potato leaves. Gene expression of chitosan treated potato leaves were determined relative to gene expression of water treated potato leaves. The relative gene expression is given in the log_2 fold change, plotted on the Y-axis. The expression of three different defense-related genes was investigated. Lines at log_2 fold changes of -1 and 1 indicate the threshold of relevant differential gene expression levels. N = 1, n = 3.

3.3.2.1. Raw data examination

According to the principal component analysis (PCA), the samples grouped by treatment or time point, respectively, which indicated good quality of the data. However, the grouping of the different replicates was not as strong as expected, indicating a high similarity between the samples. For further analysis, only genes allocated to certain functions are considered and

discussed, excluding both genes of unknown functions and genes coding for ribosomal RNAs (rRNAs).

3.3.2.2. Differentially expressed genes

Differential gene expression exclusive to 2 hpt is limited to few genes, either related to plant defense processes or not specifically assignable to certain functions. Defense-related differentially expressed genes (DEG) included a WRKY transcription factor (PGSC0003DMG400009103) and a leucine-rich repeat receptor like kinase (LRR-RLK) (PGSC0003DMG400017713), both known to be involved in recognition of disease and forwarding of corresponding signals. Further DEG were coding for an extensin (PGSC0003DMG400001380) and a proline-rich cell wall protein (PGSC0003DMG400009783), both involved in cell wall synthesis, a gene coding for a PRA1 family protein (PGSC0003DMG401031172) and a gene coding for a leucoanthocyanidin dioxygenase. The upregulation 2 hpt was overall weak, never exceeding a 1.4-fold change compared to H_2O-treatment (Figure 30).

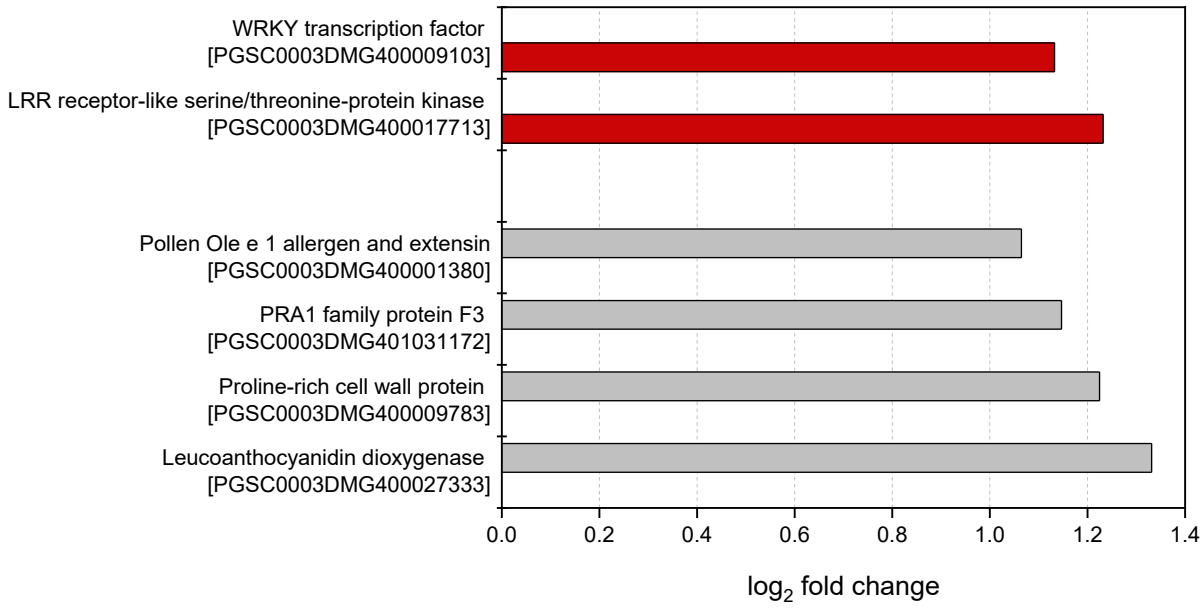

Figure 30: Chitosan-induced differential gene expression 2 hpt. X-axis shows the $\log_2$ fold changes of chitosan-treated potato leaves in comparison to H_2O-treated potato leaves. Genes assigned to plant defense are marked in red, genes assigned to other, not further specified functions are marked in grey. DEG of unknown functions or coding for rRNAs are not shown. Only genes with $\log_2$ fold change > 1 and adjusted p-value of < 0.05 were considered as significantly upregulated.

Noticeably more genes were differentially expressed 5 hpt. In total, 83 DEG were found 5 hpt, again excluding genes of unknown functions or coding for rRNAs. A total of 7 defense-related genes were observed upregulated 5 hpt in chitosan-treated leaves (Figure 31), including genes coding for photoassimilate-responding proteins (PGSC0003DMG400011087, PGSC0003DMG400014347) and genes coding for proteins, increasing the resistance to different abiotic stresses caused by osmosis (PGSC0003DMG400041193), salt and drought (PGSC0003DMG400004311).

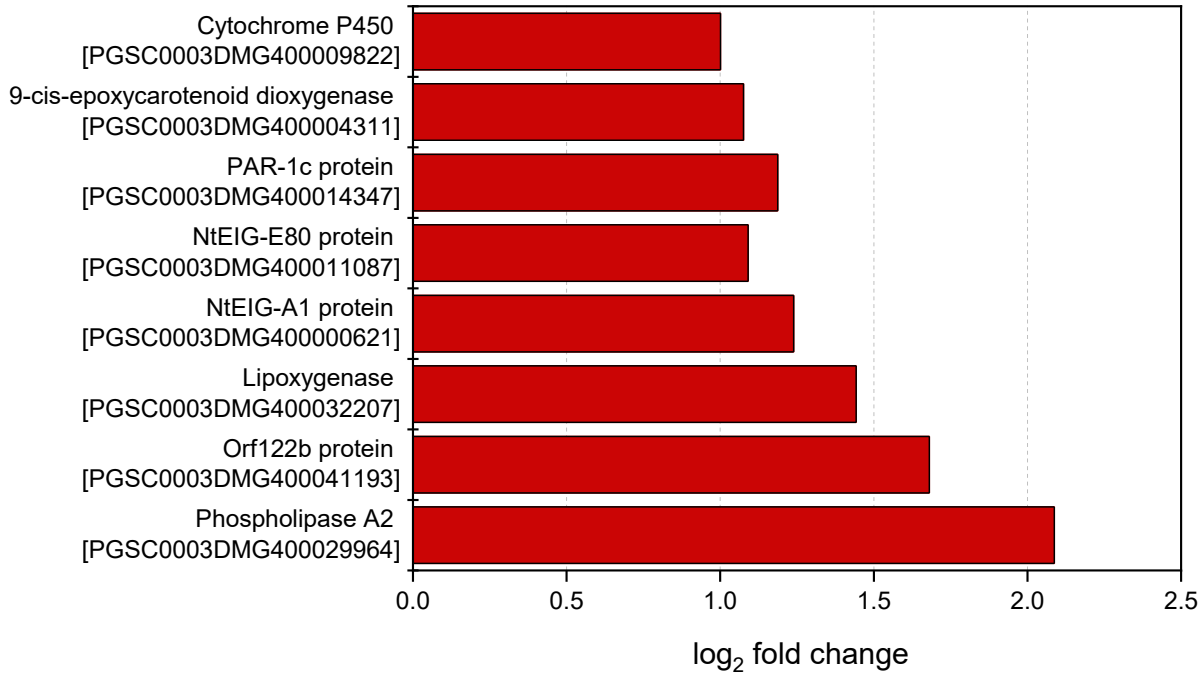

Figure 31: Chitosan-induced differential gene expression 5 hpt (defense-related). X-axis shows the log_2 fold changes of chitosan-treated potato leaves in comparison to H_2O-treated potato leaves. DEG of unknown functions or coding for rRNAs are not shown. Only genes with log_2 fold change > 1 and adjusted p-value of < 0.05 were considered as significantly upregulated.

Besides defense, primary metabolism was activated 5 hpt, which was indicated by the differential gene expression of genes allocated to either mitochondria or chloroplasts. On one hand, NADH oxidoreductase and dehydrogenase genes (PGSC0003DMG400030943, PGSC0003DMG400003375, PGSC0003DMG400013204, PGSC0003DMG400020931, PGSC0003DMG400021388) were upregulated, coding for important mitochondrial proteins involved in respiration (Figure 32).

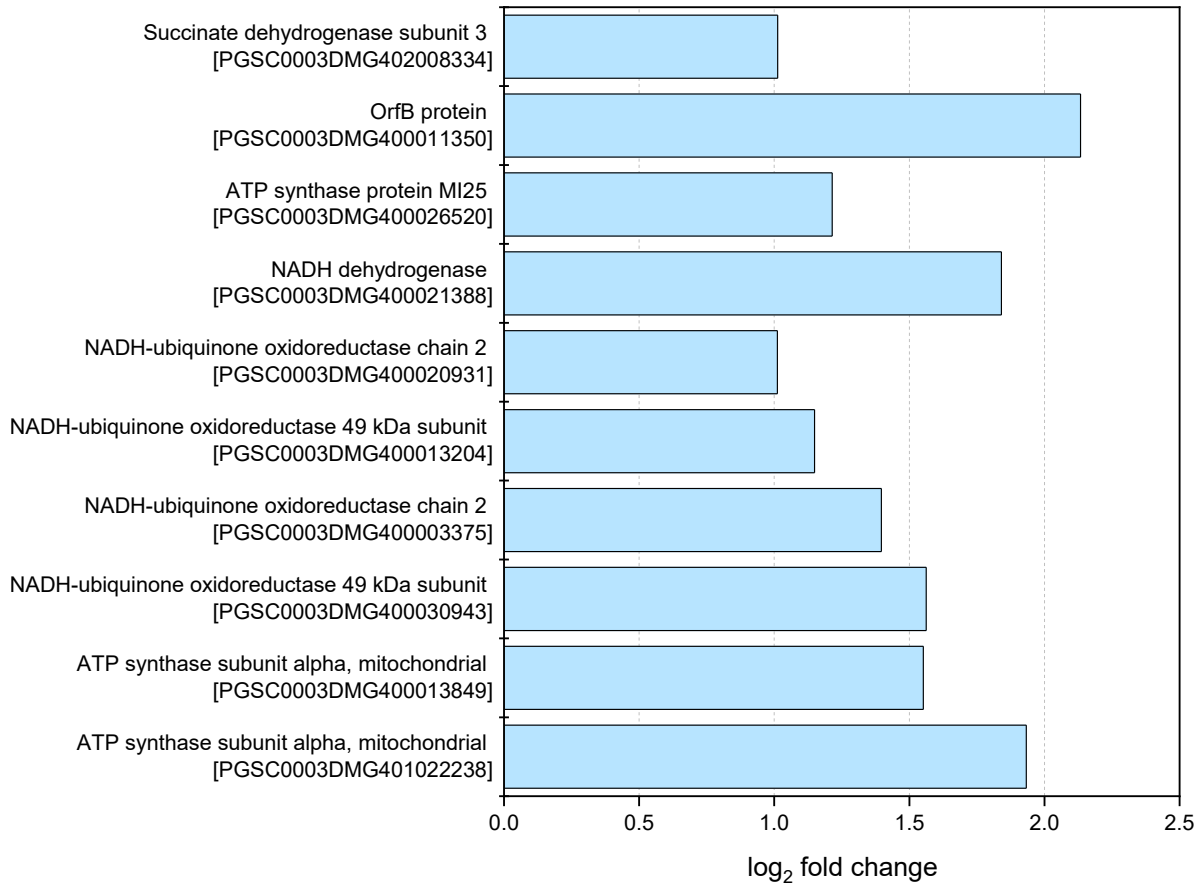

Figure 32: Chitosan-induced differential gene expression 5 hpt (mitochondrium-related). X-axis shows the log_2 fold changes of chitosan-treated potato leaves in comparison to H_2O-treated potato leaves. DEG of unknown functions or coding for rRNAs are not shown. Only genes with log_2 fold change > 1 and adjusted p-value of < 0.05 were considered as significantly upregulated.

On the other hand, photosynthesis-related genes were upregulated, including genes coding for the photosystem II subunit D1 (PGSC0003DMG400004211), the cytochrome b_6f complex f subunit f (PGSC0003DMG400002905) of the cytochrome b_6f complex and for several ATP synthase subunits (PGSC0003DMG401022238, Q27S44, PGSC0003DMG400013849, PGSC0003DMG400008476). The overall strongest differential gene expression was observed for photosystem and cytochrome b_6f complex related genes with fold changes between 2 and 3 (Figure 33).

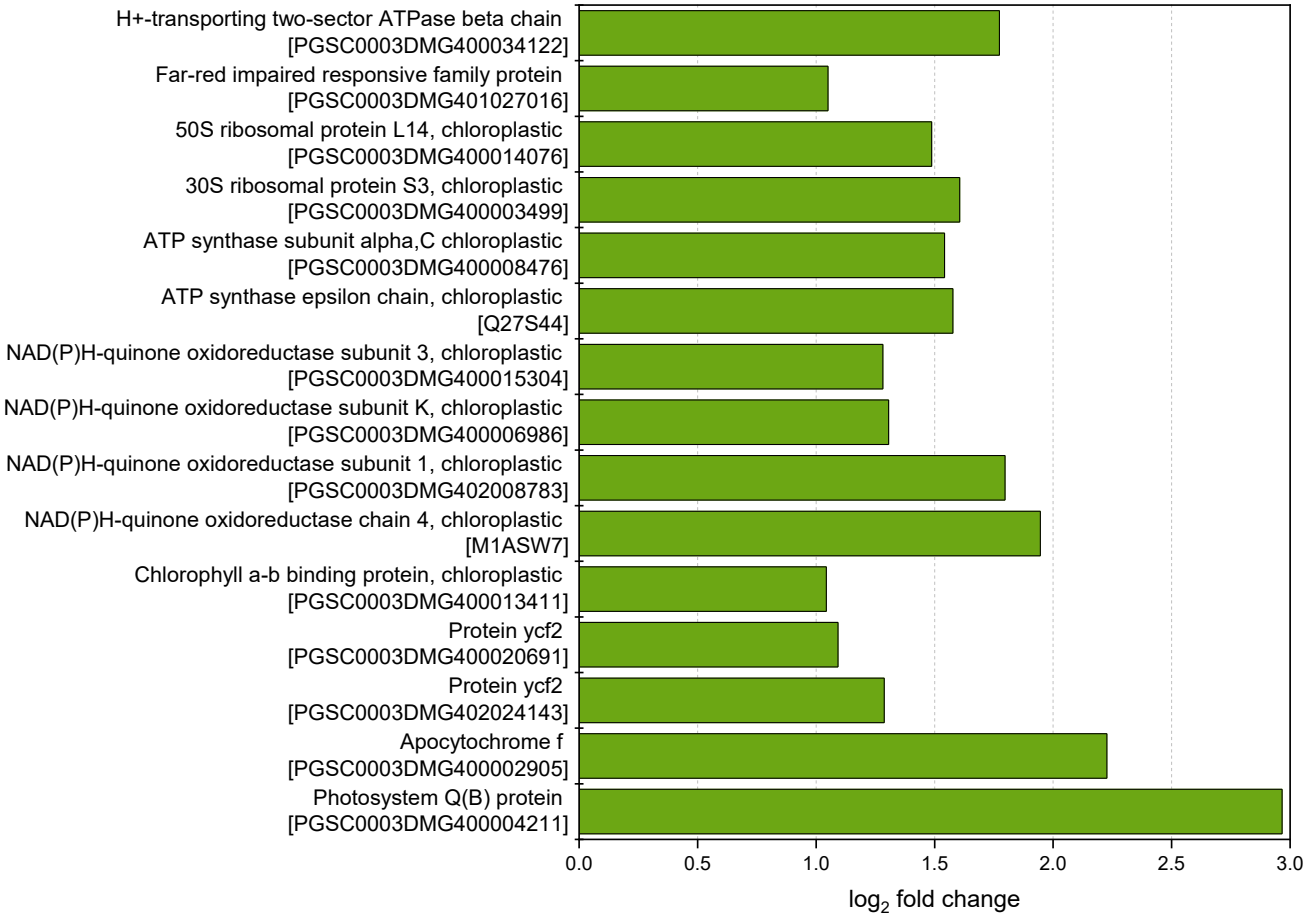

Figure 33: Chitosan-induced differential gene expression 5 hpt (chloroplast-related). X-axis shows the log_2 fold changes of chitosan-treated potato leaves in comparison to H_2O-treated potato leaves. DEG of unknown functions or coding for rRNAs are not shown. Only genes with log_2 fold change > 1 and adjusted p-value of < 0.05 were considered as significantly upregulated.

Besides the exclusive upregulation 2 and 5 hpt, a total of 12 genes were upregulated at both time points, indicating a both fast (2 hpt) and long lasting (5 hpt) gene expression (Figure 34). These DEG were hence considered as of high importance. 7 of those 12 genes could be clearly assigned to the light phase of photosynthesis with genes coding for both photosystem I (PGSC0003DMG400012033, PGSC0003DMG400015960, PGSC0003DMG400005372) and II (PGSC0003DMG400017258, PGSC0003DMG400046303) subunits as well as for the cytochrome b_6 subunit (PGSC0003DMG400019419) of the cytochrome b_6f complex. All these genes were observed higher upregulated 5 hpt in comparison to 2 hpt.

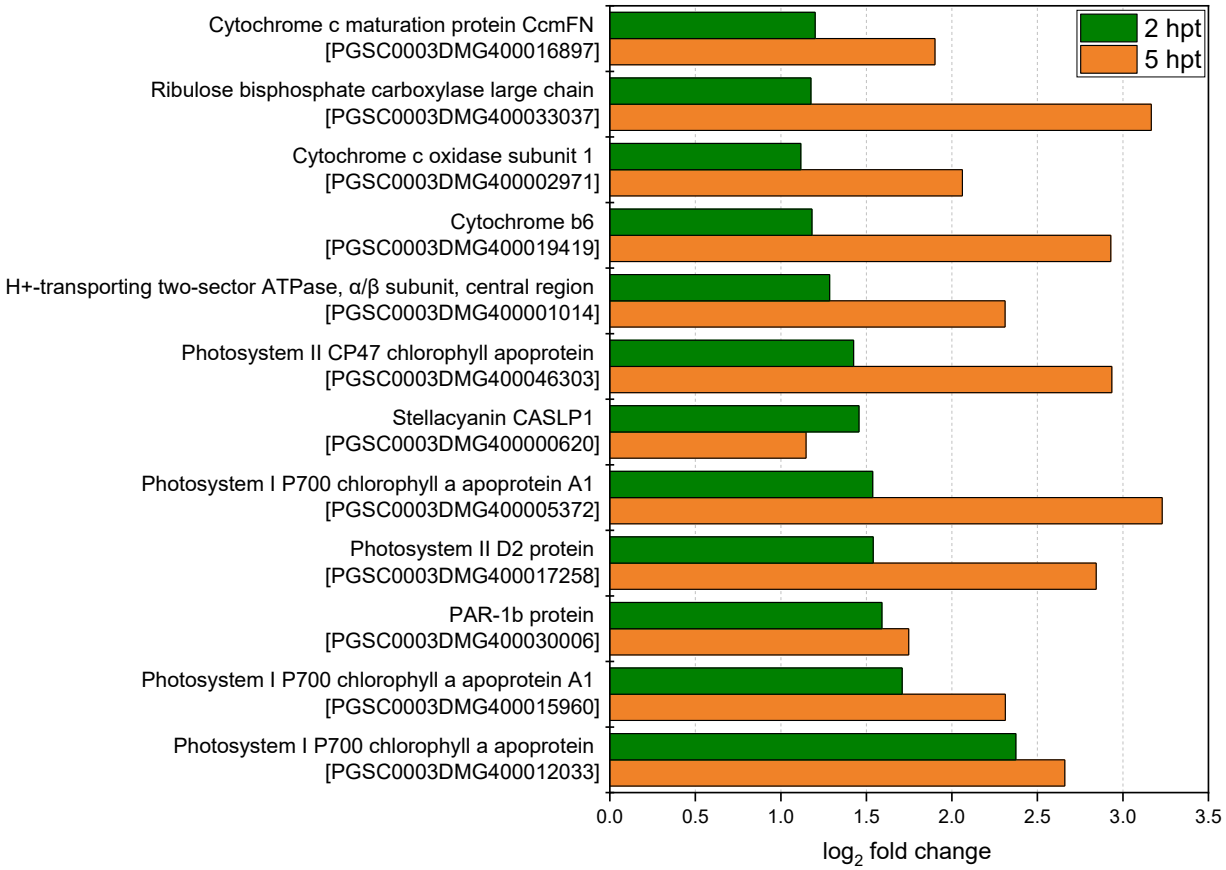

Figure 34: Chitosan-induced differential gene expression at both 2 and 5 hpt. X-axis shows the $\log_2$ fold changes of chitosan-treated potato leaves in comparison to H_2O-treated potato leaves. Green bars indicate gene expression 2 hpt, orange bars indicate gene expression 5 hpt. DEG of unknown functions or coding for rRNAs are not shown. Only genes with $\log_2$ fold change > 1 and adjusted p-value of < 0.05 were considered as significantly upregulated.

Especially 5 hpt, chitosan treatment triggered a number of clearly assignable genes, with most genes being involved in primary metabolism. Photosynthesis-related genes were of particularly prominence, being differentially expressed both 2 and 5 hpt, often with fold changes exceeding 2 or even 3. This observation aroused interest in whether chitosan treatment could activate photosynthesis in plants, which will be discussed further in the discussion chapter.

3.3.2.3. Enrichment analysis

To further elucidate gene functions, all DEG were classified according to GO terms via enrichment analysis. This analysis enabled to identify terms or gene functions that were statistically significantly overrepresented (adjusted p-value < 0.05) among the DEG (Figure 35). In this thesis, the analysis was cut down to GO terms which included at least two DEG and furthermore to genes which were overrepresented both 2 and 5 hpt, as these DEG considered

of high importance as mentioned before. The analysis resulted in a total of 6 overrepresented GO categories, of which 4 were involved in photosynthesis (thylakoid, GO:0009579; photosystem I, GO:0009522; photosynthesis, GO:0015979 and photosynthesis, light reaction, GO:0019684). Further overrepresented categories included genes for membrane components (GO:0016021) and electron carrier activity (GO:0009055).

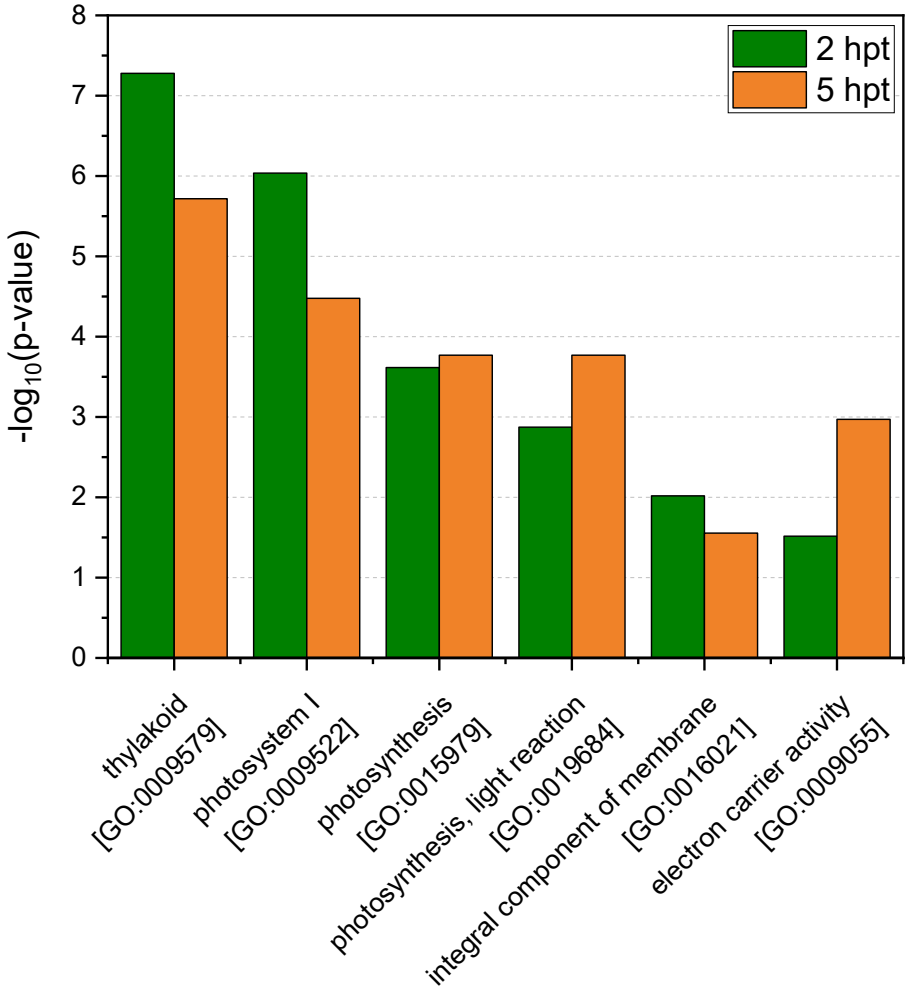

Figure 35: Enrichment analysis via GO classification. Y-axis plots the -$\log_{10}$(p-value) of the enriched GO categories which is also known as significance score. Green bars show the significance scores of enriched GO terms 2 hpt, orange bars show the significance scores of enriched GO terms 5 hpt. Only GO categories that included at least two DEG in both time points were included in the figure.

3.3.2.4. KEGG pathway annotation

To further allocate the photosynthesis-related DEG to specific structures of the photosynthetic apparatus, the DEG were assigned to certain functions using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map for the photosynthetic light reaction in potato (Ogata et al., 1999). According to the KEGG assignment, the observed photosynthesis-related DEG code for major subunits of all main light reaction components photosystem I and II, the cytochrome b_6f complex and the ATP synthase (Figure 36). Interestingly, all genes are located in the chloroplast genome (Rogalski et al., 2015), indicating that chitosan-induced gene expression is not limited to nucleic genes.

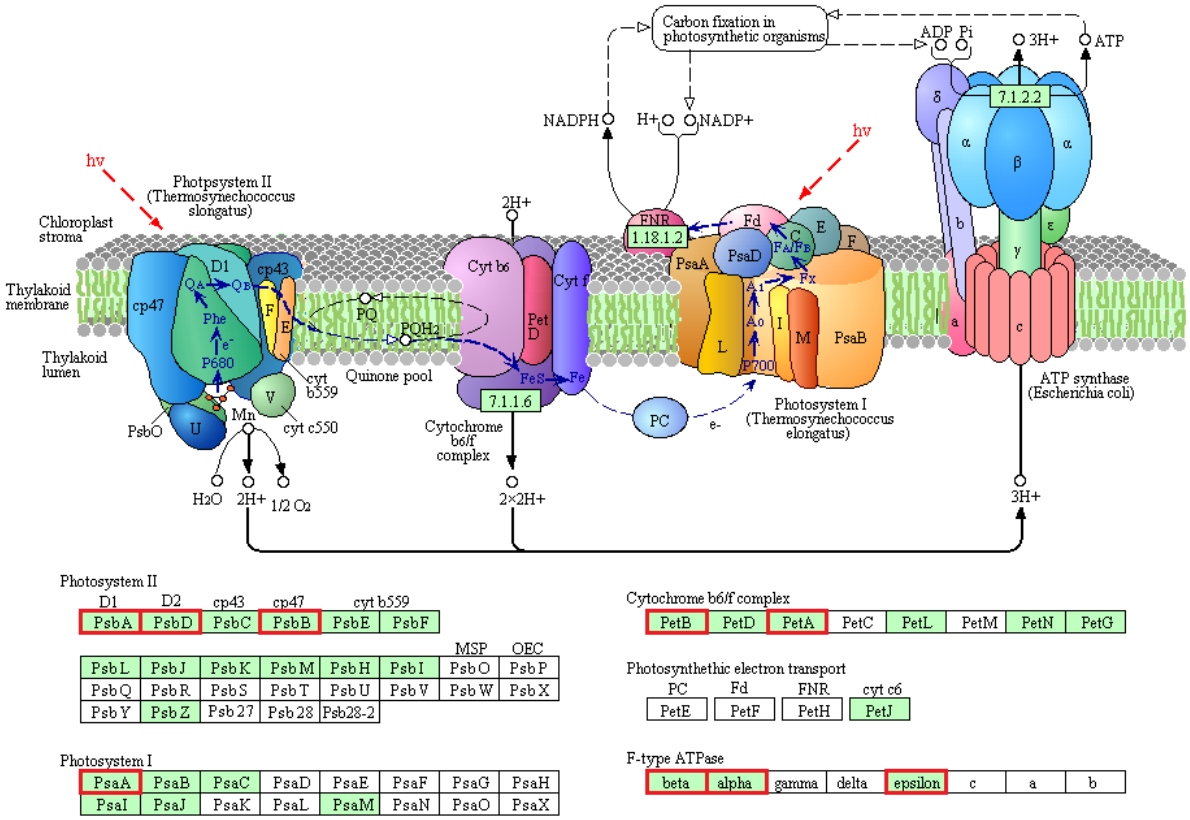

Figure 36: KEGG pathway map of the photosynthetic light reaction in potato. Upregulated genes observed in this study are marked in red boxes. Chloroplast-encoded genes are indicated in green, nucleus-encoded genes are indicated in white. Permission to use this KEGG pathway map (entry sot00195) was granted (Ogata et al., 1999; Kanehisa et al., 2017, 2019).

Chapter IV

Discussion

4. Discussion

In this chapter, both antifungal and eliciting activities of chitosan will be first described separately by interpreting the achieved results and furthermore embedded into already published literature. Eventually a conclusion will be drawn concerning how the observed findings fit into what is already known and might contribute to broaden the knowledge of both the mode of action and the applicability of biologically active chitosan.

4.1. Antifungal activity of chitosan

Fungicides are perpetually exposed to constant change. Inorganic fungicides dominated the market for centuries and are still used in both conventional and organic agriculture. For example, metal-based inorganic fungicides target fungal enzymes and thus inhibit spore germination and mycelium growth (Byrde et al., 1956), while sulfur-based inorganic fungicides oxidize to sulfurous acid, which is believed to inhibit spore germination as well (Tweedy, 2012). As such element-based antifungal activity is non-degradable, resistance development towards inorganic fungicides is limited. However, efficiency of inorganic fungicides is accompanied by changes in the microbial community on arable land, which in turn might negatively influence plant growth and yields (Zubrod et al., 2015). Organic fungicides on the other hand are a large heterogeneous group of chemicals, designed to target lots of different functions like cell division, protein synthesis and DNA. However, such compounds are susceptible for degradation and hence fungal resistance, which is continuously watched by the Fungicide Resistance Action Committee (FRAC). Fungicide resistance is a big issue since the 1970s which provided more and more observations and evidence for reduced sensitivity of different pathogenic fungi towards chemicals that were applied to combat their spread (Lucas et al., 2015). Resistance mechanisms include alterations of the fungicide target site, intracellular metabolization of fungicidal chemicals, export of fungicides from the fungal cells and reduced uptake of fungicides through membranes (Deising et al., 2008; Lucas et al., 2015). Development of such mechanisms is preferred on organisms with short dormant periods, large populations and both sexual and asexual life cycles and is furthermore favored by fungicides with a single mode of action, as resistance is usually based on single mutations occurring within a population under selection pressure (Tucker et al., 2015). Ironically, resistance-inducing mutations are often caused by the fungicide itself, acting as a selection medium for insensitive individuals which subsequently multiply and form resistant populations. Chitosan, as a potential, eco-friendly successor of commonly used fungicides, was shown to display antifungal activity

towards a broad range of pathogenic fungi (Verlee et al., 2017). As its mode of action remains elusive, but different chitosans display different efficacy in terms of antifungal activity (Sahariah & Másson, 2017), multiple modes of action might be assumed, making chitosan a promising candidate to combat both developments of fungicide resistance and environmental toxification while maintaining an effective control of fungal pathogens.

4.1.1. Chitosan as conidium germination and mycelium growth inhibitor

The antifungal activity of a chitosan was observed to be strongly dependent on its DP. Comparing such results with other studies is still often hampered by the usage of commercially available chitosans with poor physico-chemical characterization. Furthermore, studies on the size-dependent antifungal activity of chitosans is often condensed in the term low molecular weight chitosan (LMWC). However, this term is not well-defined and does not allow comparisons of different LMWC without further information about their properties. For example, a chitosan of 70 kDa was treated as LMWC in (Alburquenque et al., 2010), while a chitosan of 24 kDa was already considered as medium molecular weight chitosan (MMWC) in (Hernández-Lauzardo et al., 2008). Since the Mw of a chitosan is not only dependent on its DP, but also on its F_A, simple comparisons of different LMWC is even more impeded. Furthermore, the terms oligomers and polymers are not defined and free to interpret. Vincent Eijsink and coworkers decided to restrict the term polymer to chitosans with a DP above 100 (Aam et al., 2010), while others set the limits differently. This makes it even more important to clearly characterize the chitosan used and indicate its characterized properties. Apart from this, antifungal activity of chitosan is not only dependent on its physico-chemical properties, but also highly species-dependent. This once again shows that comparisons between different studies are only possible to a very limited extent. Nevertheless, the following chapter attempts to embed the here achieved results in the available knowledge.

In general, investigations concerning the antifungal activity of very small COS are rare and are rather focused on DPs larger than 30. Lee Hadwiger and coworkers did pioneering work in this regard, showing that the antifungal effect of fully deacetylated COS start to emerge with DP 4 and 5, with a sharp increase in activity starting with DP 6 (Kendra & Hadwiger, 1984). The antifungal activity of DP 4 and 5 were only achieved in high concentrations (250-500 µg/mL), while only 63 µg/mL of DP 6 had to be applied as MIC (Kendra & Hadwiger, 1984). Likewise tested fully acetylated COS, comparable to the COS used in this study, were observed to be completely ineffective regarding antifungal activity. Independent of the mode of action of chitosan, all common theories require positively charged GlcN units to some extent (Goy et al.,

2009; Lopez-Moya et al., 2019), which explains the inactivity of COS on *F. graminearum*. Small COS with DPn of 4 and 8 and an F_A between 0.05 and 0.07 (*i.e.* paCOS) were observed ineffective against several *Candida* species with antifungal activity starting with a DPn of 12. Furthermore, the antifungal activity tended to increase with increasing DPn (up to 116) (Sergey N. Kulikov et al., 2014). The authors hypothesize that paCOS may interfere with fungal adhesins and regulatory mechanisms for hyphal growth, in which the effectivity of chitosan is dependent on how well it can interact with those fungal mechanisms. Hence, paCOS of low DP < 10 might be too small to successfully interfere with the fungus. However, this does not explain the growth-promoting activity of paCOS observed in this work. It is known since the 1980s that chitosan is a cell wall component of the *Fusarium* genus (Lee A. Hadwiger & Beckman, 1980). Furthermore, chitosan utilization to support cell wall integrity was for instance described for *Cryptococcus neoformans*, which deacetylases nascent chitin molecules for cell wall incorporation (L. G. Baker et al., 2007). Hence, an uptake of paCOS into the fungal cytosol or even direct integration into the growing cell wall of *F. graminearum* would be likewise conceivable.

Both F_A 0.15 (*i.e.* 75/5, 75/20 and 75/100) and F_A 0.2 (*i.e.* 651 and 661) chitosans showed DP-dependent antifungal activities, with smaller chitosans displaying higher effectivity towards growth inhibition and spore germination of *F. graminearum*. This anti-proportional size-dependent effect was also observed on different *Candida* species, were the antifungal activity of chitosan decreased when using polymers (DPn ~300 and DPn > ~3000) (Sergey N. Kulikov et al., 2014). Likewise, DPn ~100 chitosan was observed superior in terms of inhibiting mycelium growth of *Rhizopus stolonifera* in comparison to DPn ~135 and DPn ~170 chitosan, however, the latter affected sporulation and germination more effectively than DPn ~100 chitosan (Hernández-Lauzardo et al., 2008). These results were furthermore confirmed and expanded recently by testing a paCOS series (DPn 17-62, F_A 0.15), again on different *Candida* species, where it was reported that an intermediate DPn (31 and 54) resulted in most effective growth inhibition, while smaller (DPn 17) and in particular larger (DPn 62) chitosan molecules had decreased antifungal activity (Ganan et al., 2019). Analogously, an optimal DPn between 23 and 40 (both F_A 0.15) was determined to inhibit germination of *Mucor piriformis* and *Botrytris cinerea*, whereas both smaller and larger chitosans had less inhibitory activity (Rahman, Hjeljord, et al., 2014).

In conclusion, the observation that an intermediate DPn (*i.e.* no small paCOS with less than 10 units, no polymers with more than ~500 units) appeared in numerous studies and seems to be

species-independent. However, a possible explanation was not found yet, apart from the simple suggestion that the higher activity is a result from better solubility of smaller chitosan molecules in water (Rhoades & Roller, 2000; Badawy & Rabea, 2011). The antifungal effect of chitosan is believed to be mostly dependent on (I) the cell wall composition of the target organism (Allan & Hadwiger, 1979) and (II) the polycationic strength of the chitosan, as it was shown that a low pH - *i.e.* more positively charged GlcN units - favors the antifungal activity of chitosan (Alburquenque et al., 2010; M. Kong et al., 2010; Ganan et al., 2019). This would not only include interactions of chitosan and negatively charged cell wall and membrane components, resulting in cell leakage and cell death, but also interactions with fungal nucleic acids as already postulated (L. A. Hadwiger et al., 2011; Lopez-Moya et al., 2019; Shih et al., 2019). It is of general acceptance that chitosan polymers cannot pass through cell walls or membranes (Raafat et al., 2008; Vinsova & Vavrikova, 2011). Consequently, chitosan must not exceed a certain size to exert the postulated effects. This could explain the observed DP-dependency of the antifungal activity of chitosan, including the here achieved results on *F. graminearum*. This hypothesis was furthermore supported by the 661-related results with a shift to an excess of polymers reduces its antifungal activity while a shift to an excess of oligomers enhanced it equally. These results provide clear evidence regarding the increased antifungal activity of smaller chitosan molecules in comparison to large chitosan molecules. As already mentioned above, it thus can be assumed that the interaction of chitosan with fungal structures is more effective with smaller molecules, probably due to an increased surface-to-volume ratio. It was furthermore shown that several family members of *Fusarium* express and secrete chitosanases (Shimosaka et al., 1993; Huaiwei Liu et al., 2010), without completely understanding their functions. Overexpression of a *F. solani* chitosanase resulted in decreased pathogenicity, whereas knock-down of this chitosanase even increased the virulence (Huaiwei Liu et al., 2010). This provided evidence of the importance of a well-balanced expression of fungal chitosanases for the pathogenesis of *Fusarium*. It thus can be assumed that chitosanases are likewise expressed by *F. graminearum*, enabling degradation of chitosan molecules if secreted. Interestingly, it was observed that fungal susceptibility to chitosan is dependent on the ability to produce chitinases and chitosananses, whereby strains producing both types of enzymes show higher resistance than strains producing only either chitinases or chitosanases (Aktuganov et al., 2018). This leads to the assumption that chitinases and chitosanases not only provide tools for fungal growth, but also contribute to the resistance towards extracellular chitosan fragments by complete degradation. It is likely that this was originally evolved as self-protection from own cell wall fragments, but now additionally serves as genuine resistance

mechanism. Based on what has been observed, the antifungal activity of chitosan might first increase due to the activity of extracellular chitosanolytic enzymes before being removed by successful complete degradation to small oligomers without any remaining antifungal potential.

The results concerning F_A-dependent antifungal activity are generally still contradicting to some extent. While low F_A chitosans were observed to be most effective against a broad range of both gram-positive and gram-negative bacteria (Omura et al., 2003; Jung et al., 2010; Younes et al., 2014), investigations concerning fungi are not as clear and seem to be more species-dependent than for bacteria. For example, a low F_A chitosan displayed generally higher antifungal activity against *C. albicans*, *F. oxysporum*, *Aspergillus fumigatus* and *A. parasiticus* (Tsai et al., 2002), while no influence of Mw or F_A was observed with *A. niger* (Younes et al., 2014). As the cell wall of fungi, in contrast to any other biological kingdom, consists of a complex matrix of chitin, chitosan and glucans, enriched with proteins (Gow et al., 2017), it can be assumed that the antifungal activity of chitosan is somehow buffered by chitin, which does not allow any interactions with chitosan. As the chitin content in fungal cell walls is highly varying (Abo Elsoud & El Kady, 2019), with different fungi expressing different classes of chitin synthases (Lenardon et al., 2010), it is likely that the antifungal activity of chitosan is different for each fungal species. Hence, different amounts of chitin in the fungal cell wall could not only determine the degree of interaction between chitosan and other fungal cell wall components, but also the potential of a chitosan molecule to pass the cell wall, which becomes more difficult with larger molecules. Consequently, as the here used F_A series had a DPn of 1300, the non-existent F_A-dependent activity could be additionally linked to the decreased activity of polymer chitosans as described above.

Besides, chitosan mixtures were observed to have synergistic activity in comparison to the individual chitosans. Concerning the 75/20 and 75/100, which showed either intermediate or no antifungal activity, a combination of both chitosans resulted in a complete inhibition of mycelium growth of *F. graminearum*. Synergism was even stronger when mixing an inactive concentration of CPS 4 paCOS (30 µg/mL) with inactive concentrations of 75/5. All tested concentrations of 75/5 resulted in synergistic activity; both 5 and 10 µg/mL of 75/5 mixed with 30 µg/mL even resulted in complete growth inhibition of *F. graminearum*. Antimicrobial synergistic activity of chitosan and a second compound - mostly a fungicide - is already well-known and frequently described in literature. Accordingly, a mixture of chitosan and peppermint oils are described to inhibit anthracnose diseases in mango in a synergistic manner (K. Á. R. de Oliveira et al., 2017). Furthermore, chitosan was observed to synergistically

enhance the activity of antibiotics against *P. aeruginosa* (Muslim et al., 2018). With greatest relevance to this study, combinations of paCOS and larger chitosan oligomers (DPn 9-206, F_A 0.15) with five different commercially available fungicides resulted in synergistic antifungal activity against *B. cinerea*, *Alternaria brassicicola* and *Mucor piriformis* in both *in vitro* and *in vivo* studies as well as in field trials (Rahman, Shovan, et al., 2014). It was shown that all combinations of chitosans with different fungicides were at least additive, with some combinations being strongly synergistic, which is in accordance with the observations made in this thesis. Another study showed a synergistic effect of chitosan in combination with Fluconazole on the proliferation of yeast cells (Jaime et al., 2012). The authors of both studies suggest that synergism of chitosan and fungicides are due to different modes of actions. For example, a combination of chitosan and Teldor, a commercial fungicide whose active ingredient fenhexamid inhibits ergosterol biosynthesis (Duben et al., 2002; Leroux, 2007), could have synergistic antifungal activity as a result of an increased cell wall permeability due to chitosan, which enables Teldor to faster reach the fungal cell membrane (Rahman, Shovan, et al., 2014). In fact, chitosan-caused cell wall disruption might not only be the main mode of action, but also the general reason for synergistic activity of chitosans and fungicides. While synergism between chitosan and other compounds are often described as mentioned above, this study is only the second report of synergistic activity of chitosan combinations after (Attjioui, 2018). The first study investigated the antifungal activity of a combination of a small chitosan polymer (DPn 90) with paCOS (DP 2-17) with F_A 0.1 each. The interaction of both chitosans was observed to be strongly synergistic, especially when using small concentrations of the polymer (Attjioui, 2018). These results are in acceptance with the findings in this study, where combinations low concentrations of a small polymer (75/5) with paCOS (CPS 4) resulted in synergistic antifungal activity. The background of the synergistic activity of chitosan mixtures is not understood yet. However, synergism is more likely to occur if the individual components of a mixture perform different modes of action on their targets, as described for combinations of chitosans and fungicides (Rahman, Shovan, et al., 2014). Hence, it can be assumed that chitosans with different physico-chemical properties display different modes of action on their target tissue. It was stated that chitosan polymers are more likely causing membrane disruption than chitosan oligomers (Kauss et al., 1989). One possible antifungal mechanism of chitosan mixtures could thus include cell wall and membrane deformations by chitosan polymers, which enables chitosan oligomers to enter leaking cells and interact with proteins and nucleic acids. As chitosans with intermediate DP (with tendency to smaller molecules) were determined to exhibit strongest antifungal activities, another hypothesis is the enzymatic degradation of

chitosan polymers via fungal chitosanolytic enzymes (Huaiwei Liu et al., 2010) to more active oligomers. This would not only enhance the overall antifungal activity of an appropriate chitosan mixture, but also prolong its effect due to an ongoing supply of nascenting chitosan oligomers from polymer molecules, before being completely hydrolyzed and thus inactive. This theory is also stated in (Attjioui, 2018) and would describe a synergistic activity of a chitosan mixture with both individual chitosans displaying the same mode of action. Despite of not fully understanding the reason for synergism of chitosan mixtures yet, it could be shown that their usage can significantly contribute to an antifungal activity. Instead of finding a certain chitosan with desired physico-chemical properties, future investigations might rather focus on the determination of a chitosan mixture. In the end, this also generally highlights the importance of the dispersity of a chitosan concerning its biological activity (Attjioui, 2018).

4.1.2. Gene expression changes in response to chitosan

Potential gene expression changes of *F. graminearum* in response to chitosan exposure was analyzed via 8 different genes, categorized into either pathogenesis-related or ergosterol-related genes. The following chapter serves to briefly introduce the different genes before discussing the influence of chitosan on their expression in *F. graminearum*.

The pathogenesis-related gene group consisted of the genes os2, ve1, tri6, pks12 and aurO. While os2 and ve1 display more general functions in pathogenicity and virulence, tri6, pks12 and aurO are genes specifically involved in mycotoxin synthesis. Os2 (osmosensor 2) is a mitogen activated protein kinase (MAPK) involved in trichothecene - a family of mycotoxins - production upon osmotic stress (Ochiai et al., 2007). It was shown that presence of os2 is required for infection and symptom development on soybean plants, supported by the observation that os2 expression provided resistance against soybean phytoalexins (Sella et al., 2014). Ve1 belongs to velvet-domain-containing proteins, a protein group forming the so called velvet complex, responsible for fungal secondary metabolites including antibiotics (Gerke & Braus, 2014; Martín, 2017). Ve1 was found to be required for morphological development, colony hydrophobicity, toxin production and general pathogenicity in *F. verticillioides*. Furthermore, ve1 seems to regulate catalase expression and hence, oxidative stress resistance (Lan et al., 2014). In tomato, the gene product of ve1 acts as an effector molecule which activates ETI towards *F. oxysporum* via ve1 recognition (de Jonge et al., 2012). Tri6 belongs to a family of trichothecene biosynthesis genes, whereby tri6 was found to act as transcription factor, regulating both mycotoxin production and through that, pathogenicity in general (Seong et al., 2009). Besides its function in toxin production, tri6 was observed to regulate the

expression of a broad range of other genes and was hence described as a global transcription factor (Nasmith et al., 2011). Both pks12 and aurO belong to the PKS12 gene cluster of *F. graminearum* (Westphal et al., 2018), responsible for fusarin biosynthesis, another class of mycotoxins, mainly produced by *Fusarium* species (Cambaza, 2018). In this cluster, pks12 resembles a polyketide synthase (Rugbjerg et al., 2013), while aurO codes for an oxidoreductase (Frandsen et al., 2006; Connolly et al., 2013). While pks12 provide an intermediate product for rubrofusarin synthesis, aurO is part of an extracellular enzyme complex that converts rubrofusarin into aurofusarin (Frandsen et al., 2011).

Erg4, erg6 and erg11 are genes involved in ergosterol biosynthesis. Ergosterol is the major sterol of fungal cell membranes, analogously to chloresterol in mammals. In general, sterols are essential membrane lipid components of eukaryotic organisms that influence different kinds of membrane parameters including permeability, fluidity and general stability (Z. Liu et al., 2019). Hence, ergosterol biosynthesis is a popular target for fungicides (Hata et al., 2010; K. Mazu et al., 2016). All three genes code for proteins directly belonging to the ergosterol biosynthesis pathway, with erg11 coding for the cytochrome P450 lanosterol C-14α-demethylase, involved early in the pathway, erg6 coding for the C-24 sterol methyltransferase acting in the middle of the pathway (Fan et al., 2013) and erg4 coding for the sterol C-24 reductase, catalyzing the final step of ergosterol biosynthesis (X. Liu et al., 2013).

Treating *F. graminearum* mycelium with 661 resulted either in non-significant gene expression changes compared to control treatment or to downregulation of all investigated genes. In case of upregulation (tri6 and erg4, 24 hpt), it could be rather seen as as non-differential gene expression due to the high standard errors. Considering this, 661 caused downregulation of all pathogenesis-related genes both 3 and 24 hpt. However, the same gene expression pattern could be observed for pH 6.5 exposed *F. graminearum*. In case of pH 3, downregulation was lower at 3 hpt (as mentioned before, upregulation was more likely to be denied due to high standard deviations), but clearly enhanced 24 hpt. Regarding the ergosterol biosynthesis genes, both 661 and pH 6.5 treatment resulted in downregulation of erg6 and erg11, but not of erg4. Gene expression of *F. graminearum* exposed to pH 3 showed less downregulation for erg6 and erg11, but stronger upregulation for erg4. However, these results were even more affected by high standard deviations. Based on the results, it can be assumed that the observed gene expression is closely linked to the pH. The gene expression profile of 661 treated *F. graminearum* closer represents the gene expression of pH 6.5 exposed *F. graminearum* since the final pH of 661-supplemented CM was determined as 5.3 and thus, closer to 6.5 than to 3. It is known that

F. graminearum requires acidic pH for tri gene expression and hence trichothecene biosynthesis (Merhej et al., 2010); however, gene expression of tri6 as a trichothecene biosynthesis related transcription factor seemed not to be affected by pH in this study. As it is known that gene expression of tri genes as well as actual production of mycotoxins is dependent on the observed time point and growth state of *F. graminearum* (Gardiner et al., 2009), tri6 might be required at different time points than the selected ones. It was furthermore shown that the virulence of *Fusarium* species is dependent on fusaric acid, lowering the surrounding pH and by this stimulating disease symptoms on host cells (V. K. Singh & Upadhyay, 2014; López-Díaz et al., 2018). On the other hand, development and growth of *F. oxysporum* were found to decrease with alkalinization of the culture medium, without affecting its sporulation (Gheorghe et al., 2015). Therefore, treating *F. graminearum* with chitosan might help to increase the surrounding pH and thus counteracting against acidification, which is required for its virulence. Regarding this results, it would make sense to investigate the expression of virulence genes involved in acidification, since these genes should be highly overexpressed in a alkaline-buffered pH surrounding (Fernandes et al., 2017). Although 661 was not capable of significantly downregulate the investigated pathogenesis-related or ergosterol biosynthesis related genes, an important and noteworthy aspect is that on the other hand, chitosan did not seem to trigger stress-induced gene expression which is usually linked to the production of toxic secondary metabolites (Schmidt-Heydt et al., 2008; Ponts, 2015). This can be considered as useful information concerning chitosan as fungicide, since the application of fungicides is often accompanied by an increase in mycotoxins (D'Mello et al., 1998; Popiel et al., 2017). Depending on the mycotoxin concentration, the crops might be saved, but unusable for consumption. Despite not being confirmed in the present work, studies about a dual effect of chitosan, inhibiting both mycelial growth and mycotoxin production of different *Fusarium* species are described (Ferrochio et al., 2014; Zachetti et al., 2019). Altogether, the achieved results should be treated with caution, as they suffered from high standard deviations and only few biological replicates. Furthermore, two studies investigating the expression of ergosterol-related genes of *Saccharomyces cerevisiae* in response to paCOS treatment resulted in contradictory results, with one study observing upregulation and one study observing downregulation of ergosterol biosynthesis (Jaime et al., 2012). Hence, the influence of chitosan on ergosterol biosynthesis can only be assumed and has to be further analyzed.

How chitosan is able to alter the fungal gene expression profile is unknown. It has been suggested that chitosan directly interacts with cellular DNA and thus preventing RNA synthesis (L. A. Hadwiger et al., 2011). However, gene expression alterations as a response of the fungus

to chitosan perception or even active update (Palma-Guerrero et al., 2009) is also conceivable. For example, chitosan was shown to induce stress and cell wall integrity genes in *S. cerevisiae*, possibly as a repair mechanism. Matching this, *Neurospora crassa* was observed to express a class 3 lipase involved in lipid replacement as one main response to chitosan (Lopez-Moya et al., 2016). These studies might provide further evidence for the cell wall and membrane perturbing activity of chitosan as its main mode of action (Zakrzewska et al., 2005).

Overall, the non-increased expression of adverse stress genes in chitosan treated *F. graminearum*, together with the previously shown mycelium growth and spore germination inhibition effect of chitosan, provide once again evidence for chitosan being a promising alternative for conventional fungicides.

4.1.3. Chitosan as new big seller on the fungicide market?

Combining the observations from both antifungal activity assays and gene expression studies, it can be concluded that chitosan can easily keep up with existing fungicides. Chitosan strongly inhibited the growth of *F. graminearum*, especially when applied in mixtures. Furthermore, it could be shown that chitosan did not trigger mycotoxin production, at least concerning the studied genes, which confirmed previous studies (Ferrochio et al., 2014; Zachetti et al., 2019). This makes chitosan a powerful compound, as conventional fungicides often come along with drawbacks like stress-induced mycotoxin production of the targeted fungus.

As mentioned, chitosan was observed to be effective against a broad range of fungi. However, it can be assumed that the efficiency of chitosan is not only dependent on its physico-chemical properties, but furthermore on the cell wall composition of its target (Palma-Guerrero et al., 2010; Abo Elsoud & El Kady, 2019). Therefore, it is of paramount interest to determine which chitosan works best on which pathogenic fungus in order to establish chitosan as fungicide - or at least fungicide component. Especially in combination with fungicides, the antifungal activity can benefit from both the direct efficacy of chitosan on fungal growth and development and its reinforcement of the efficiency of the fungicide (Rahman, Shovan, et al., 2014). This allows a reduction in fungicide concentration, which is especially desirable in case of metal-based fungicides like copper - or, as shown here, a complete replacement of conventional fungicides with chitosans and chitosan mixtures.

Another important aspect to consider is fungicide resistance. As already mentioned above, fungicide resistance is one of the biggest issues in modern agriculture. As resistance development is usually a result of adaptation towards a fungicide, the fungicide itself acts as a

selection medium for resistant individuals, then forming new resistant populations (Deising et al., 2008). Accordingly, chitosan treatment could eventually result in resistance with chitosan itself fueling this action. For instance, resistance against paCOS was found in yeast, which responded to paCOS treatment with transcriptional changes that reduced membrane permeability (Jaime et al., 2012). Likewise, it was shown that chitosan resistance in *Staphylococcus aureus* is dependent on an enhanced production of cell wall polymers and an overall increased positive cell surface charge that reduces chitosan binding (Raafat et al., 2017). Chitosan resistance however might show certain advantages over resistance to conventional fungicides. First, fungicide resistance is more likely to occur towards compounds with a single and distinct mode of action. This usually results in mutation-based qualitative resistance, leading to a complete tolerance towards the fungicide. As long as no definite single mode of action is postulated for chitosan, but it remains treated as compound with several putative mode of actions, chitosan treatment would rather result in quantitative resistance, which still allows fungal growth to a certain extent, depending on the strength of the resistance (Deising et al., 2008). Quantitative resistance still allows adjustment of the fungicide (*e.g.* by increasing the concentration or slightly changing the physico-chemical properties of chitosan), while qualitative resistance implies complete inactivity. Second, if different fungicides share the same mode of action, a qualitative resistance to one fungicide would subsequently lead to resistance towards the other fungicides, a phenomenon known as cross-resistance (Leroux, 1992; M. Hahn, 2014). Suspecting different modes of actions for chitosan, cross-resistance is unlikely to happen. Plus, chitosan was shown not to confer resistance to other fungicides that target the fungal cell walls, despite of showing the same activity (Jaime et al., 2012). This would further allow chitosan to be used in combination with fungicides that may have lost their antifungal activity if applied alone, but recover if applied together with chitosan (Jaime et al., 2012).

Albeit resistance might not be avoided, chitosan represents a strong compound as antifungal agent - if not by superseding conventional fungicides at least by supporting their actions in a way that not only serves the successful repression of pathogens threatening crop plants, but also the environment.

4.2. Eliciting activity of chitosan

The ability of external molecules to trigger reactions in plants and thus act as elicitors is already known for at least a century. For example, the hypersensitive reaction (HR) was first described as early as 1915 by Elvin C. Stakman, discovering a rapid cell death as a plant response to rust fungus infection (Stakman, 1915). Based on this, studies on plant elicitors more and more

revealed both the molecular mechanisms of elicitation and types of molecules with the potential to act as elicitors. The ability of sugars to act as elicitors for plant responses were firstly discovered in the 1980s, elucidating not only the influence of carbohydrates as regulatory molecules (J. K. Sharp, McNeil, et al., 1984), but also first hints about structure-function relationships (J. K. Sharp, Albersheim, et al., 1984). At the same time, Noriyuki Doke observed that potato tuber infection with *P. infestans* caused superoxide generation at the plant plasma membrane (Doke, 1983b), which could also be induced only by *P. infestans* cell wall preparations (Doke, 1983a). Today it is well known that elicitors cause a broad range of plant responses, one of the earliest known ones always being the oxidative burst. Since chitosan was discovered to display eliciting activity on plants (Pearce & Ride, 1982; Moerschbacher et al., 1986), it does not surprise that this observation fueled the already great interest in chitosan research concerning its versatility in plant protection and fertilization.

4.2.1. Chitosan-induced oxidative bursts in plants

In this study, the eliciting potential of chitosan was analyzed in respect to F_A and DP. Despite of existing studies dealing with structure-function relationships of chitosans in terms of plant responses, the aim of this study was to deepen this knowledge by using a broad range of different, well-defined chitosans, ranging from small oligomers to large polymers and from fully acetylated to more than halfway through de-acetylated molecules. Furthermore, this study investigated the eliciting potential of chitosan combinations for the first time. As seen for the antifungal activity, the potential of a chitosan to elicit plant responses is crucially dependent on its physico-chemical properties. In this study, chitosans with F_A ranging from 0.0 to 0.6 and DPn ranging from 2 to ~2000 were screened for eliciting activity. Overall, best eliciting activities could be observed with chitosans of intermediate DP and F_A of 0.4 or 0.5. Both too small and too large, but especially chitosans with very low F_A, did not display high eliciting activity on potato leaf discs.

Concerning the F_A, an optimum curve could be generated with the highest activity with F_A 0.4 or 0.5, respectively. Basically, one major mode of chitosan perception in plants is believed to be through PRRs in the plant plasma membrane, which both trace the molecule and forward a signal within the plant cell (Iriti & Faoro, 2009; Saijo et al., 2018). However, as no distinct chitosan receptor could be described yet, chitosan perception is assumed to be taken over by chitin binding receptor complexes like CERK1 in combination with LYP in rice and LYK in *Arabidopsis* (B. Liu et al., 2012; Cao et al., 2014). Considering this, receptor-related chitosan perception could explain the preference for higher F_A chitosans eliciting stronger oxidative

bursts, as higher acetylated chitosan molecules closer resemble chitin. Maximum eliciting activity with chitosan polymers of intermediate F_A was already observed in earlier studies on wheat leaves (Vander et al., 1998) and *Arabidopsis* (Gubaeva et al., 2018), confirming the here observed findings. Furthermore, decrease in eliciting activity with an F_A beyond 0.5 was also observed in both studies. The authors of the wheat leaves study state that chitosan polymers are expected to be degraded by apoplastic wheat leaf endochitinases (Ride & Barber, 1990), which would not only influence the eliciting activity but might furthermore explain the deceased activity of F_A 0.6 chitosan, in case being more susceptible for chitinases in general. By breaking down the chitosan polymer into smaller polymers or oligomers, the eliciting activity might be significantly altered as discussed later. Another explanation for decreased eliciting activity of high F_A chitosan polymers could be hydrophobic interactions of GlcNAc units within the chitosan molecule. It is known that chitin chains tend to aggregate and agglomerate, which is observed to attenuate with deacetylation (Popa-Nita et al., 2010; Roy et al., 2017). Secondary structures of chitosan molecules caused by hydrophobic interactions of GlcNAc units might prevent either binding or to the corresponding receptors dimerization of receptor subunits and thus lower or completely withdraw eliciting activity. Binding of chitosan to cell membranes is not only linked to electrostatic interactions, but also to hydrophobic interactions of GlcNAc residues with membrane fatty acids and other uncharged components (Pavinatto et al., 2007). Therefore, decreased eliciting activity of high F_A chitosans might additionally indicate that receptor perception is at least the major mode of action of chitosans on plant cells next to receptor-independent, physico-chemical interactions.

COS (DP 4-6) did not result in eliciting activity on potato leaf discs. As only two concentrations were tested with the higher concentration leading to higher RLU, it can be assumed that eliciting activity might be observable by further increasing the COS concentration. Comparable COS of fully acetylated DP 3-6 molecules were tested for eliciting activity on *Araucaria angustifolia* and where likewise observed to be unable to trigger an oxidative burst (Dos Santos et al., 2008). COS were furthermore observed to be active on wheat leaves with DP $\geq$ 7 (Vander et al., 1998). Based on these findings, perception of COS seems to be dependent on their size, with larger DP strongly influencing their ability of triggering oxidative bursts. This statement was furthermore confirmed on *Arabidopsis* seedlings, only responding to COS with a minimum DP of 6 (Gubaeva et al., 2018). The authors additionally showed that fully deacetylated chitosan oligomers (DP 3-8) did not trigger an oxidative burst in *Arabidopsis* seedlings (Gubaeva et al., 2018). This clearly provides evidence for the importance of acetylated units for the perception of chitin/chitosan oligomers. It has been hypothesized that at least five acetyl groups are

required to enable both binding of the oligomer and dimerization of the receptor molecules to fully enable perception and signal forwarding in rice cells (Hayafune et al., 2014; Hanae Kaku & Shibuya, 2016), which was subsequently proven by the eliciting activity of enzymatically produced paCOS fulfilling this requirement (Cord-Landwehr et al., 2016). A later hypothesis reduced the number of required acetyl groups to four (Gubaeva et al., 2018). Assuming that the receptor-based perception of COS in potato does not differ much from rice and *Arabidopsis* plants, the lack of eliciting activity of a COS mixture with DP 3-6 is likely to be explained by a too short size.

The commercially available CPS 4 paCOS series (DP 2-12, F_A 0.1) showed eliciting activity in a concentration range between 10 and 750 µg/mL with an optimal concentration of 250 µg/mL. Eliciting activity of paCOS was also observed on wheat leaves in a DP range between 3 and 8 (Dos Santos et al., 2008). However, as the CPS 4 paCOS was determined to have an average F_A of 0.1, is highly unlikely that it consists of a lot of oligomers with at least five acetyl groups. Hence, it can be assumed that either the hypothesis of (Hanae Kaku & Shibuya, 2016) is not universally applicable or the eliciting activity of paCOS is not limited to receptor-dependent oligomer perception. On the other hand it was shown that chitosan-induced elicitation of oxidative bursts in *Arabidopsis* seedlings is highly dependent on the presence of AtCERK1 (Gubaeva et al., 2018). Furthermore, the usage of potato leaf discs promotes exposure of both wounded and intact cells to chitosan, which provides additional interest in the hypothesis of receptor-independent plant responses to chitosan treatment.

As a hydrolysis product, 661 comprises of both polymers and oligomers and thus consequently displays a high dispersity of 5. Hence, strong differences between unseparated 661 and its separated fractions in terms of eliciting activity could not be observed in a significant manner. However, the oligomer fraction seemed to be slightly more active than the polymer fraction and the unseparated 661. The most striking observation was the narrow concentration range in which the polymer fraction displayed eliciting activity. Concerning receptor-dependent perception, this result could be explained by receptor blocking of large chitosan molecules in high concentrations, which is apparently not as problematic with smaller molecules. This blocking effect might either be caused by single or multiple chitosan molecules, both preventing successful receptor dimerization due to the claimed space of the molecule(s). Considering receptor-independent activity, a high concentration of large chitosan molecules could be potentially toxic to the plant cell. For instance, it was shown that chitosan induces programmed cell death in pea leaves (Vasil'ev et al., 2009) and soy bean cells (Zuppini et al., 2004).

Especially the results of the latter study fits well into the here observed findings, as the cell death induced by chitosans in a concentration of 200 µg/mL resulted in cell death without any H_2O_2 production, but rather might be caused by a fast plasma membrane disturbance (Zuppini et al., 2004). As both the maximum eliciting activity and the activity range of the 661 oligomer fraction is slightly enhanced compared to unseparated 661, it can be assumed that larger molecules, which are present in unseparated 661, generally contribute to either receptor blockage or a higher risk of cell death. This was furthermore confirmed by shifting the composition of 661 to either an excess of oligomers or polymers, which resulted in higher eliciting activity for increasing oligomer excess, while an increase in polymer excess gradually decreased the eliciting activity down to inactivity.

Neither concentration range nor maximum oxidative burst concentration differed in the eliciting activity of 75/5, 75/20 and 75/100, although the DPn ranged from 170 to ~1000. However, increasing the DPn further to ~1700-2000 as tested with 90/1000 and 90/3000, a severe impact on both parameters could be observed. A significantly less concentration was required for 90/1000 or 90/3000 to achieve the maximum oxidative burst activity. Furthermore, the concentration range was narrowed down strongly, with 50 µg/mL already resulting in no oxidative burst activity and hence might block receptors or lead to cell death as described before. As charge interactions become more likely with increasing DP, especially when using low F_A chitosans (in this case 0.15), application of 90/1000 and 90/3000 could cause membrane disturbance and eventually cell leakage, already in low concentrations. Likewise, chitosan perception through receptors might be prevented by the sheer size of one chitosan polymer molecule. As already mentioned for the antifungal activity of chitosans, the activity of chitosan polymers might be furthermore limited due to reduced solubility in water (Rhoades & Roller, 2000; Badawy & Rabea, 2011). Another aspect that must be taken into account is the constitutive secretion of chitinases by plant cells as an important resistance mechanism against fungi (Punja & Zhang, 1993). These chitinases not only directly damage pathogenic fungi through cell wall degradation, but furthermore provide chitin fragments acting as elicitors for plant PRRs (H. Kaku et al., 2006; Wan et al., 2008). It is highly likely that plant chitinases deriving from the potato leaf discs degrade the applied chitosan, which might contribute to amplification of the oxidative burst response. However, especially when using large chitosan polymers, the mass of emerging oligomers might either lead to too much charge interactions or prevention of receptor-dependent chitosan perception due to receptor blocking. Either effect could explain the decreased or erased eliciting activity of chitosan polymers in too high concentrations. The assumption of chitosan degradation via plant chitinases is furthermore

supported by the fact that only polymer chitosans displayed eliciting activity in very low concentrations. While large chitosan molecules could be broken down to a sufficient amount of smaller chains, chitosan oligomers seemed to be present in insufficient amounts for both direct receptor-dependent eliciting activity and chitinase substrate to deliver an adequate number of molecules. Supporting this hypothesis, it has been shown in previous experiments that oxidative bursts triggered by polymeric chitosans are slightly delayed in comparison to bursts triggered by oligomeric chitosans, but longer lasting (unpublished results). This is an additional hint to the activity of plant chitinases that hydrolyze the chitosan to smaller molecules, which then become elicitors for plant receptors. As too small chitin or chitosan oligomers are believed to be eliciting inactive as described before, chitinase degradation of already small molecules might further contribute to their inactive nature.

Probably the most considerable observation is the synergistic activity of a chitosan mixture, which resulted in significantly higher eliciting activity in comparison to the individual chitosans. Whereas application of 75/5 showed mediocre oxidative burst induction, both 90/1000 and 90/3000 alone did not trigger significant oxidative burst signals in the used concentrations. However, combining 75/5 with each of the large polymeric chitosans resulted in synergism. In contrast to antifungal activity, synergistic activity of chitosan as plant elicitor has not been described yet. However, as assumed for combinations of chitosans and fungicides, the synergistic activity of chitosan mixtures on plants might be likewise assigned to different modes of actions of each component (Rahman, Shovan, et al., 2014). For example, one chitosan could trigger receptor-mediated oxidative burst while the other chitosan causes plasma membrane stress, enhancing the oxidative burst response, simultaneously. Another possible explanation is provided by the already mentioned degradation of chitosans via plant chitinases. Using different chitosans likely results in different chitinase products, which potentially mimics multiple attacks of different organisms, causing the plant to enforce its defense responses. Related to this, a recent study showed that plants successfully deal with a dual herbivore attack through both local and systemic defense responses (Kiełkiewicz et al., 2019).

In conclusion, the achieved results confirm earlier studies, stating that the biological activity of chitosans on plants is dependent on their physico-chemical properties. It could be shown that small chitosan oligomers are active in a broader concentration range, while large chitosan polymers are only active in a narrow concentration range. This is likely caused by chitinase activity on the chitosan molecules, leading to molarity increases, which in turn might contribute to receptor blockage. Furthermore, large chitosan molecules could also prevent their perception

through receptors by the formation of secondary structures. Another important result is the inactivity of high chitosan concentrations – especially of polymeric chitosans, which might be a result of cell death caused by plasma membrane disruption. In addition to these findings, the synergistic activity of a chitosan mixture provides evidence for different modes of action for chitosans with different physico-chemical properties. Hence, it is strongly suggested that the different theories of the mode of actions of chitosans on plants may be treated equally conceivable, instead of assuming that only one mode of action of chitosan is possible.

4.2.2. Chitosan-induced gene expression

Chitosan treatment triggered significant differential gene expression of rather few genes. In comparison, hundreds of genes were observed to be differentially expressed in chitosan-treated strawberry fruits (Landi et al., 2017). This significant difference might be assigned to the fact that ChitoPlant (name changed to ChiProPlant recently), a commercial chitosan-containing plant stimulant from ChiPro GmbH (Bremen, Germany) was used for strawberry treatment. As this product is labeled as "chitosan-based", other ingredients are likely to contribute to transcriptomic changes after its application. The low number of DEG observed in this study might thus be the result of using a pure, well-defined chitosan. All DEG will be discussed in more detail according to their functional groups in the following subchapters.

4.2.2.1. Photosystems

Regarding photosystem (PS) II, *psbD*, the gene coding for the D2 protein, one of its two main subunits, was upregulated (PGSC0003DMG400017258). The fold change of *psbD* was almost doubled 5 hpt (2.8) compared to 2 hpt (1.5). PSII enhancement was furthermore accompanied by upregulation of *psbA*, the gene coding for the D1 protein, which represents its second main subunit (PGSC0003DMG400004211). Together, D1 and D2 form the heterodimer reactive center core of PSII (Marder et al., 1987). The *psbA* gene showed the highest upregulation of all genes with significant fold changes. As it was shown that induction of additional *psbA* gene copies is associated with photoprotection of PSII in cyanobacteria (Kiss et al., 2012) and higher plants (F. Wang et al., 2016), this observation could indicate photoprotection activity in potato as a response to chitosan. The susceptibility of PSII to photoinhibition, the light-dependent reduction of photosynthetic capacity, is well known and well-studied (Samuelsson et al., 1985). However, as all leaves – independent of their treatment – faced the same environmental conditions during incubation, significantly higher fold changes of *psbA* and other PS-related genes may indicate that their upregulation is not directly associated to photodamage. Additionally, upregulation of genes coding for auxiliary proteins like the DegP2 and FtsH

proteases, which support PSII degradation and replacement in cases of photoinhibition (Haußühl et al., 2001; Nixon et al., 2004), could not be observed. Still, as there is evidence about protection of PSI, which in rare cases can also suffer from photodamage, by regulating the PSII activity (Tikkanen et al., 2014), upregulation of PSII related genes could be allocated to a general precaution mechanism of the potato. As a third main component of PSII, *psbB* was upregulated via chitosan treatment (PGSC0003DMG400046303). *PsbB* encodes the PSII chlorophyll-binding protein CP47, which forms the inner light-harvesting complex of PSII together with the *psbC*-product CP43 (Barber et al., 1997; Bricker & Frankel, 2002). Co-expression of the two core protein genes *psbA* and *psbD* together with *psbB* can be explained by their close collaboration in which CP47 is suggested to be located in the center of the D1/D2 heterodimer, transferring excitation energy from the PSII light-harvesting complexes to the D1/D2 dimer (Luciński & Jackowski, 2006). This observation is furthermore supported by a common pathway model for the PSII core polypeptide synthesis, after that expression products of *psbA*, *psbD* and *psbB* genes form a protein complex intermediate during biosynthesis of the PSII core before being attached to CP43 (de Vitry et al., 1989). Upregulated genes related to PSI were three different variants of *psaA* (PGSC0003DMG400012033, PGSC0003DMG400015960, PGSC0003DMG400005372), coding for the P700 chlorophyll a apoprotein A1. As for PSII, a protein heterodimer forms the reaction center of PSI, encoded by the genes *psaA* and *psaB* (Busch & Hippler, 2011). This dimer binds P700, the primary electron donor of PSI, consisting of a chlorophyll a molecule, and initiates NADP reduction via electron transfer (Webber & Lubitz, 2001). The generally higher and longer upregulation of PSII components could be explained by their shorter lifetime due to oxidative damage, as PSII is the strongest known natural oxidizing agent, responsible for the conversion of H_2O to O_2 (Vrettos & Brudvig, 2002). It was also shown that the half-life times of PSI components are significantly higher in comparison to PSII components (30-75 h for PSI, 1-11 h for PSII proteins) (Yao et al., 2012), thus general recovery of PSII might also influences the here observed higher fold change values of corresponding genes.

4.2.2.2. Cytochrome b_6f complex and ATP synthase

The cytochrome b_6f complex is the smallest of the three photosynthetic membrane protein complexes. It mediates the electron transfer between PSII and PSII by accepting electrons from plastoquinol to plastocyanin (plastoquinol-plastocyanin oxidoreductase), accompanied by proton transfer from the chloroplast stoma into the lumen (Kurisu et al., 2003). The complex is a homodimer with each monomer consisting of eight or nine subunits, depending on the species

(H. Zhang & Cramer, 2004). It is mainly built of four large essential subunits, the chloroplast-encoded subunits PetA (cytochrome f), PetB (cytochrome b_6), PetD (subunit IV) and the nuclear-encoded subunit PetC, also known as the Rieske protein (Mark Aurel Schöttler et al., 2015). In photosynthesis, two electrons are transferred from plastoquinol to cytochrome b_6f complex. The first electron is accepted by PetC (Rieske protein), the second one by PetB (cytochrome b_6). Both electrons are then transferred to PetA (cytochrome f), eventually reducing two plastocyanins with the transfer of one electron per molecule (Baniulis et al., 2008). In this study, genes coding for cytochrome f (*petA*, PGSC0003DMG400002905) and cytochrome b_6 (*petB*, PGSC0003DMG400019419) subunits were observed to be significantly upregulated. Expression of *petB* was upregulated at both time points, with an almost 3-fold upregulation 5 hpt. Upregulation of *petA* could only be observed 5 hpt. Although there is evidence about a lifetime of the cytochrome b_6f complex of at least 24 h (X.-S. S. Gong et al., 2001), no data is available about the lifetime of certain subunits. However, the higher upregulation of *petB*, coding for the cytochrome b_6 subunit, could point out a shorter half-life of this subunit. As cytochrome b_6 is located on the reducing site of the complex, taking electrons from plastochinol, cytochrome b_6 might be exposed to oxidative damage from the plastosemiquinone radical, the intermediate product of plastochinol after losing the first electron to the Rieske protein (Pospíšil, 2009). These findings also fit into the accepted biogenesis theory for the cytochrome b_6f complex in which cytochrome b_6 (*petB*) and subunit IV (*petD*) are assembled first before inserting cytochrome f (*petA*) into the membrane (Mark Aurel Schöttler et al., 2015). However, significantly different gene expression of *petD* could not be observed in this study. Overall, upregulation of essential cytochrome b_6f complex subunits can be assigned to photosynthesis induction as abundance and efficiency of this complex severely determines the electron flux rate (Anderson, 1992).

The chloroplast ATP synthase is the last protein complex in the light-dependent photosynthesis reaction chain in which finally the proton-motive force of the beforehand built up proton gradient is used to create ATP (photophosphorylation). Chloroplast ATP synthases (cF_1F_0 synthases) mainly consists of two subcomplexes, the stroma-located catalytic head F_1 and the membrane-bound motor F_0. The F_1 part consists of three heterodimers of α- and β-subunits ($\alpha_3\beta_3$ subcomplex) which form the moving part of the head and the subunits γ and ε that are attached to subunit c of F_0 (A. Hahn et al., 2018). Subunit c forms the rotor of the ATP synthase through which the protons pass during ATP production (Stock, 1999). Other subunits of F_0 are subunit a and the peripheral stalk, acting as a stator between F_0 and F_1 (J. E. Walker & Dickson, 2006). In the process of ATP synthesis, protons first bind and pass through the c-subunits of F_0,

which eventually causes the F_1 head to rotate. This rotation causes ADP and P_i to bind to $\alpha_3\beta_3$ of F_1 subunits, forming ATP. In total, three ATP molecules are created and released per complete rotation (360 °) of the F_1 head. This rotational catalysis model was first described by Paul Boyer in 1997 (Boyer, 2002). ATP synthases execute the final step of the photosynthetic light reaction by producing energy for the subsequent light-independent reactions to finally convert carbon dioxide into sugars. In this study, upregulation of three different genes encoding α-subunits (*atpA*, PGSC0003DMG401022238, PGSC0003DMG400013849, PGSC0003DMG400008476), as well as one gene encoding each a β-subunit (*atpB*, PGSC0003DMG400034122) and a ε-subunit (*atpE*, PGSC0003DMG400025106) respectively was observed. This upregulation only occurred 5 hpt and was on average weaker (1.5 to 2 fold) compared to upregulation of the cytochrome b_6f complex (2.2 to 2.9 fold) or photosystems (1 to 3 fold). The induction of ATP synthase related gene expression might provide evidence for a crosstalk of ATP synthase and cytochrome b_6f complex in potato. This co-regulation of both protein complexes to ensure proton balance and thus controlling ATP and NADPH production was already discussed and investigated in other plant species (Mark A Schöttler & Tóth, 2014). For example, it was shown that tobacco plants with reduced ATP synthase accumulation but unchanged cytochrome b_6f abundance and functionality display strong lumen overacidification, overall leading to photosynthesis repression and thus smaller and weaker phenotypes (Rott et al., 2011). However, transcriptome analysis of chitosan-treated potato leaves at later time points could display higher expression of ATP synthase genes to avoid this state.

4.2.2.3. Nonlinear photophosphorylation

Apart from the linear electron transport from PSII over cytochrome b_6f and PSI, which eventually reduces NADP to NADPH, a non-linear, cyclic electron transport exists between PSI and the cytochrome b_6f complex (Joliot & Joliot, 2006). In cyclic electron transport, electrons are transferred from PSI over different electron carriers back to the cytochrome b_6f complex and finally back to PSI to refill the electron gap. During this process, more protons are carried into the chloroplast lumen which can eventually be used to generate more ATP without the accumulation of NADPH, which accepts the electron from PSI in linear electron transport (Shikanai, 2014). Accumulation of reducing agents can be furthermore avoided by using NADPH as electron donor directly to reduce plastoquinone to start the cyclic electron transport (Munekage et al., 2004). Different models for cyclic electron transport have been discussed in higher plants. One prominent model is the PGR5-PGRL1-dependend pathway in which electrons are transferred via the proton-gradient regulation protein PGR5 to a putative

ferredoxin-plastoquinone reductase (PGRL1), which subsequently transfers electrons to plastoquinone (Hertle et al., 2013). Another putative model includes a type I NADPH dehydrogenase (NDH) complex which is homologous to the respiratory complex I in mitochondria, but encoded in chloroplast *ndh* genes (Burrows et al., 1998). As one of the major functions of cyclic electron transport, NDH complex increases ATP production by an increased proton import into the thylakoid lumen and thus enhancing ATP synthase activity (Strand et al., 2017). Right after its discovery, it was shown that chloroplasts of higher plants contain functional NDH complexes of large size but low abundance, possibly taking part not only in cyclic electron flow but also in stress management and even crosstalk between chloroplasts and general cellular metabolism (Burrows et al., 1998; G. N. Johnson, 2011). It was shown that NDH-mediated cyclic electron transfer plays a crucial role in C_4 plants, as their ATP demand relative to NADPH is higher than in C_3 photosynthesis (Ishikawa et al., 2016). However, NDH complex activity was also measured *in vivo* in C_3 plants to compensate ATP deficits (Joet et al., 2002). Today, it is known that the NDH complex in higher plants consists of a supercomplex of several subcomplexes, whereby the main two subcomplexes A and M are encoded by 11 plastid-encoded genes (Shikanai, 2016). In this study, evidence was found for four different NDH complex subunit coding genes upregulated in response to chitosan 5 hpt. Two of those genes encode for ndhI (PGSC0003DMG401011339) and ndhK (PGSC0003DMG400006986), that form the plastoquinone-binding subcomplex A together with ndhJ and ndhH (Burrows et al., 1998; Strand et al., 2017). Both corresponding genes were upregulated 1.9 and 1.3 fold, respectively. The other two upregulated genes (PGSC0003DMG402008783, 1.8 fold and PGSC0003DMG400015304, 1.3 fold) could not clearly be assigned to certain subunits, but potentially code the missing ndhJ and ndhH subunits of subcomplex A. Nuclear-encoded subunits and protein factors are also believed to be present in NDH subcomplexes (Ifuku et al., 2011) whose expression was not observed in this study. As NDH complex activity is known to prevent oxidative stress in chloroplasts caused by temperature, humidity, drought and other abiotic fluctuations (Yamori & Shikanai, 2016; Essemine et al., 2017), it can be assumed that chitosan triggers the potato to prepare for a broad range of abiotic and biotic stresses through NDH complex induction, possibly helpful during upcoming pathogen threats or environmental changes.

4.2.2.4. Mitochondrial respiratory chain components

Alike the electron transport chain in the photosynthetic light reaction including the above described NDH complex, the respiratory chain in the mitochondria was partly triggered by

chitosan treatment. Upregulation of different protein complexes including the ATP synthase could be observed at both time points, whereas the upregulation was stronger at 5 hpt. 2 hpt, three genes encoding for proteins related to cytochrome c oxidase (complex IV) or cytochrome c directly (PGSC0003DMG402008771, PGSC0003DMG400002971, PGSC0003DMG400016897), as well as one ATP synthase subunit gene (PGSC0003DMG400001014) were upregulated. This upregulation was relatively weak; however, same genes were upregulated 5 hpt with overall higher fold changes up to over 2. Additionally, two other ATP synthase related genes showed upregulation 5 hpt. *OrfB* (PGSC0003DMG400011350) is a conserved gene in plant mitochondrial genomes, encoding for the ATP8 protein which is part of F_0 of the mitochondrial ATP synthase (Sabar et al., 2003). Analogously, the *orf25* gene (PGSC0003DMG400026520) was also upregulated 5 hpt. Like *orfB*, *orf25* was observed to be located in the ATP synthase F_0 motor, however, their functions in plants is not understood yet (Heazlewood et al., 2003). In contrast to 2 hpt, where only complex IV and complex V (ATP synthase) related genes were observed to be upregulated, upregulation of complex I and complex II related genes was exclusive to 5 hpt. Five different complex I (NADH dehydrogenase) subunit genes (PGSC0003DMG400030943, PGSC0003DMG400003375, PGSC0003DMG400013204, PGSC0003DMG400020931, PGSC0003DMG400021388) were upregulated between 1 and 1.8-fold in response to chitosan treatment. Furthermore, a slight upregulation of a complex II (succinate dehydrogenase) subunit 3 gene (PGSC0003DMG402008334) could be observed. In total, chitosan treatment of potato leaves led to upregulation of all protein complexes of the mitochondrial respiratory chain except for complex III. The respiratory electron transfer in plant mitochondria is not only important for energy supply via ATP synthesis, but can respond to different metabolic states of plant cells if altered due to environmental changes (Schertl & Braun, 2014). It is also known that not only the electron chain of the light reaction, but also the mitochondrial electron chain can react to light stress, *e.g.* supporting the chloroplasts to deal with excess NADPH (Yoshida et al., 2011). However, as mentioned earlier, as all leaves were incubated equally, significant upregulation of corresponding genes is more likely to be a response directly to chitosan than to light stress. As several studies investigated the participation of mitochondria in producing ROS to regulate plant stress (Møller, 2001; Gleason et al., 2011; S. Huang et al., 2016), expression of mitochondrial respiratory chain related genes could be assigned to defense responses as discussed further below.

4.2.2.5. Defense-related genes

Direct activation of genes clearly assigned to defense-related processes were rather limited to 2 hpt. Only a LRR-receptor like kinase (PGSC0003DMG400017713) and one type of WRKY transcription factor (PGSC0003DMG400009103) were upregulated. Nevertheless, LRR receptor like kinases (LRR-RLKs) play central roles in signaling during pathogen perception (Afzal et al., 2008) whereas WRKY transcription factors display a major transcription factor family in plants, regulating a broad range of processes including biotic and abiotic stresses (Phukan et al., 2016). The here observed LRR-RLK was for instance also found to be upregulated in the wild potato *Solanum commersonii* after *Ralstonia solanacearum* infection (Zuluaga et al., 2015). Despite the stronger upregulation after infection in comparison to the upregulation after chitosan treatment (2.7 and 2 vs. 1.2), chitosan treatment of potato seems to slightly induce defense in the plant which might help against upcoming infections. Interestingly, downregulation of both a glutaredoxin family protein (PGSC0003DMG400008952) and an ethylene responsive factor (PGSC0003DMG400017233) was observed 5 hpt. As both protein families are involved in infection recognition and especially subsequent signaling (Rouhier et al., 2008; P. Y. Huang et al., 2016), it can be assumed that chitosan treatment particularly leads to the gene expression related to early defense responses. These early responses might not be required anymore at later time points, either due to the expression of corresponding downstream genes or due to the recognition of the potato leaf not suffering from a real infection. LRR-RLKs are also known to be involved in ROS signaling (Eckardt, 2017) which will be discussed in the next chapter. Dozens of WRKY transcription factors were studied and identified in potato in 2013 by Huang et al. and furthermore classified into different groups (HUANG & LIU, 2013). This study was later continued by Zhang et al., showing that the expression of WRKY transcription factors is often linked to drought, heat, infections and other biotic and abiotic stress conditions (C. Zhang et al., 2017). Based on their classification, the WRKY transcription factor found in this study is classified as StWRKY22, located on chromosome 3 and grouped into group III, the group that contains WRKY transcription factors influencing disease resistance (Y. Wang et al., 2015; Y. Huang et al., 2016). Upregulation of both LRR-RLK and StWRKY22 clearly indicate that chitosan can trigger defense responses in potato leaves within the first few hours after treatment. It is conceivable that this LRR-RLK is important for chitosan perception and signal forwarding, possibly through mitogen-activated protein (MAP) kinases as described for chitin perception in rice and *Arabidopsis* plants (Kawasaki et al., 2017). Few other upregulated genes, functioning not directly in disease responses, might influence resistance however indirectly and support the hypothesis of chitosan eliciting the potato defense system.

Both extensins (PGSC0003DMG400001380) and proline-rich cell wall proteins (PGSC0003DMG400009783) are known to be involved in plant cell wall biosynthesis; however, there is evidence that the latter might also be necessary in cases of stress, assisting the plant to deal with alterations in cell volume and turgor and also participating in other defense-related actions (Kavi Kishor, 2015). Furthermore, a PRA1 family protein gene was found to be upregulated (PGSC0003DMG401031172). PRA (prenylated Rab acceptor) proteins are transmembrane proteins and known to regulate vesicle trafficking (Alvim Kamei et al., 2008). Recent studies also suggest their involvement in plant immune responses, showing that overexpressing a PRA1 protein in tomato reduces resistance to fungal pathogens by decreasing intracellular trafficking and degradation of a certain PRR (Pizarro et al., 2018). The likewise upregulated gene coding for a leucoanthocyanidin dioxygenase (PGSC0003DMG400027333) is known to be involved in the biosynthesis of flavonoids, a group of secondary metabolites acting among other functions in responses against UV light and pathogen infection (Falcone Ferreyra et al., 2012). A phospholipase A_2 (PLA_2) gene (PGSC0003DMG400029964) was upregulated 2-fold 5 hpt. PLA_2 enzymes catalyze the cleavage of fatty acids and lysophospholipids from the glycerol backbone of membrane phospholipids. These products can regulate several processes in plants, including plant growth, plant development and stress responses (Lee et al., 2005). For example, it was shown that PLA plays a role in elicitor-induced oxidative bursts in soybeans via NADH oxidase activation by PLA hydrolysis products (Chandra et al., 1996). Several other genes were found significantly upregulated 5 hpt. *Orf122* (PGSC0003DMG400041193, 1.7-fold) was found to be induced at salt-stress conditions in barley leaves within the first 24 h after stress induction (Ueda et al., 2004). Furthermore, a gene coding for a lipoxygenase was upregulated (PGSC0003DMG400032207, 1.4-fold), catalyzing an intermediate step of the jasmonic acid synthesis, a plant hormone known to be involved in plant responses to wounding and systemic resistance (Siedow, 1991; Schaller & Stintzi, 2009). Also, two genes similar to elicitor-inducible genes from tobacco (*NtEIGs*) were upregulated. It was reported that *NtEIG-A1* (PGSC0003DMG400000621, 1.2 fold) shows similarities to stellacyanins, a group of plant-specific copper binding proteins that are involved in primary defense responses (Nersissian et al., 1998; Takemoto et al., 2003). Correspondingly, another stellacyanin-like protein (PGSC0003DMG400000620), showing similarities to stellacyanin CASLP1, found in infected leaves of *Capsicum annuum* (H. Y. Kong et al., 2002), was observed to be upregulated at both time points. As stellacyanins are described as defense-related proteins, the expression pattern of the stellacyanin found in this study demonstrates the ability of chitosan activating early resistance mechanisms in the potato, as the upregulation of the found

stellacyanin decreased from 2 hpt (1.5-fold) to 5 hpt (1.1-fold). The second upregulated *NtEIG*-similar gene was *NtEIG-E80* (PGSC0003DMG400011087, 1.1 fold) which has been described as a photoassimilate-responsive protein (Takemoto et al., 2003). This study also reported that NtEIG-E80 displays high identity to PAR-1c, another photoassimilate-responsive protein (Takemoto et al., 2003), which was also found to be upregulated in chitosan-treated potato (PGSC0003DMG400014347, 1.2-fold). Expression of photoassimilate-responsive proteins was found systemically in potato virus X infected tobacco plants, but also in response to high levels of glucose and sucrose transport through leaves of healthy plants (Herbers et al., 1995).

Altogether, quite few DEG were observed to have a function directly allocated to plant defense. However, as mentioned before and being further discussed below, plant defense in response to chitosan treatment is rather triggered indirectly through a general increase in metabolic activity, mainly through primary metabolism and especially through electron driven processes.

4.2.2.6. Electron-driven disease management

As described before, chitosan treatment led to significant upregulation of gene expression related to electron carrying proteins in the chloroplast and the mitochondrium. Apart from the first conclusions about a general ATP synthesis trigger, this chapter should introduce an alternative perspective about what chitosan could induce in the potato leaves. Although both the respiratory chain and the light reaction ultimately lead to ATP production, it is the electron transfer itself that can play a crucial role in plant immune responses as electrons have the ability to form ROS. ROS are unavoidable byproducts of photosynthesis, respiration and other metabolic processes and function as both signaling molecules and antimicrobial agents (Choudhury et al., 2017). One way to produce ROS is via electron transfer to atmospheric oxygen (O_2), resulting in gradual reduction to superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). As especially the mitochondrium and the chloroplast display the main driving forces for metabolism of plant cells via electron transfer, both organelles also have the highest potential to provide ROS, not only as byproducts, but as deliberately accumulated and mediated signal molecules (Foyer & Noctor, 2003). Another cellular component capable of providing ROS is the apoplast. Apoplastic ROS are mainly produced by membrane-bound NADPH oxidases (respiratory burst oxidase homologs, Rboh) by electron transfer from NADPH to atmospheric oxygen, resulting in superoxide. It is mainly produced in response to pathogen attacks but also plays a role in developmental and metabolic functions (Bolwell & Wojtaszek, 1997). In the chloroplast, the primary ROS product is superoxide and, as follow product, hydrogen peroxide, produced via Mehler reaction at PSI (Das & Roychoudhury, 2014).

Chloroplast ROS is usually formed during reduced carbon fixation which mainly occurs in stress situations (Takahashi & Murata, 2008). It was also reported that photosynthesis-derived ROS production is activated in chloroplasts in response to PAMP perception (Serrano et al., 2016). However, this finding was accompanied by a downregulation of photosynthetic gene expression, while the opposite was observed in this study. Thus, it is more likely that ROS production in potato chloroplasts in response to chitosan treatment is more due to a general metabolic shift, which leads to decreased carbon fixation as described above. It was shown that chloroplasts not only participate in ROS production in the event of infection, but even mediate and amplify ROS signals deriving from the apoplast (Joo, 2005). Plant mitochondria on the other hand regulate mitochondrial ROS (mROS) mainly via complex I and complex III (Q. Chen et al., 2003). The concentrations of mROS are usually kept low and are detoxified by several antioxidant systems, however, stress conditions can lead to repressed mROS control and finally in their release into the cytoplasm which might also participate in the regulation of overall cellular functions (Navrot et al., 2007). ROS production at complex I can be controlled more easily as this complex releases oxidants in the proximity to antioxidant defense systems. In contrast, complex III released ROS are directed away from these defense systems (Q. Chen et al., 2003). As no upregulation of complex III genes could be observed, it can be assumed that mROS production is generally triggered, but kept under control as good as possible. In total, the formation of ROS in different cellular compartments in response to different conditions, accompanied by the export of ROS into the cytosol, eventually leads to a crosstalk of ROS in plant cells. Shapiguzov et al. 2012 described this “ROS-talk” and how different ROS influence the production of further ROS. The authors eventually conclude that ROS signaling connects a broad range of cellular processes (Shapiguzov et al., 2012). For instance, ROS signaling was reported to be involved in the communication between chloroplast and nucleus in response to high light conditions (Galvez-Valdivieso & Mullineaux, 2010). With electrons being the driving force for ROS formation, the results of this study clearly demonstrate the ability of chitosan to activate (or at least contribute to) the crosstalk between different plant cell organelles via ROS. This alteration of the redox state subsequently triggers an abundance of different processes including further gene expression, primary and secondary metabolism and direct protection against diseases (Frederickson Matika & Loake, 2014). Furthermore, the crosstalk between ROS, especially from chloroplast and mitochondrial electron chains, eventually regulates cellular redox homeostasis which ultimately provides information to the plant on current energy status for growth and general development (Foyer & Noctor, 2009).

4.2.3. Chitosan-triggered plant resistance – more than just a simple counterattack

It could be shown here that chitosan treatment of potato leaves resulted in (at least) two different responses - the fast and immediate oxidative burst, directly detectable after treatment and the activation of gene expression, which is nowadays believed to be also activated in minutes, especially for so-called immediate-early genes (Bahrami & Drabløs, 2016). As described previously, oxidative bursts in response to chitosan might either be a result of chitosan perception through receptors or a response to cell wall and cell membrane interactions with chitosan (or both). It can be suggested that the activation of gene expression is indirectly triggered by chitosan, possibly also to some extend mediated by the oxidative burst reaction. In fact, it was shown that ROS are contributing to systemic signaling in *Arabidopsis* plants (Miller et al., 2009) and that ROS accumulation and interaction with other messenger molecules eventually result in SAR (Mittler & Blumwald, 2015). Hence, an oxidative burst is far more than a simple response to a threat. It is rather a fast, effective and universal response to any unfamiliar action, leading to the production and release of ROS which in turn can not only combat these action if necessary, but furthermore mediate and activate a range of metabolic reactions and initiate crosstalk between different organelles and cells (Mittler et al., 2011; Shapiguzov et al., 2012). Additional evidence for a ROS-mediated crosstalk of cell organelles could be delivered, as genes coding for mitochondrial respiratory complexes were upregulated in response to chitosan, further contributing to an overall alarmed or generally more productive state of the plant cell (Møller, 2001). A crosstalk between PTI and photosynthesis was already described through expression of defense genes and chlorophyll fluorescence measurements, indicating that PTI decreases non-photochemical quenching and thus, activating photosynthesis (Göhre et al., 2012). Here, further evidence was provided, as chitosan - presumably treated as PAMP - triggered gene expression of photosynthesis-related genes. *Vice versa*, oxidative bursts are directly dependent on sugars, which provide reducing power via NADPH through glycolysis (Couée et al., 2006). Thus, overall, chitosan treatment seems to lead to a generally increased vigor, which is characterized by both increased resistance and increased primary metabolism. Fitting to this, rice plants were observed to show higher biomass and increased photosynthesis rates after being treated with chitosan (Phothi & Theerakarunwong, 2017). As the study measured the direct photosynthesis rate, it provides further evidence about the activation of photosynthesis not only genetically, but also on a functional level as already mentioned above. The here achieved results and the results of other studies investigating photosynthesis activation and yield increase (Akter Mukta et al., 2017; Landi et al., 2017) reveal that plant responses induced by chitosan treatment seem to be independent of the plant species.

In total, the mutual dependence of primary metabolism (photosynthesis) and plant defense (oxidative burst) clearly indicates the importance of a certain cellular balance in regards to nutrient distribution and redox state. Any imbalance caused by biotic or abiotic stresses hence inevitably results in a broad range of responses in order to fight the stress (Gaur & Sharma, 2014).

4.3. Biologically active chitosan in plant protection – the bottom line

As discussed above, application of chitosan can both enhance resistance of plants against biotic and abiotic stress, eventually resulting in better growth, faster development and higher yields and act as an antimicrobial agent against a multitude of pathogens, ranging from small unicellular bacteria to eukaryotic fungi, oomycetes and insects. Hence, chitosan has a dual effect, both strengthening the plant and attacking pathogens directly and activating resistance in plants, which further fights intruding organisms. It could be shown that a low F_A favors antimicrobial activity while an intermediate F_A is best to elicit plant responses, most likely linked to the different modes of perception on plants and microbes. Furthermore, large chitosan oligomers (DP > 10) and small chitosan polymers (DP 100 - 1000) were observed having the best antimicrobial and eliciting activities. As it was shown that combinations of different chitosans enhance both activities in synergistic fashion, combining an antimicrobially active with an eliciting active chitosan can not only overcome the problem of different chitosans being required for either plants or pathogens, but also further enhance the overall biological activity. Based on the achieved results, it can be stated that the most suitable chitosan combination for plant protection consists of intermediate sized oligomers and a polymer. While the oligomers directly act on the cell wall of pathogens and plants and can be perceived by plant receptors, chitosanolytic enzymes progressively degrade the polymer. This results in a long-term effect of the combinations, since oligomers are constantly nascenting from the polymer. This long-term effect might be critical for disease prevention, causing pathogenic cell death under ongoing chitosan interactions, accompanied by plant defense responses. Additionally, since chitosan is likely to target different cellular components of pathogens, mostly dependent on their negative charge, its activity is spread over different attack points, not only facilitating synergistic activity, but also delaying the development of resistance. Chitosan resistance is furthermore slowed down by the fact that chitosan is a natural source, *i.a.* occurring in the fungal cell wall. Considering chitosanolytic enzymes taking over important tasks related to cell wall integrity, but also contributing to the biological activity of chitosan, complete chitosan resistance even seems more improbable.

Chitosan might very well be of even greater interest in the future, since knowledge about its effectivity in plant protection and its mode of action increases rapidly. However, as with everything, it is not all roses. Could the actual chitosan production amounts cover increasing demands? From seafood waste to clean and well-defined chitosan, chitin must go through a series of production steps (Yadav et al., 2019). First, chitin has to be isolated from the natural source. Chemical or enzymatical extraction as the second step includes deproteination, demineralization and decoloration. Eventually, chitin is transformed into chitosan, again either chemically or enzymatically before being analyzed and characterized. According to a life cycle assessment of chitosan production in India and Europe from 2018 (Muñoz et al., 2018), Indian chitosan production mainly serves agricultural purposes while European chitosan production focuses completely on a medical use. Hence, it is easily understandable that chitosan production in general not only differs in amounts, but also in desired purity. While large amounts of chitosan might be needed to be sufficiently applicable on crop fields, medical grade chitosan requires less quantity, but high purity. To keep chitosan quality and increase chitosan quantity at the same time in case of rising demands for both sectors is likely to become difficult, especially concerning both overfishing and sea pollution. Furthermore, chitosan production diverts shrimp shell waste from the animal feed market which also claims parts of the seafood waste (Muñoz et al., 2018), a circumstance that was predicted already for a long time (Roberts, 2008). Even if only addressing a single chitosan application sector like agriculture as it was conducted in this study, it remains highly questionable whether the whole planet could be supplied with chitosan.

This work could expand the knowledge about the biological activity of chitosan and provided new insights into how chitosan might act on plants and against their pathogens. It can be stated that the activity of chitosan is not at all limited to a single mode of action, but rather displays several modes of action, depending on its physico-chemical properties and the organisms treated with. A visual summary of the results of this work can be found in Figure 37. As a non-toxic, renewable and biodegradable resource and being eventually approved by the EU as basic substance compound for plant protection products, the road is finally paved for chitosan being used as fertilizer, plant strengthener, pesticide and fungicide - if not solely, at least as supplement to established products to contribute to an overall more efficient, environmentally friendly and more sustainable crop protection.

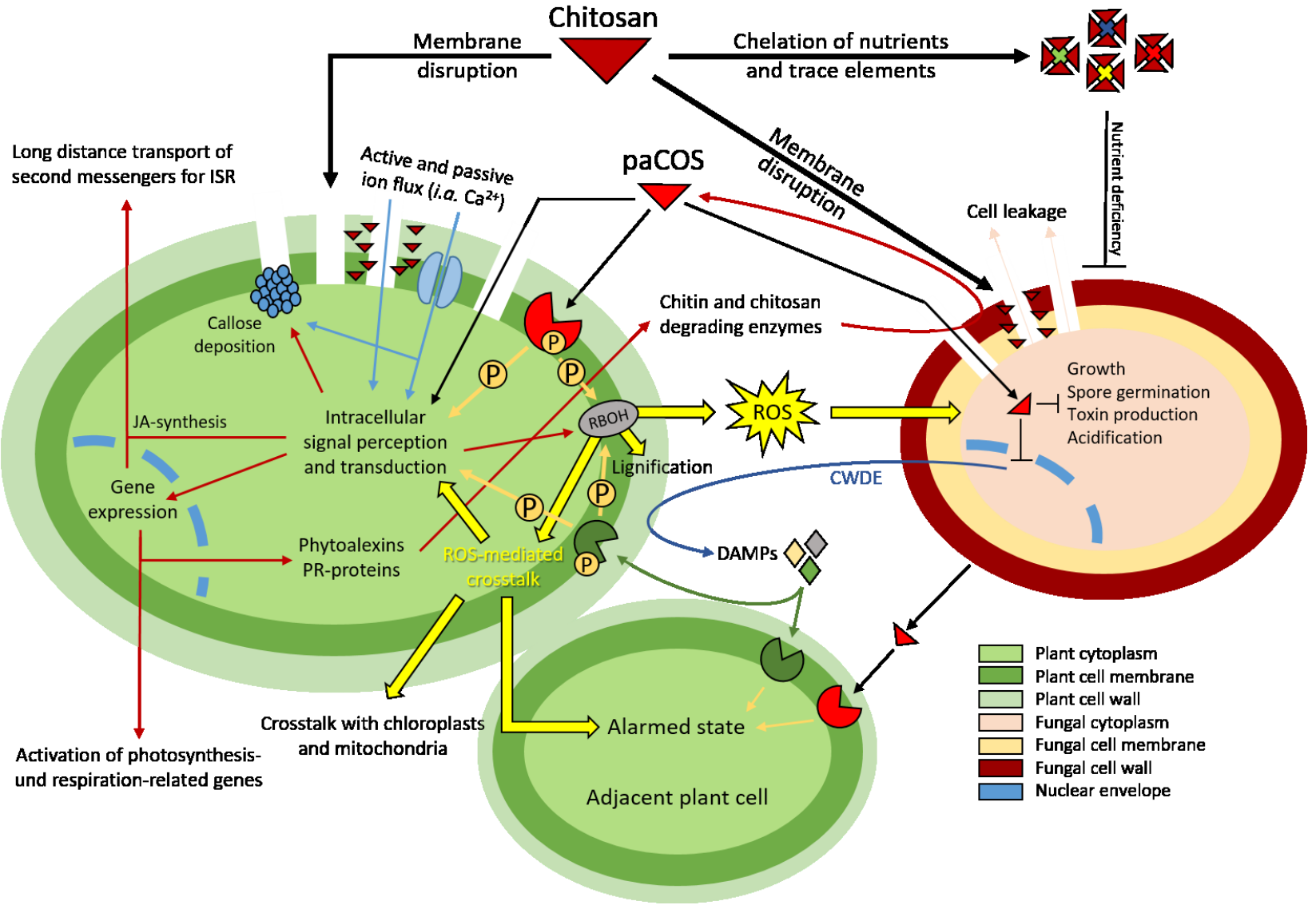

Figure 37: Final model of the modes of action of chitosan on plants and fungi. Chitosan polymers are believed to interact with negatively charged cell wall and cell membrane components, causing disruption, leakage and first intracellular stress responses. Additionally, chitosan polymers might chelate essential fungal nutrients and thus prevent their access. Via enzymatical degradation, chitosan is hydrolyzed to paCOS, which might enter the cells. Furthermore, paCOS are perceived by plant receptors, which contribute to intracellular signaling. However, the fungal cell wall is a second paCOS source as plant cells constitutively secret chitinases to perceive intruding fungi. Plant responses include ROS production via oxidative burst, callose formation at damage sites, JA-synthesis and activation of gene expression. ROS are not only fighting the pathogen, but also utilized as second messenger for intra- and intercellular crosstalk. Certain responses are likely to induce according reactions in adjacent plant cells and far away plant cells, further contributing to resistance. In fungal cells paCOS might inhibit mycotoxin production and gene expression, next to an overall growth and developmental inhibitory effect.

Chapter V

Summary and outlook

5. Summary and outlook

The aim of this work was to shed more light on the potential role of chitosan towards its action on plants and pathogens and thus contribute to sustainable plant protection. It could be shown that chitosan can act in several different ways, depending on its properties as well as dependent on the treated plant or pathogen species. The influence of the characteristics of a chitosan towards its biological activity was studied sophistically, expanding the knowledge about the importance on a well-chosen Mw (*i.e.* F_A and DP) for optimal efficiency. Based on the achieved results, a further influence of extrinsic factors, *e.g.* secretion of chitin and chitosan degrading enzymes, can be suspected to additionally contribute to the bioactivity of chitosan.

It is still a long way to go to completely integrate chitosan as a globally recognized and accepted plant protection compound, but time has already begun to find alternatives to current plant protection strategies in order to become future-oriented and sustainable. After determination of the optimal Mw for plant protection, subsequent work should focus on the pattern of acetylation (P_A), which was left aside in this work. Since the first description of block wise and random orientation of acetyl groups along the chitosan chain (Kurita et al., 1977) and more recent statements about P_A determination as a crucial step for structure-activity-analyses (Kumirska et al., 2009; Weinhold et al., 2009), studies on production and activity of P_A-defined chitosans increased throughout the last decade. For example, enzymatic production of paCOS with fully defined P_A via chitin deacetylases has been described (Hamer et al., 2015; Naqvi et al., 2016), eventually allowing enzymatic production of all possible paCOS tetramer patterns (Hembach et al., 2017). These tetramers were found to be priming-active on rice cells in a recent study, mostly in case of a GlcNAc unit at the non-reducing end and a GlcN unit at the reducing end, stating that a yet unknown paCOS receptor might be responsible for priming induction by small chitosan oligomers that would not result in eliciting oxidative bursts (Basa et al., 2019). Based on this knowledge, it appears that using mixtures of random P_A chitosans for bioactivity screening inevitably lead to weak reproducibility, as the activity is likely to differ from pattern to pattern. As a random P_A is present in chemically produced chitosans by default, future studies might focus on defined acetylation patterns and even more, chitosan production might require either shifting to enzymatic processing or careful separation and enrichment of specific desired molecules to secure reliable activity of the product.

Another not yet covered aspect in this thesis is nanotechnology. Nanoparticles are used for plant disease management for some time already, either alone as protectants or as nanocarriers for

pesticides or RNAi molecules (Worrall et al., 2018). Chitosan nanoparticles were successfully tested against all types of plant pathogens including bacteria, fungi and viruses (Kashyap et al., 2015), either alone or carrying *e.g.* copper as fungicide (Brunel et al., 2013; R. C. Choudhary et al., 2017). Chitosan nanoparticles are said to have enhanced features compared to their molecule counterparts through their potential to controlled-release active compounds into the plant (Cota-Arriola et al., 2013). Additionally, it is believed that chitosan easily absorbs to the epidermis of leaves, hence facilitating the uptake of active compounds into plant cells (Malerba & Cerana, 2016). The huge drawback of nanomaterials is the lack of long-term trials and field trials of nanoparticle-based pesticides and the associated unpredictable risk to the environment that emanate from their application (Worrall et al., 2018). The slow release of active compounds from nanoparticles might contribute to a long lasting plant protection, but also might lead to a sustained damage of the plant microbiome in soils due to a higher persistence of nanoparticles in comparison to sole molecules (Kah et al., 2013). Although nanopesticides are not generally prohibited in the European Union, but treated as regular plant protection products according to Regulation (EC) No. 1107/2009 (http://data.europa.eu/eli/reg/2009/1107/oj, assessed on 29.07.2019) and despite of ongoing risk assessments (G. W. Walker et al., 2018; Villaverde et al., 2018), acceptance for nanoparticles remains low without clear evidence about their environmental fate.

In this work, not only the direct responses of chitosan-treated plants and pathogens could be further enlightened, but also the knowledge about gene expression changes in response to chitosan was expanded. With next-generation sequencing techniques including whole transcriptome sequencing becoming more and more feasible due to dropping prices, upcoming chitosan research should pursue studies on genomics to get further information about the action of chitosan on gene expression. Expanding the here conducted studies to other plants and pathogens could reveal different genetic responses of different species to chitosan treatment. On the other hand, transcriptomic comparisons of gene expression responses to different chitosans (*e.g.* differential gene expression induced by either polymers or oligomers) might contribute to a better understanding of the different modes of actions a chitosan molecule can display depending on its physico-chemical properties. Furthermore, future approaches might investigate whether the transcriptomic changes are also reflected on the functional level, *e.g.* through protein detection or direct photosynthesis measurements.

True to the beauty of science, this work may have opened more doors than closed, but still could make a contribution to enlighten the potential of chitosan for sustainable plant protection.

Chapter VI

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6. References

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