Towards *Fusarium* resistant oat

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30 hec

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FOREWORD

The experimental part of this thesis was conducted during 12.10.2009 – 28.5.2010 at the Department of Plant and Environmental Sciences of Gothenburg University.

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Finally I would also like to thank all my friends and my mom for supporting me all the time.
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<tr>
<td>ALA</td>
<td>α-Linolenic acid</td>
</tr>
<tr>
<td>ATA</td>
<td>Alimentary toxic aleukia</td>
</tr>
<tr>
<td>DAS</td>
<td>Diacetoxyscirpenol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
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<td>Deoxynivalenol</td>
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<td>Fusarium head blight</td>
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<td>Reverse transcription polymerase chain reaction</td>
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1. Introduction

Fungal infections of cereal crops are gaining more focus then ever due to the rising levels of mycotoxins in grains. Infected grains have less economic value and pose a health risk when ingested by people or animals. Ongoing research has concentrated on the connection between different growing conditions, climate, variety and mycotoxin levels. However little research has been on the genetical mechanisms behind fungal resistance.

Several studies implicate that there is a chemical cross talk between the fungi and the host plant mediated by, among others, oxylipins. Oxylipins are formed by the oxidation of polyunsaturated fatty acids catalyzed by lipoxygenases (LOX). Plant oxylipins function as signals in defense responses and development whereas the fungal oxylipins are needed for normal growth and for production of secondary metabolites, including mycotoxins.

Previous studies showed that 9-LOX catalyzed oxylipins induce conidiation and mycotoxin production in fungi and also that 9-LOX gene disrupted transgenic maize showed increased resistance to Fusarium and reduced levels of mycotoxin contamination. Inspired by these results the aim of this thesis was to test if similar results could be achieved in oat.

Oat was chosen as a model organism due it’s high nutritional value and health enhancing properties and due to the fact that high levels of Fusarium mycotoxins can frequently be found in oats.
2. *Avena Sativa* - Oat

*Avena Sativa* commonly known as oat, is a hexaploid monocot cereal grain crop cultivated for human and animal consumption. Oats have 3 diploid genomes (AA, CC, DD) of which A and C are from two distinct species and the D genome is similar with the A genome (Rines *et al.*, 2006). The size of the hexaploid genome of oat is $1.6 \times 10^7$ kb, more than five times larger than the human genome (Genamics™, 2006).

Oats are considered as a high-quality food and feed with the highest protein level (12 – 20%, dehulled) and a superior amino acid profile with the highest levels of globular proteins amongst any cereal (Rines *et al.*, 2006).

Oats also contain relatively high levels of β-glucan fibers which are beneficial for digestion and have proven cholesterol lowering properties (Brown *et al.*, 1999; Davidson *et al.*, 1991; Ripsin *et al.*, 1992). Because of this, oat is the only cereal that has a health claim both in the US and within the EU.

In addition oat seeds contain compounds with antioxidative properties such as tocopherols, inositol phosphates and avenanthramides that are unique to oat (Shewry *et al.*, 2008).

Moreover oats are naturally gluten free, which is a requirement for people with gluten intolerance and celiac disease (Celiac Disease Fondation, 2010). Among others a recent study made in Norway (Guttormsen *et al.*, 2008) points out that most adults with celiac disease can tolerate oats.
Unfortunately, oat cultivation, as well as wheat and barley, are presently threatened by fungal infections, especially by *Fusarium* species, which produce harmful toxins. If nothing is done to limit these infections with mycotoxin contamination of the seeds as a consequence, less oats will be consumed leading to reduced oat consumption, which in turn would negatively affect the health status of the population, reducing life quality and increasing the cost for the health system.

### 3. Fungal infections

Fungal infections are a serious threat to global food production. These infections also contribute to sustainability problems, since they cause economical, environmental and even social problems world-wide. The economical aspect is easy to calculate simply by estimating the overall yield loss caused by fungi. In addition, fungal infections reduce the weight of the kernels and even the quality of the grains. The environmental aspects are linked to yield loss because to be able to produce the same amount of uninfected grains a bigger area is needed and in addition fungicides often have to be used. Social effects as a consequence of fungal infections are probably the most difficult part to estimate, even tough they can be devastating due to the toxins produced. Several fungal toxins are poisonous and ingestion can lead to chronic toxicoses that even can be fatal.

#### 3.1 *Fusarium* Head Blight

One of the most devastating fungal diseases of small grains like oat, wheat and barley is *Fusarium* Head Blight (FHB), also called scab. FHB is caused by several species of fungi called *Fusarium*. The most economically relevant species are *F.*
graminearum and F. culmorum. (Foroud et al., 2009; Parikka et al., 2008; Parry et al., 1995; De Wolf, 2003; Hollingworth et al., 2004).

The fungus infects susceptible cereal crops prior to and during kernel development. When the cereals are infected at the early flowering stage, the crops fail to produce kernels. Later infection results in smaller and discolored kernels. Most Fusarium thrives in relatively humid and wet conditions with an optimum temperature of 25°C and FHB occurs in these conditions during anthesis and grain development stages (Parry et al., 1995; Abramson et al., 1987; McMullen et al., 1997 and 2008; Paulitz, 1999; Hollingworth et al., 2004; De Wolf, 2003).

3.2 FHB symptoms

Initial symptoms of a Fusarium infection are discoloration of the kernels and spikelets. The fungi may infect only parts of the cereal and kernels (Figure 1.). Diseased kernels are smaller than uninfected ones and the color is usually light tan to brown or even pinkish. They appear bleached and they are shriveled compared to healthy green kernels and they are often referred as tombstones. These tombstones are lighter in weight and smaller in size (Parry et al., 1995; Abramson et al., 1987; McMullen et al., 1997 and 2008; Paulitz, 1999; Hollingworth et al., 2004; De Wolf, 2003).

A delayed kernel infection can result in plumper kernels that appear to be healthy, but they are not. If the weather conditions are favorable for the fungi, it will produce orange to pink spore masses on the spikelets and glumes (Parry et al., 1995; Abramson et al., 1987; McMullen et al., 1997 and 2008; Paulitz, 1999; Hollingworth et al., 2004; De Wolf, 2003).
3.3 Disease cycle

The fungi persist and grow on infected crop residues of small grains and corn. During favorable weather conditions in the spring, perithecia develop to produce ascospores. The mature ascospores are discharged and dispersed by wind, rain and insects onto the heads and other parts of the cereal crops. If the weather conditions and the growth phase of the plant are favorable for the fungi and if the spores are deposited on or inside the flowers and spike tissues, mycelium growth will be initiated and the susceptible plant infected (Subramaniam et al., 2009).

After a brief biotrophic phase growth on the florets the fungus initiates the final phase of the infection of the crop and develops into *Fusarium* head blight. After this the fungus starts to produce new spores for further colonization opportunities (Subramaniam et al., 2009; Parry et al., 1995; Abramson et al., 1987; McMullen, et al., 2009).
Due to less tillage of fields in modern agricultural praxis, crop residues are not broken down during the rest season of the field. Unfortunately, this serves as an energy source for fungi and increase its survival and possibility to infect crops during the next growth season (Parry et al., 1995; Abramson et al., 1987; McMullen et al., 1997 and 2008; Paulitz, 1999; Hollingworth et al., 2004; De Wolf, 2003).

3.4 Management

Developing functional FHB-management strategies is essential for reducing the occurrence of the disease and for breaking the disease cycle. Based on the disease cycle, there are three different components necessary for a possible FHB outbreak. These components are the inoculum source (type of fungus, number of spores, etc), developmental stage and health of the susceptible host and weather conditions favorable for the fungi.

The inoculum source is usually present in the soil as ascospores or it can travel from surrounding fields with wind and insects. There are several strategies that the farmer can use to reduce the amount of inoculums harbored in the soil. Several studies have shown that crop rotation, tillage and chemical or biological fungicides are the most effective in this task (Dill-Macky and Jones, 2000; Gilbert and Fernando, 2004; Horsley et al., 2006; Jones, 2000; Khan et al., 2006; Miller et al., 1998; Paul et al., 2007). Tillage reduces the crop debris needed for inoculum survival over the winters.
For long term management of inoculums the best option is to grow FHB resistant cultivars, which in principle works the same way as crop rotation, i.e. if a non host crop is grown in the field before the susceptible ones infection is reduced (Foroud and Eudes, 2009). If there is no energy source for the survival of the fungi there will not be any ascospores either that can attack if a sensible crop should follow.

This leads to the second target of disease components. As mentioned before crop rotation and resistant cultivars could partly overcome the *Fusarium* problem. However the availability of registered resistant cultivars is limited to a few with only moderate resistance to FHB. So far breeding toward FHB resistance has had very little success due to a large environmental factor that complicates the selection process. Available varieties with some resistance on the other hand tend to have quite poor agronomical properties, which makes them not favored among farmers (Foroud and Eudes, 2009; Bjørnstad and Skinnes, 2008).

The third component necessary for FHB is favorable weather conditions, which at least theoretically makes it possible to develop forecasting models for precautionary management of FHB (Foroud and Eudes, 2009). In addition, if farmers would be willing to grow early ripening oat and therefore harvest earlier, they would more likely avoid late autumn rainy conditions.

One way to limit damage caused by FHB would be to apply all the above management strategies. However the most effective strategy would be to combine highly resistant varieties with good agronomical properties. This would make the variety more favorable among farmers since it would reduce the source of inoculum and make the oat less susceptible to weather conditions. In other words everyone wins, since the farmers get a better yield without costly and possibly harmful fungicides and the consumers get a chemical and mycotoxin free high quality oat.
3.5 FHB in oat

Generally plants have two prevalent types of resistance against pathogens such as *Fusarium*. Type I is a generalized resistance against infections, where the pathogen is unable to infect the plant and in type II resistance the pathogen is able to infect the plant but it is unable to spread beyond the infection site (Subramaniam *et al.*, 2009, Foroud and Eudes, 2009).

Several comparative inoculation trials made in Norway and Canada show that oats are relatively less infected by *Fusarium* than wheat or barley (Bjørnstad and Skinnes, 2008). A possible explanation for this phenomenon is the oat's long pedicels separating individual spikelets in the panicle. However oats tend to accumulate higher levels of mycotoxins in Finland and Norway (Parikka *et al.*, 2008), which might be caused by the more marginal growth conditions in the Nordic. An addition to the climate conditions oats are usually harvested later than wheat and barley in the autumn and therefore are exposed for longer growth periods in humid and rainy conditions, the optimal conditions for *Fusarium* growth.

4. Mycotoxins

Mycotoxins are biologically active secondary metabolites produced by fungi. These secondary metabolites have different properties and effects against other organisms. Some have antibacterial effects against other microorganisms, some are active against plants (phytotoxins) and others are harmful for animals (mycotoxins) (Smith *et al.*, 1985). Despite the large variety of mycotoxins produced, it is still not clear why fungi produce these secondary metabolites.
*Fusarium* species also produce several different mycotoxins of which the trichothecenes group has the strongest association with chronic and fatal toxicoses of humans and animals. Alimentary toxic aleukia (ATA) is one of the most known conditions caused by *Fusarium* mycotoxins. Other *Fusarium* mycotoxin groups are fumonisins, zearalenones, fusarins, beauvercina and enniatins, butelonide and equisetin (Desjardins *et al.*, 2007).

There are two different ways to be exposed to mycotoxin. The most likely situation is via primary mycotoxicosis where the ingested food is contaminated with mycotoxins. In most of the cases these sources are cereal-based products. The other possibility is called secondary mycotoxicosis and when the toxins are spread through food chain. This means that we can encounter mycotoxins even via meat and dairy products if the animals were fed with contaminated feed (Smith *et al.*, 1985).

### 4.1 Trichothecenes

Trichothecenes are a large family of mycotoxins including the most important toxins related with cereal grains such as diacetoxyscirpenol (DAS), T2- toxin (T-2), nivalenol (NIV) and deoxynivalenol (DON) (Desjardins *et al.*, 2007).

*Fusarium* trichothecenes are tricyclic sesquiterpenoid compounds and they act as inhibitors for ribosomal protein synthesis.

There are four different types of trichothecenes that have been identified from trichothecene-producing fungi. These types are called A, B, C and D. Types C and D trichothecenes are not associated with FHB. Type A and B are the major two groups of mycotoxins responsible for FHB and related health concerns (Foroud and Eudes, 2009).
Type A includes T-2 and HT-2 toxins (HT-2), which are mainly produced by *F. sporotrichioides* and *F. poae*. Type B comprise the toxins DON and NIV, which are the preferred toxins of *F. graminearum* and *F. culmorum*. Mycotoxins of type A are shown to have a much higher toxicity in mammals than type B, which has more phytotoxic effects. For example T-2 has been reported to be around ten times more toxic than DON, which is the most prevalent toxin associated with FHB (Foroud and Eudes, 2009).

Exposure to trichothecenes can lead to growth retardation and reproductive dysfunction in mammals and in plants trichothecenes inhibit seedling growth and regeneration. Additional impacts of the toxic compounds include disruption of nucleic acid synthesis, mitochondrial function, membrane integrity and cell division. Trichothecenes have also been shown to induce apoptosis of animal cells and the toxins may also induce programmed cell death in plants (Foroud and Eudes, 2009).

### 4.2 Production of mycotoxins

The production of mycotoxins in grains is highly dependent on the weather conditions, the most important being temperature and humidity. Humidity affects the water activity of the grain and also the amount of free water available for the fungi. The optimal relative humidity for fungal growth is around 25%. However environmental conditions are not the only factors affecting the production of the mycotoxins but several other factors such as the host plant genome and a variety of molecules can serve as inhibitors or enhancers for mycotoxin production (Smith *et al.*, 1985).
Interestingly fungal biomass growth does not correlate with mycotoxin content in the kernels. The kernels might appear healthy and still have a high mycotoxin level. The opposite also occurs, i.e. the kernel is fully diseased but very low or no mycotoxins are produced in it. This makes the prevention and elimination of mycotoxin contamination more complicated (Parry et al., 1995; Abramson et al., 1987; McMullen et al., 1997 and 2008; Paulitz, 1999; Hollingworth et al., 2004; De Wolf, 2003).

There still no consensus why fungi produce mycotoxins. Some studies claim that it increases their virulence and others say that they are related to sporulation and morphogenetic changes. However it is proven that mycotoxins are not needed for biomass growth, because usually the production of mycotoxins begin after the log growth phase (Smith et al., 1985).

4.3 Cross-talk between fungi and host

Several studies implicate that there is a possible cross talk between the fungi and the host plant. It has been showed by Gao et al., (2007, 2008, 2009) that host plant oxylipins interfere with fungal development and secondary metabolism. In their study they produced a transgenic maize (Zea mays) in which the 9-LOX gene had been disrupted. The created transgenic maize had reduced levels of several 9-LOX catalyzed hydroperoxides. The results of their first study (2007) clearly show that conidiation and production of the mycotoxin fumonicin B1 by Fusarium verticillioides were drastically reduced in the infected kernel compared to wild type maize. However the inactivation of the gene did not effect fungal vegetative growth. The study conclude that maize 9-LOX derived oxilipins are required for reproductive development and secondary metabolism but not for vegetative growth in F. verticillioides.
5. Lipid peroxidation

Lipid peroxidation is common to all biological systems. In plants lipid peroxidation occurs in both developmentally and environmentally regulated processes and as responses to abiotic and biotic stresses. Formation of oxidized polyunsaturated fatty acids (PUFA's), also called as oxylipins, is a main reaction in lipid peroxidation. Oxylipins can form both through autooxidation and by enzymatic activities. The catalytic enzymes that initiate the biosynthesis of the oxylipins are called lipoxygenases (LOXs) (Feussner and Wasternack, 2002). Non-enzymatic peroxidation occurs during oxidative stress by the formation of reactive oxygen species (ROS) (Müller, 2004).

5.1 Oxylipins

Plant oxylipins are primarily formed by the oxidation of linoleic acid (LA, 18:2) and α-linolenic acid (ALA, 18:3), the most abundant PUFAs. The biosynthesis of the oxylipins starts by the formation of hydroperoxy fatty acids catalyzed by LOX enzymes (Feussner and Wasternack, 2002). The formed hydroperoxy fatty acids are further metabolized by six different pathways causing a large structural variety of the oxylipins.

This great diversity of the oxylipins indicates a greater biological role in plants. These molecules serve among others as signaling molecules in development and in defense against microbes, insects and fungi (Blée, 2002; Howe and Jander, 2008; Göbel and Feussner, 2009).
In fungi the molecular roles of the oxylipins are different from plants. Fungi oxylipins are referred to as psi-factors (for precocious sexual inducer). Psi-factors are needed for normal growth, regulation of sporogenesis and for biosynthesis of secondary metabolites, including mycotoxins (Brodhagen and Keller, 2006; Brodowsky and Oliw, 1993; Hamberg et al., 1994; Su et al., 1998).

5.2 Lipoxygenase

In plants LOX enzymes catalyze the oxidation of free polyunsaturated fatty acids, primarily linoleic (C18:2) and linolenic (C18:3) acids, either at position 9 or 13 of the carbon chain of the fatty acid. They are therefore referred to as 9-LOX and 13-LOX. The resulting products are either 9-hydroperoxy and 13-hydroperoxy PUFAs. The oxylipins derived from linolenic acid are called 9- or 13-HPODE and the oxylipins derived from linolenic acid (Figure 2) are called 9- or 13-HPOTE. These oxylipins of the two different LOXs enter separate metabolic pathways resulting in different fatty acid peroxides with different roles in the signaling events (Feussner and Wasternack, 2002, Gao et al., 2007, 2008, 2009).

**Figure 2. LOX reaction of linolenic acid.**
The colored number refer to the carbon atom number and the box points out the reaction position of the LOX enzymes. Source: Feussner and Wasternack, 2002.
EXPERIMENTAL PART

6. Materials and Methods

This Master's thesis project is part of a larger project aiming at the development of *Fusarium* resistant oat varieties. Previously, an expressed sequenced tag (EST) database was developed for oat (Bräutigam *et al*., 2005). In addition an oat TILLING (Targeting Induced Local Lesions IN Genomes) population was created by EMS mutagenesis of oat seeds (Chawade *et al*., 2010). This TILLING population is unique in the word and open ways to identify rare mutations by genotypic and phenotypic screening since the variation in the population is high.

Taking advantage of these tools, and by using '3 and '5 prime RACE starting from an oat EST sequence, two putative full-length *LOX* gene sequences were identified. By BLAST search analysis, the ESTs were identified as *LOX* genes. They were denoted Ascid009_5B1_M2_3B3 and Ascid067_5A4_M3-7_3A3 and later on referred to as AsLOX1 and AsLOX2 respectively. The sequences served as the starting point for this project. The oat genome is not yet sequenced due to its very large size.

The overall aim of the experimental work was to clone full-length LOX-coding sequences, determine their LOX-activity and screen the TILLING-population for mutants in *LOX* gene sequences.

For all the sequencing purposes Belinda was chosen as a model plant since it is an oat variety that is grown commercially in many places in Sweden.
As a basic lab procedure, samples were kept on ice during reaction preparations to avoid degradation of the samples by RNase, DNase and protease activity. For dilution purposes, triple distilled water was used.

DNA and RNA concentrations were measured in 1µl droplets with a NanoDrop 2000c Spectrophotometer from Thermo Scientific, using the appropriate software settings for the measurements. The same equipment and software were used to measure the optical density (OD$_{600}$) of cell cultures with a standard 1 cm cuvette.

6.1 DNA and RNA extraction

Total DNA and RNA were extracted from leaves from Belinda oat grown in climatised growth chambers. QIAGEN's Rneasy® Plant Mini Kit or Dneasy® Plant Mini Kit were used for the extractions. The extraction preparations were performed according to the appropriate kit's protocols. After extraction the extracts were stored in -20°C.

When necessary a DNAse treatment was performed on the extracted total RNA to avoid genomic DNA contamination. The treatment was performed with the Rnase-Free DNase Set designed for the Rneasy® Plant Mini Kit.

6.2 DNA electrophoresis

DNA samples were analyzed by gel DNA electrophoresis technique where 0.8-1% TBE agarose gel was prepared by standard procedures. To obtain the best separation and visualisation of the fragments, 5µl of DNA samples were mixed with 1µl of 6X Blue or Orange Loading Dye and 1µl of 10X Gel Star and loaded into the wells of the agarose gel. Also a DNA ladder mixed with Gel Star was
added to the gel to be able to specify the sizes of the analyzed DNA fragments.

The running conditions varied between 70V-130V based on the size of the fragments and the desired separation efficiency. All electrophoresis runs were made with BIORAD PowerPac 300 power supply.

Gel samples were visualized under UV-light with ChemiGenius Bio Imaging System (Syngene) with their GeneSnap software.

6.3 Designing of primer pairs

Primers for sequencing and cloning of the two LOX-genes were designed to cover the full open reading frames (ORF) based on the original sequences AsLOX1 and AsLOX2 with the software MacVector designed for Mac OS X or with an internet tool called Primer3. The designed primers were ordered from Eurofins MWG Operon (Germany).

PCR reaction conditions were optimized by gradually changing the annealing temperatures for the primers but keeping all other parameters constant. The amplifications were analyzed by gel electrophoresis. The used primers for the gene fragments and their optimal annealing temperatures are listed in Table 1.
Table 1. Primer pairs used for amplification of *AsLOX1* and *AsLOX2*-gene fragments.

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<tr>
<th>Oligo name</th>
<th>Sequence (5’ - 3’)</th>
<th>Length of fragment (bp)</th>
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<td>AsLOX2R5.2</td>
<td>TTAGATGGAGATGCTATTGGGGATT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The first forward and the last of the reverse primers were used to amplify the full-length genes. The new primer pair combination was tested for the optimal annealing temperature in the same way as the fragments. The used primers and temperatures are listed in Table 2.
Table 2. Primer pairs used for isolation of the full length genes, *AsLOX1* and *AsLOX2*.

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence (5' - 3')</th>
<th>Length of gene (bp)</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascid009_5B_F1</td>
<td>ATGTTCGGATTCGGATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascid009_5B_R4</td>
<td>CAGATGGAGATGCTGTTTG</td>
<td>2630 (+1)</td>
<td>57.9</td>
</tr>
<tr>
<td>AsLOX2F1.1</td>
<td>ATGTGTGGCGGCTGGATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AsLOX2R5.2</td>
<td>TTAGATGGAGATGCTATTGGGATT</td>
<td>2589</td>
<td>58.8</td>
</tr>
</tbody>
</table>

6.4 PCR

All PCR reactions were carried out in a thermal cycler made by BIORAD, model C1000™ Thermal Cycler. For each PCR reaction a PCR reaction kit iTaq™ DNA Polymerase from BIORAD was used. Reagents were thawed and kept on ice prior to adding them to nuclease-free, thin-walled (Eppendorf) PCR tubes. For multiple reactions a master mix was prepared for maximal equality between reactions and for minimizing reagent loss. The reaction components are listed in Table 3.

Table 3. PCR reaction components.

<table>
<thead>
<tr>
<th>Reaction set up per tube (25µl):</th>
<th>Volume:</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X iTaq Buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.75µl</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>19.125µl</td>
</tr>
<tr>
<td>iTaq DNA Polymerase</td>
<td>0.125µl</td>
</tr>
</tbody>
</table>
For the PCR reactions a typical thermal cycling protocol was used where the polymerase was activated during 1 cycle of 3 minutes at 95°C. PCR amplification was done in a loop of 40 cycles, of 30 seconds at 95°C (denature), 30 seconds at the optimal annealing temperature (anneal) and 1 minute/kb at 72°C (extend). After the loop the samples were kept at 12°C until the program was stopped.

6.5 RT-PCR

RT-PCR reactions were performed the same way as PCR reactions using the same equipment. For the reaction set up the kit SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase from Invitrogen™ was used. The reactions were prepared according to Table 4.

Table 4. RT-PCR reaction components.

<table>
<thead>
<tr>
<th>Reaction set up per tube (25µl):</th>
<th>Volume:</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Reaction Mix</td>
<td>12.5µl</td>
</tr>
<tr>
<td>RNA</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.5µl</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>9.5µl</td>
</tr>
<tr>
<td>Platinum® Taq Mix</td>
<td>1.0µl</td>
</tr>
</tbody>
</table>

For the RT-PCR reactions a typical thermal cycling protocol was used where the cDNA synthesis was initiated during 1 cycle of 30 minutes at 55°C. After that 1 cycle of denaturation for 2 min at 94°C. PCR amplification was done in a loop of 40 cycles, of 15 seconds at 94°C (denature), 30 seconds at the optimal annealing temperature (anneal) and 1 minute/kb at 68°C (extend). After the loop a final extension cycle was initiated for 5 min at 68°C and the samples were cooled down and kept at 12°C until the program was stopped.
6.6 DNA purification and isolation

To be able to clone and subclone the correct genes and DNA fragments the DNA samples had to be purified from other fragments and vector pieces. First the samples were run on electrophoresis gels and the correct bands were identified by their sizes. Purification of the DNA molecules were performed by cutting out a band of the expected size from the gel. The cutting was performed under low intensity UV-light to avoid the breakdown of the DNA molecules using a sterile scalpel to avoid contamination. The DNA was extracted from the agarose gel with QIAGEN's QIAquick® Gel Extraction Kit, according to the manufacturer’s protocol.

6.7 Digestion with restriction enzymes

Various restriction enzymes were used to verify the presence of the appropriate insert in the cloning vector and to excise fragments. Final expression vectors were also mapped with suitable restriction enzymes to determine the orientation of the insert in the vector, which is vital for protein expression.

Most of the digestions were performed according to Fermentas's Molecular Biology Catalog & Product Application Guide 2010-2011, Fast DNA digestion protocol. Cleavage products were analysed by gel electrophoresis.
6.8 Cloning for sequencing

For all sequencing purposes the TOPO TA Cloning® Kit for Sequencing and TOP10 *E.coli* cells were used. The kit is designed to enable the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector for sequencing.

The supplied TA Cloning® vectors are already linearized and are supplied with Single 3' deoxythymidine (T) overhangs. This makes the ligation of the PCR product into the TA Cloning® vector more efficient as long as the PCR products has the deoxyadenosine (A) at the 3' ends derived from the *Taq* polymerase nontemplate-dependent terminal transferase activity. The vector map with the insertion site for the DNA fragment is shown in Figure 3.

![TOPO cloning vector map](source: TOPO TA Cloning® Kit for Sequencing cloning manual) The map shows the construct of the vector with the insertion site of the PCR product, restriction sites, promotors and the antibiotic resistance genes.
Long time storage and gel extraction of the PCR products degrades the A overhangs, which lowers the transformation efficiency of the plasmid. To counteract this phenomena it is possible to activate the transferase activity by adding a small amount of Taq polymerase into the PCR product and heat it to 70°C for 10 minutes in the thermal cycler.

### 6.8.1 TOPO® Cloning Reaction

For the ligation of the vector 0.5µl of the fresh PCR or re-tailed product was mixed with 0.5µl of the cloning kit's Salt Solution and 0.5µl of the TOPO® vector and was diluted with 1.5µl of sterile water to obtain 3µl of total volume. The mixture was incubated for about 5 minutes at room temperature. After incubation the mixture was placed on ice until the cell transformation.

### 6.8.2 Transforming One Shot® Competent Cells

The transformation of the TOP10 cells were performed essentially according to the protocol with the exception of the incubation times, which were further optimized.

2µl of the plasmid (TOPO® Cloning Reaction) was added to each transformation of One Shot® TOP10 Chemically Competent E. coli. The cells were mixed gently by tapping the tubes and were incubated on ice for 5 minutes. The cells were heat-shocked for 30 seconds at 42°C and transferred immediately to ice. 250µl of room temperature SOC medium were added and the tubes further incubated for ca 30 minutes at 37°C in a water bath shaker.

50µl from each transformation mixture were spread on pre-warmed selective LB plates containing 50µg/µl of Ampicillin or Carbenicillin. Alternatively, tubes were centrifuged for 2 minutes at 8000 rpm with soft mode settings, cells resuspended
plates spread with 50µl of the concentrated cell cultures.

Plates containing the transformation mixes were sealed with parafilm and incubated at 37°C overnight. The next day colonies were counted and picked for further analysis. Plates were transferred to 4°C for storage.

6.8.3 Analysis of colonies

Individual colonies were picked with sterile toothpicks and resuspended in Falcon tubes with 3ml LB medium containing the same amount of antibiotics as used in the plates. Cells were incubated at 37°C overnight with shaking.

The next day the samples with growth (cloudy LB), were spinned for 6 minutes at 5100g at 4°C. The supernatants were removed by pouring and pipetting.

The plasmids were eluted with QIAprep® Spin Miniprep Kit made by QIAGEN according to the manufacturer's protocol.

To be able to verify the presence of the DNA fragment in the cloning vectors, 1µl of the eluted plasmids were digested with FastDigest® EcoRI restriction enzyme in 1µl of 10X FastDigest® buffer from Fermentas and diluted with 8µl of sterile water. Digestion samples were incubated at 37°C for 20 minutes and transferred to ice. Samples with the correct insert size were identified by gel electrophoresis. The concentration of the corresponding plasmids were measured and around 1500ng of the plasmids were transferred into new Eppendorf tubes and diluted with EB buffer (QIAGEN) to a final volume of 15µl. The diluted samples were sent for sequencing to Eurofins MWG Operon in Germany. The remaining plasmids were stored at -20°C.
6.9 Subcloning

For protein expression the gene of interests were cleaved out from the sequencing vectors with appropriate restriction enzymes and inserted the genes into the expression vector. The final construct is shown in Figure 4.

![Gene constructs of AsLOX1 and AsLOX2.](image)

**Figure 4. Gene constructs of AsLOX1 and AsLOX2.**
The two construct are made with a small piece of the TOPO sequencing vector digested with EcoRI and inserted the insert in the expression vector. AsLOX1 also have a SalI restriction site that is suitable for orientation check.

To ensure efficient expression and easy purification of the recombinant proteins the GST gene fusion system was chosen (Amersham Biosciences, 2002). In this system the included pGEX vector is designed to express the recombinant proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus in *E. coli* bacterial cells. This GST carrier protein can be cut off from the recombinant protein by enzymatic cleavage with PreScission Protease. To maintain the right encoding frame of the genes after digestion with EcoRI, the vector version pGEX-6P-1 (GE Healthcare, Life Sciences) was chosen. The map of the vector is shown in Figure 5.
The subcloning was initiated by digesting about 2.5µg of the chosen plasmid and separately 1µg of the expression vector with EcoRI restriction enzyme. After digestion, a small amount of the cleavage products and the undigested plasmids and vectors were run on gel to ensure full digestion. The rest of the digested plasmids were loaded on a gel with bigger wells to be able to load as much samples as possible. The genes of interest were cut out of the gel and were extracted with standard procedures. All samples were kept on ice to avoid degradation.

Meanwhile the digested vectors were dephosphorulated for 30 minutes at 37°C to avoid self ligation. The dephosphorulated vectors were chloroform extracted and precipitated to get rid of the phosphatase enzymes and to change the buffer of the samples.

Figure 5. Vector map of pGEX-6P-1
The map shows the construct of the vector with the restriction sites, promotors and the antibiotic resistance gene.
For inserting the cleaved genes into the vector 5 different ligation mixtures were prepared according to Table 5, where x represents either no insert at all, 4µl or 6µl of the extracted inserts.

Table 5. Ligation reaction set up.

<table>
<thead>
<tr>
<th>Ligation mixture per reaction (10µl)</th>
<th>Volume:</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X T4 DNA Ligase Buffer (Fermentas)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>T4 DNA Ligase (Fermentas)</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Vector</td>
<td>1.0µl</td>
</tr>
<tr>
<td>Insert</td>
<td>x µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>7µl - x µl</td>
</tr>
</tbody>
</table>

The ligation reactions were incubated at room temperature for 20 minutes and the ligase activity was inactivated by heating the samples to 70°C for 5 minutes.

Transformation of the E.coli cells were done as described above in the cloning protocol with 1µl of the ligation mixture.

To ensure the right orientation of the inserts in the plasmids, a set of colonies were picked for analysis with the standard procedures for plasmid elution. The concentration of the eluted plasmids were measured and 10µl – 15µl of plasmids were digested with SalI and the samples were analysed by gel electrophoresis. The orientation could be identified based on the SalI restriction site creating different size of digestion fragments as shown in Figure 4.

Two of the right oriented plasmids were chosen for protein expression.
6.9.1 Cloning for protein expression

Cloning for protein expression was done in two different strains of *E.coli*, BL21 and BL2-CodonPlus (BL21CP) competent cells. The CodonPlus strain has an extra plasmid to facilitate more effective protein expression of eukaryotic proteins due to different codon preferences in bacteria and plant cells. The extra plasmid contains genes that encode tRNAs that mainly hinder efficient expression of the protein. Therefore the BL21CP cells requires an extra antibiotic stress (25µg/µl of Chloramphenicol) to ensure the presence of the plasmid in the cell cultures.

Transformation of the BL21 strain was done as described above in the cloning protocol with 1µl of the plasmids with right orientation and one transformation with an empty expression vector. The transformation of the BL21CP requires prior to transformation the addition of 2µl of diluted β-mercaptoethanol with an incubation time of 10 minutes.

6.9.2 Protein expression with E.coli

To ensure the exponential growth phase of the cell cultures about 0.5ml of over night culture were inoculated into 50ml of fresh LB media with appropriate antibiotics according to the strains requirements in each sterile flasks. The cell cultures were shaken in 37°C until the optical density (OD₆₀₀) was between 0.4 and 0.6 with the optimum of 0.5. At this point a 1ml control sample was taken of the cultures and spun for 1 minute at maximum speed (14000g) with a table top centrifuge.

Meanwhile to induce the protein expression in the cultures, IPTG was added to a final concentration of 0.4mM. The cultures were incubated for 2 hours in 37°C in the shaker incubator.
The supernatant were removed from the control samples and the pellet was re-suspended in 75µl of NuPage buffer with 50mM DTT. The samples were heated on a heat block at 75°C for 5 minutes and after that were cooled down on ice and centrifuged at 4°C at maximum speed for 10 minutes. 20µl of the supernatant was transferred into a new Eppendorf tube and were frozen for storage.

After the 2 hours incubation new control samples were taken and treated the same way as described above. The remaining cell cultures were poured into centrifugable tubes and were centrifuged at 5000g for 15 minutes at 4°C. The supernatants were discarded and the cell pellets were frozen for storage.

6.9.3 Preparing cell extracts

The frozen cell pellets were thawed on ice and resuspended in 10ml of 100mM Tris-HCl pH7.4 with 300mM NaCl. After resuspension, the cells were lysed with a french press and the lysed cell extracts were centrifuged for 30 minutes at 14000g at 4°C

6.10 SDS-PAGE

To analyze the protein expression 15µl of the control samples of the protein extraction with loading dye were loaded on a pre-made polyacrylamide SDS-PAGE gel. The separation was performed at 150V for 1.5 hours. After the electrophoresis the gels were stained Coomassie Brilliant Blue for over night and after staining, the gels were destained a few times for optimized visualisation of the protein bands.
6.11 Testing the LOX activity

For normalizing the activity assay the protein concentration of the cell extracts were determined by the Bradford assay. Samples of 5µg, 10µg and 50µg of the measured proteins concentration were incubated in 800µl of 100mM Tris-HCl pH7.4, 0.1% Tween-20 and 300mM NaCl solution and either 100µM linoleic acid (18:2) or 100µM linolenic acid (18:3) in glass tubes for 30 minutes at room temperature. The fatty acids were dissolved at 10mM in ethanol. After incubation, 2ml of methanol containing 20mg of SnCl₂ were added to the samples and incubated for 10 minutes at room temperature. After this 5µg of internal standard (17:0) in methanol and 1ml of chloroform were added. After vortexing, the samples were incubated in a cold room at 4°C for 10 minutes. 1ml of chloroform and 0.95ml of distilled water and 50µl of acetic acid were added to the samples and vortexed. The phases were let to separate and the lower phase was transferred to a new glass tube, dried under N₂ and dissolved in 0.5ml of ethyl acetate for storage.

Before GC-MS analysis, the dried samples were extracted with diethyl ether and derivatised to methyl esters with diazomethane. The samples were injected into the GC-MS and were let to run for about 15 minutes.

The peaks with presumed retention time for the oxylipins and fatty acids were identified based on the mass to charge ratio of the peaks.
7. Results

7.1 Sequences of *AsLOX1* and *AsLOX2*

Different PCR conditions and primer combinations were tested and the resulting products analyzed by gel electrophoreses. In several cases fragments of the expected sized were produced. In some cases temperatures gradients in the annealing steps were used to optimize the reactions. Reaction products of the expected sizes were excised from the gels and cloned without further purification. All cloned fragments were verified by restriction enzyme mapping and DNA sequencing.

A typical gel samples of the RT-PCR gradient optimization is shown in Figure 6.

![Figure 6. Gel electrophoresis picture from RT-PCR amplified genes](image)

Lanes 33, 34, 35 show amplification using *AsLOX1* primers and lanes 36, 37, 38 using *AsLOX2* primers. Lane to the left show size markers. The expected band is supposed to be between 2500bp and 3000bp and the numbers indicate the sample number for the genes amplified by RT-PCR.
Figure 6 shows the effect of different annealing temperatures in the RT-PCR reaction. In this case the clearest bands are obtained with the highest temperatures.

The obtained sequence data were analyzed with an internet based tool provided by NCBI. First the sequences were cleared from vector contamination, which was done with VecScreen. Then the sequences were compared to the original sequences of *AsLOX1* and *AsLOX2* with BLAST alignment tool and were reverse complemented when needed.

A typical output from the BLAST alignment tool is shown in Figure 7.

![Figure 7. BLAST alignment of PCR amplified *AsLOX1* fragments.](image)

Colored boxes on top indicate the given score based on the similarity of the analysed fragments (thin horizontal red lines) to the query sequence *AsLOX1* (thick horizontal red line). The thin, vertical black lines intersecting the gene fragments indicate an insertion in the cloned sequences compared to the query sequences. Numbers under the query line indicate size of the fragments in bp.

The analyzed sequences of PCR amplified *AsLOX1* fragments show two insertions compared to the query sequence of *AsLOX1*. The similarity of the cloned sequences to the query sequence were in the region of 94%- 100% on the nucleotide level.
In the case of the *AsLOX2* fragments, the results are similar to *AsLOX1*. The sequences reveal six different insertions in the cloned DNA compared to *AsLOX2*. And the similarity of the sequences were in the same range as in *AsLOX1*.

To be able to produce the recombinant proteins, full length sequences of the genes had to be produced. To obtain this, we attempted to amplify the sequences from total RNA with RT-PCR. The analysis was done the same way as with genomic DNA.

Results from the alignment analysis reveal the same inserts as in PCR amplified fragments in both of the genes. To ensure that the results were not caused by genomic DNA contamination in the RNA, the extracted total RNA samples were DNase treated and RT-PCR products re-cloned.

The results after the DNase treatment show no insertions. Furthermore the obtained sequences aligned to the query sequence of the genes with minor nucleotide differences to the query sequence.

After extensive analysis and confirmation of the correctness of the fragments of the genes and the specificity of the primers, the full length genes were amplified with RT-PCR from DNase treated RNA, cloned, sequenced and analyzed the same way as the fragments.

Comparing the full length clone *AsLOX1* sequences to the query sequences, revealed that the last nucleotide of the full length clone was missing. The missing nucleotide was an deoxyadenosine (A) and it was not included in the reverse primer for *AsLOX1*. Luckily the first nucleotide of sequencing vector was an A and the EcoRI restriction site after the stop codon, therefore it includes the nucleotide in the cleavage product. New primers were not designed.
In all 12 positive full length clones of \textit{AsLOX1} were sent for sequencing and the results were once again similar to the fragments with no insertions.

Comparison of the \textit{AsLOX2} sequences to the query revealed an other mistake made with the design of the primers. \textit{AsLOX2} was missing 3 nucleotides, the whole stop codon. The sequencing vector did not add the required nucleotides for creating a stop codon and therefore the reverse primer had to be redesigned to be able to produce an active protein.

The new reverse primer was tested with the original forward primer only once with High Fidelity RT-PCR and DNase treated RNA. The reaction did not produce a product. Due to time limitation the experiment were continued with only the gene \textit{AsLOX1}.

### 7.2 Subcloning results

Subcloning of the genes were done in several different ligation concentrations and with different volumes added to the transformation. Results from the plates revealed that the ligation mixtures with lower insert concentration produced more colonies. Also the lower volume of the ligation mixture added to the cells performed better.

Digestion of the extracted plasmids revealed a much lower plasmid count than obtained from the sequencing clones. During cloning we noticed that smaller amounts of plasmids added to the cells produced more colonies on the selective plates.
Out of 10 colonies of the *AsLOX1* subclones with optimized transformation concentrations, 9 had the gene and 8 of them were in the correct orientation. Two of the clones were chosen for protein expression.

### 7.3 Protein expression of AsLOX1

A typical result from the protein expression experiments are shown in Figure 8.

By comparing the band patterns on the SDS-PAGE gel prior and after induction it can be deduced that the expression of the recombinant proteins worked and both of the *E.coli* strains produced the expected size proteins.

![Figure 8. E. coli LOX gene expression experiment](image)

SDS-PAGE results from *AsLOX1* with strain BL21 (left) and BL21 Codon Plus strain (right). Lanes 1, 3 and 5 are samples prior to induction and lanes 2, 4 and 6 are after 2 hours of induction. The arrows indicate the gene fusion proteins (130kDa) and the GST protein (26kDa) alone. The intensity of the colors indicate the amount of proteins in the samples.
7.4 GC-MS results

Some of the results from the activity assay analyzed with GC-MS is showed in Figure 9 and in Figure 10. Based on the produced peaks from the internal standard (17:0) and the used fatty acid (18:2) the analysis worked as expected.

Figure 9. GC-MS analysis of LOX enzyme reaction products

TOP (Vector only): Reaction products derived from linoleic acid (18:2) without a LOX enzyme. BOTTOM (AsLOX1): Reaction products derived from linoleic acid (18:2) with AsLOX1 enzyme. The time axis (x) indicates the retention time (specific to each molecule) and the y-axis indicates the abundance of the molecules.

From Figure 9 it is difficult to say if there is a peak produced by the oxylipins. The peak is expected to be visible between 10 and 11 minutes. The two scans clearly show some differences in the peak profiles within the control expression.
(vector only) and the actual protein expression (AsLOX1), however not at the correct retention time zone. Therefore a new scan was run with higher sensitivity for the expected peaks to make sure that the impurities of the samples do not mask the wanted peaks. The more sensitive run produced the same result with no peak at the expected time or mass profile. Results are shown in Figure 10.

![Figure 10](image-url)

**Figure 10. High resolution GC-MS analysis of LOX reaction products**

TOP: Reaction products derived from linoleic acid (18:2) with AsLOX1 enzyme with higher sensitivity for the produced oxylipins. BOTTOM: Zoomed in section from the same results (TOP). The arrow indicates the placement of the expected oxylipin peak. The time axis (x) indicates the retention time (specific to each molecule) and the y-axis indicates the abundance of the molecules.

The presented results indicate that the expressed protein did not produce any oxylipins since there was no peak with the correct mass to charge ratio with the correct retention time. The results show no activity at all. Results were similar from the other samples with no activity.
8. Discussions

In this work we cloned two oat LOX genes and tested the activity of one full length clone in an *E. coli* expression system.

Sequencing results obtained from RT-PCR revealed that the extracted total RNA had genomic DNA contamination and therefore had to be DNAses treated to obtain the real encoding sequences of the genes. After DNAses treatment results show that the two LOX genes are existing in the genome and the full length genes are clonable.

Results obtained by PCR from genomic DNA show inserts in both of the genes. Two inserts in *AsLOX1* and six inserts in *AsLOX2*. Alignment results revealed 3 different version within the inserts. These findings suggest that the isolated genes have at least three different version in the genome. The insertions are likely to be introns, however there is no proof that the putative LOX genes are from the three different genomes of oat. They might be as well from one gene family in one of the genomes.

Protein expression results show that the two different *E. coli* strains produced slightly different results. This was expected since the strain BL21 Codon Plus has the extra plasmid to facilitate effective expression of eukaryotic protein. The results show that BL21CP was more effective in producing the wanted recombinant protein, which is about 130 kDa and the empty GST produced the 26 kDa protein with no band for the recombinant gene. BL21 also produced the wanted protein but also much more smaller and unwanted proteins. These might be a results of insufficient amount of tRNA needed for the protein and therefore the translation was terminated prematurely.
Based on these results the samples obtained the strain BL21CP was tested for the LOX activity. The GC-MS results show that the recombinant protein had no activity at all. There are several possible explanations for this. One possibility is that the assay did not work as was expected due to the fact that unpurified crude cell extracts were used for the assay. A more possible explanation is that the expressed proteins were not in an active form due to wrong folding patterns and therefore the assay did not produce any oxylipins. This might be also caused by the fusion protein located in the C-terminus that might interfere with the active sites of the enzyme. An other explanation could be that the *E. coli* produces so called inclusion bodies that interfere with the expressed recombinant protein with an inhibition effect.

According to some studies made on rice 9-LOX genes (Shirano and Shibata, 1990; Mizuno *et al.*, 2003) the produced proteins were only in an active form if the expression was incubated in lower temperatures. In additions to the rice *LOX* genes also *Arabidopsis thaliana* *LOX* genes expressions were performed in lower temperatures but with High Five insect cells instead of *E. coli* (Bannenberg *et al.*, 2009).
9. Conclusions

Since both of the genes *AsLOX1* and *AsLOX2* were amplifiable and clonable, it proves that the putative 9-LOX genes exists in the oat genome. Sequencing results also reveal 3 different version of both of the LOX genes.

The obtained genomic sequences can be used as templates for future MALDI-TOF screenings. Utilizing the differences in the inserts it is possible to design gene specific primers for screening purposes, that ensure more accurate results.

Unfortunately the activities of the putative LOX genes are not confirmed. However the activities are confirmable by optimisation of the expression system and the activity assay with purified proteins.

10. Suggestions for further work

After confirmation of the 9-LOX activities using GC-MS the screening process for mutants in the oat TILLING library can be initiated. The screening will be performed using an in-house developed MALDI-TOF based method for mutant identification at the genetic level. The effect of the mutations can then be confirmed by infecting the mutant with a *Fusarium* strain and comparing it to wild type oat. The goal will be to develop a commercially viable oat variety with *Fusarium* resistance.
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