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Primary analyses of catalases in *Arabidopsis thaliana*

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Abstract

Catalase is involved in the protection of the cell against reactive oxygen species (ROS), though the detoxification of hydrogen peroxide (H_2O_2) in the peroxisomes. If not destroyed or controlled ROS can lead to damage of the cell and even kill the cell. H_2O_2 can also act as a signaling molecule and in this way be a natural part of the organism's way of surviving in the environment. This can put catalase in a different light, with the possibility of having a more complex and wider function than previously thought. The full role and function of catalase is therefore yet to be discovered. Studies have shown that catalase can have some activity outside the peroxisomes, in the cytosol or in the mitochondria. This raises the suspicion if catalase could have an interaction with cytosolic proteins, such as DJ-1. DJ-1 has an interaction with SOD and GPX. SOD and GPX are involved in the conversion of O_2^- to H_2O_2 and further to H_2O. This is also the main role of catalase. It is therefore believed that catalase can in this manner have an interaction with DJ-1. DJ- 1, called PARK 7 (in human beings) is one of the main Parkinson`s disease related protein. Parkinson`s disease is a disorder that blocks post- synaptic signaling and leads to neurodegeneration. This is caused by environmental and genetic factors that might lead to the accumulation of ROS in the cell. This accumulation (if not handled by the cell) causes mutations in the genes involved and disrupts the pathway of dopamine transfer across neurons with the outcome of aggregation of Lewy Bodies.

In this thesis the catalases in *Arabidopsis thaliana* were analyzed. Catalase localization and interaction with DJ-1 and other ROS related proteins were analyzed. Knock out mutant plants and plants with over- expressed catalase were observed with regard to their phenotype. Levels of catalase in plants of different age and in different plant tissue were also checked. Out of the three variants of catalases in *A. thaliana*, AtCat3 had the highest expression level (relative stable over time), AtCat1 had the second highest expression level (decreasing over time) and AtCat2 had the lowest expression (nearly not detectable). AtCat3 had its highest expression in leaf, stem and seed couples (measured after 4 weeks of growth in soil) and AtCat1 had its highest expression in stem and leaf (measured after 4 weeks of growth in soil). Expression of AtCat2 in different plant tissue after 4 weeks of growth in soil was too low to measure. Catalase expression in flower was relatively low for both AtCat1 and AtCat3.

By doing research on the genes involved in Parkinson`s disease, the mechanism behind the disorder can fully be discovered and this can make it possible to understand the disorder on a deeper level.

Acknowledgments
Abstract

Table of content

1.0 Introduction

1.1 Parkinson`s disease

Parkinson's disease is a chronic neurodegenerative disease and belongs to a group conditions called motor system disorder. The disorder usually affects people over the age of 50. The main symptoms of parkinsonism are trembling in hands, arms, legs, jaw and face, rigidity or stiffness, bradykinesia (or slowness of movement) and impaired balance and coordination[1,2]. The symptoms of this motor system disorder are a result of the loss of dopamine- producing brain cells[3]. Dopamine is a neurotransmitter that is produced in several different areas of the brain, including the substantia nigra and ventral tegmental area. As a hormone dopamine inhibits the release of prolactine from the anterior lobe of the pituitary[4]. Dopamine structure can be seen in figure 1.1.

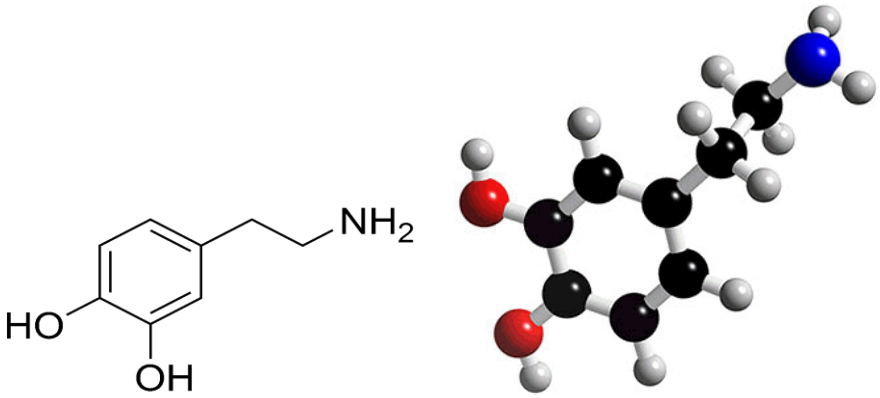


Figure 1.1: The structure of dopamine[5] and the bullet structure of dopamine[6].

Currently there is no cure for the disease and treatment consists of controlling the symptoms. Treatment consists of the medicament levodopa combined with carbidopa[7]. The medicament carbidopa delays the transformation of levodopa into dopamine until it reaches the brain. Nerve cells can then convert levodopa to dopamine and replenish the brain`s supply[7].

[1] www.ninds.nih.gov/disorder/parkinsons_disease/parkinsons_disease (viewed : 3/3-12)
[2] www.ninds.nih.gov/conditions/parkinsons_disease (viewed : 3/3-12)
[3] www.news-medical.net/helth/what-is-dopamine.aspx (viewed : 5/3-12)
[4] www.news-medical.net/helth/what-is-dopamine.aspx (viewed : 5/3-12)
[5] http://clinicallypsyched.com/dopamine-model-drug-addiction-schizophrenia-treatment/
[6] http://www.chm.bris.ac.uk/motm/dopamine/dopamineh.htm
[7] http://www.ninds.nih.gov/didorders/parkinsons_disease.htm (29/05-2012)

It is important to do research on the mechanisms under laying the disorder to be able to understand the disorder and maybe develop a better treatment.

1.1.1 Genetic and environmental factors involved in Parkinson`s disease development

Different factors can cause development of PD. These factors are generally divided into genetic and environmental factors. Genetic factors can for example be genetic mutations and environmental factors can be toxins in the environment, such as neurotoxin components.

In a normal state, dopamine is released in the presynaptic neuron and this result in signaling to the postsynaptic neuron. This happens through D1 and D2 type dopamine receptor (fig. 1.2, Normal state of dopamine transfusion). This leads to a cascade of signaling and activation stages which leads to formation and activation of PKA (Girault J-A. and Greengard P. 2004). Genetic mutations, environmental toxins and neurotoxins can cause mitochondrial oxidative stress and the release of ROS (Bossy-Wetzel E. *et. al.* 2004). This can lead to for example apoptosis and misfolding of α- synuclein (Yasuda T. and Mochizuki H. 2010) (fig 1.2, Parkinson`s disease state). This leads to proteosomal degradation. The main function of proteosomes is to degrade unneeded or damaged proteins by cutting their peptide bonds[8]. The mechanism of proteosomes is a part of a major mechanism which can control and regulate concentration of particular proteins and degenerate misfolded proteins[9]. When the cell no longer can degenerate misfolded proteins (like in the Parkinson's disease state, fig 1.2) this lead to no transfer of dopamine across the neurons (fig. 1.2, Parkinson`s disease state). Genetic mutations of α- synuclein and parkin disrupt the pathway and leads to further accumulation into Lewy Bodies. Lewy Bodies are tiny, spherical protein deposits found in nerve cells. They are named after the doctor that first discovered them in 1912 and there are two types of Lewy bodies; one classical and one cortical[10]. When dopamine transfer across the neurons are blocked, this leads to a post- synaptic signaling block and this again lead to

[8] http://en.wikipedia.org/wiki/Proteasome (28/05-12)
[9] https://wasatch.biochem.utah.edu/chris/publications/55.pdf (29/05-12)
[10] http://alzheimers.org.uk/site/scripts/documents_info.php?documentID=113 (29/05-12)

neurodegeneration. See figure 1.2 for graphic illustration of normal state of dopamine transfer and Parkinson`s disease state.

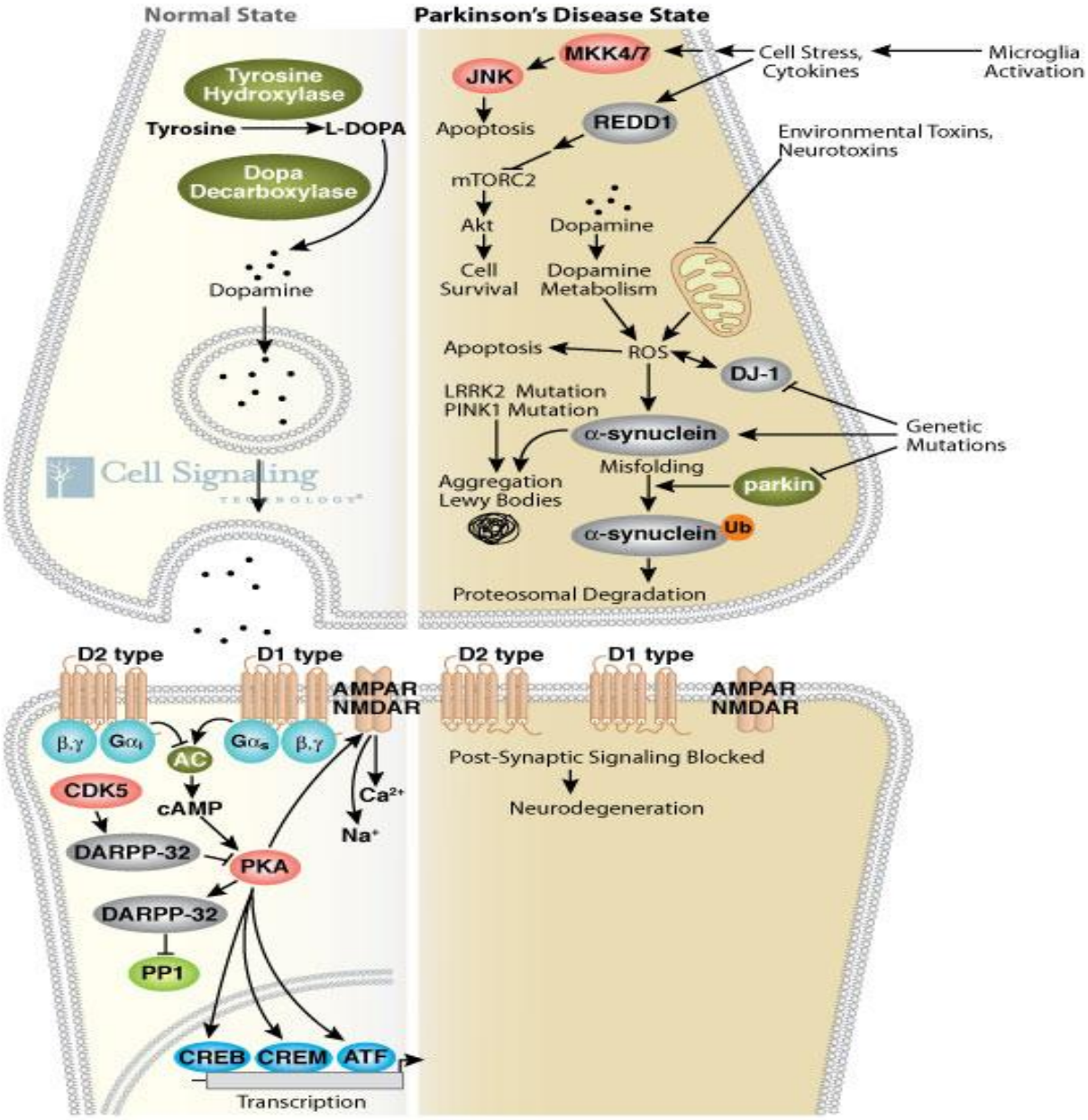


Figure 1.2: Normal state of dopamine transfusion vs. Parkinson`s disease state[11].
Illustrates how Parkinson`s disease develops in the neurons, and the main genes involved. The blocked post- synanptic signaling lead to neurodegeneration.

Much has been learned in the last years about the genetics of familiar Parkinsonism.

However, far less is known about the genetics involved in the sporadic cases of the disease.

Most cases of Parkinsonism are sporadic and occur to people with no family history of the

[11] http://www.cellsignal.com/reference/pathway/parkinsons_disease.html (viewed : 20/3-2012)

disease. It is believed that different types of environmental factors can trigger different variations of the disease and with this give different variation of the disease (Benmoyal-Segal L. and Soreq H. 2006). Although the mechanism under laying sporadic Parkinsonism remains unclear and is not fully studied yet, sporadic cases of the disease may result from both environment and genetic factors. More research on the sporadic type of the disease is needed to be able to understand the development of the disease.

1.1.2 *Genes involved in Parkinson`s disease development*

Research is still going on to determine the full mechanism behind the development of PD. Statistics show that approximately 15% of people with Parkinson`s disease have a family history of this disorder. Different genes have been found to be involved in the development of the disorder, this for example LRRK2, PARK2, PARK7, PINK 1 and SNCA gene (Table 1.1). Mutations in these genes can cause the development of PD.

Table 1.1; Genes involved in the development of PD.[12]

Gene	Name
LRRK 2	Leucin- rich repeat kinase 2 (dardarin)
PARK 7	Parkinsons protein 7 (DJ-1)
PARK 2	Parkinsons protein 2 (E3 ubiquitin ligase)
PINK 1	PTEN inducing putative kinase 1
SNCA	α- synuclein(non A4 component of amyloid precursor)

For understanding the disorder on a deeper level the genes involved must be mapped and their function must be further studied. Table 1.1 only show some of the genes involved in development of PD. Further analyses is necessary for investigate the complex system of genes involved in the development of the disorder. One gene named PARK 7, coding the protein DJ-1 is particularly linked with PD. PARK 7 stands for parkinsonism daises (autosomal recessive, early onset) 7 and provide instruction for coding the gene DJ-1. It is localized on

[12] http://gov.nlm.nih.gov/gene (viewed : 5/3-12)

chromosome 1 position 1p36.23. This can be seen on figure 1.3. This figure display chromosome 1 and the yellow arrow indicate where PARK 7 is localized.

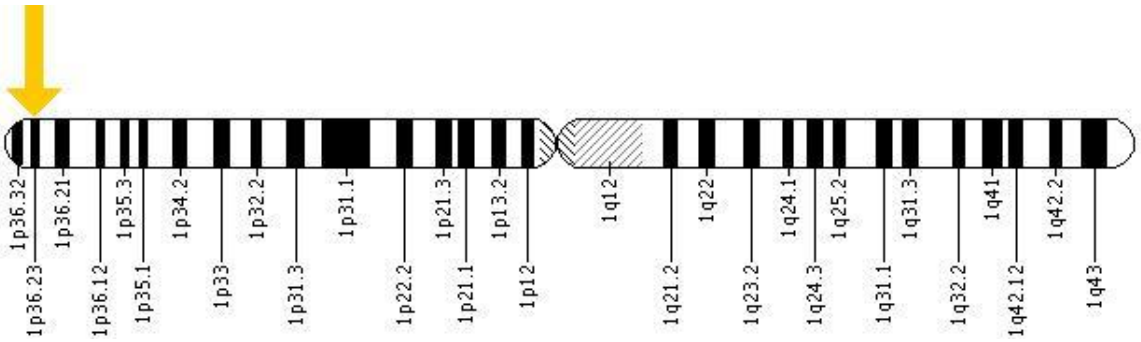


Figure 1.3: Chromosome 1. The yellow arrow indicates where PARK 7 is localized[13].

Mutations in DJ-1 are associated with Parkinson's daises. Loss of function mutations in DJ-1 causes the disease. Research have shown that either delegation of several exons that result in effective knock out (Bonifati V. *et. al.* 2003) or point mutations destabilizing the protein and causes loss of protein function might be the mechanism behind loss of DJ-1 function (Miller D.W. *et. al* 2003). Loss of DJ-1 (PARK 7) function leads to neurodegeneration (see fig. 1.2). DJ-1 functions are not fully discovered and work on DJ-1 from *Homo sapiens* and other species is still going on. Studies have shown that DJ-1 is involved in oxidative stress response (Bonifati V. *et. al*. 2003, Canet-Aviles R.M. *et. al* 2004).Oxidative stress occur when unstable molecules called free radicals raise to levels that damage or kills the cell. DJ-1 response to hydrogen peroxide (H_2O_2) with the shift in isoelectric point (Ip) to a more acidic one (Canet-Aviles R.M. *et. al* 2004, Mitsumoto A. and Nakagawa Y. 2001). DJ-1 eliminates H_2O_2 in vivo by oxidizing itself (Taira T. *et. al.* 2004). The same study also found that DJ-1 knock down by siRNA rendered SH-SY5Y neuroblastoma (susceptible to H_2O_2, MPP+ or 6-hydrodopamine) induced cell death (Taira T *et. al.* 2004). Cells containing DJ-1 mutant, including the mutant L166P, became susceptible to cell death in parallel with loss of oxidized forms of DJ-1(Taira T. *et. al.* 2004). These results clearly indicate that DJ-1 play a part in anti oxidative stress reaction. Mutations in DJ- 1 lead to cell death and neurodegeneration, which is observed in Parkinson's disease (Bonifati V. *et. al. 2003).*

[13] http://ghr.nlm.nih/gene/PARK7 (viewed : 5/3-12)

DJ-1 has a moderate homology to the bacterial proteins ThiJ and PfpI (Bandyopadhyay S. and Cookson M.R. 2004). These proteins are involved in thiamine synthesis and protease activity. ThiJ kinase is involved in the biosynthesis of thiamine and since thiamine is an essential nutrient it is believed that function has been passed down to most of the eukaryotes (Bandyopadhyay S. and Cookson M.R. 2004).The ThiJ / PfpI super family is a large protein family, suggesting that DJ-1 have other unidentified functions (Bandyopadhyay S. and Cookson M.R. 2004). DJ-1 show for example sequence homology to a number of proteins containing ThiJ domain, including protein chaperones (Quigley P.M. *et. al.* 2003) and catalase (Horvath M.M. and Grishin N.V. 2001). If DJ-1 has a role as redox- sensitive chaperone, it might help in folding newly synthesized proteins and damaged proteins into correct 3d-shape [9,14].

1.1.3 *Model organisms used for studying Parkinson`s disease*

Traditional model systems for studying PD have been rodent and primate species. Other species that have been used for studying the disease include zebra fish (*Danio rerio*), flies (*Drosophila melanogaster*), anurans (frogs and toads) and nematodes (*Caenorhabditis elegans*). For studying how gene and protein expression contribute to the development of the disease these kinds of model systems are accepted. All of the model organisms mentioned above is relatively easy to work with, compared to primate and rodent model organisms. Knowledge about zebra fish is for example rich in embryology/anatomy and genetics, this provide a good model organism for studying PD. Not only is it possible to study the genetic mechanism under laying the disease, but also the behavior of the fish (for example the swimming pattern). Studying these model organisms is valuable for studying evolutionary conserved patterns and cellular mechanisms that are relevant to PD. For the research to reach its full potential, it is important to see the work done in context with medical research.

[14] http://medical-dictionary.thefreedictionary.com

1.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant that is widely used as a model organism. The plant belongs in the family of mustard plants. The plant is native to Europe, Asia and northern parts of Africa. Different ecotypes of the plant can be found, in this thesis the ecotype wild type columbia was used. The plant is approximately 20 – 25 cm high, have most of the leaf in the base of the stem surrounding the plant. It have few leafs on the stem, a white small flower (3 mm) and each seed capsule (siliqua) contain approximately 20-30 seed. See figure 1.4 picture of *Arabidopsis thaliana*. *A. thaliana* have a life cycle under optimal conditions of 6 weeks.

A huge advantage with using *A. thaliana* as a model organism (among many others) is its high efficient when transformed with methods utilizing *Agrobacterium tumefaciens*.

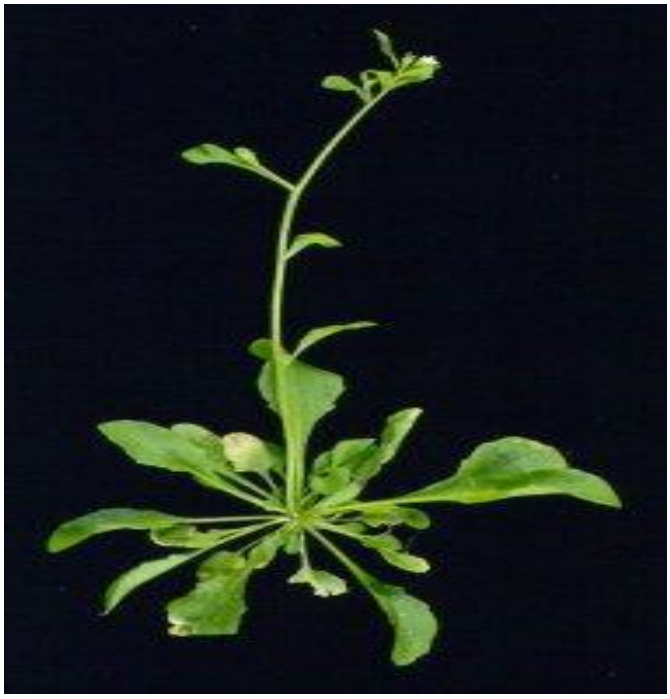


Figure 1.4: *Arabidopsis thaliana*

Arabidopsis thaliana is sometimes called "the super weed". This is because it has one of the smallest genome in the plant kingdom, which contains very little junk DNA. It also grows well with little care. It only need water and light to grow. As already said it have a rapid development at approximately 6 weeks and one plant can produce 10 000 seeds. This is way it has earned the name "the super weed".

1.2.1 *Arabidopsis thaliana* as a model organism for studying Parkinson`s disease

Traditionally a plant model organism has not been used for studying human diseases. However using *A. thaliana* as a model organism have reviled discoveries directly linked to human health and processes in human biology have been studies through use of this model organism (Jones A.M. *et. al.* 2008). The advantages of using *A. thaliana* as a model organism for studying PD are many. The *A. thaliana* genome is well known and established making it easy to obtain information from various web pages and data banks, like for example the web page NCBI or the web site TAIR. The plant has also a small adult size and a relative rapid life cycle. This makes it relative simple to study and with little effort it is possible to do identification studies and generation studies of for example mutants. The plant is easy to maintain and running costs for keeping it is low. Another aspect is that the plant has few ethical issues connected to it, making it more convenient to work with. Although *A. thaliana* is a plant model system its genome contains homolog of numerous genes involved in human disease.

A. thaliana has orthologues to all PD associated genes (but not the PARK 1 and PARK 3 genes) (Xiang Ming Xu and Simon G. Møller 2011). Studies have shown that the gene homolog to DJ-1 / PARK 7 in *A. thaliana* (AtDJ-1a) have relation to PD mechanism (Xu X.M. *et. al.* 2010). Although *A. thaliana* and human genes have very different genes, orthologs from human can be found in *A. thaliana*. Research has shown the value of *A. thaliana* as a model system particular the fields of neurological disease and cancer (Rubin G.M. *et. al* 2000). As high as 71 % of the genes involved in neurological disorders have *A. thaliana* orthologs (with a E-value less than E^06) (Jones A.M. *et. al*. 2008). This seen in context with the more traditional model organisms for neurological disorders like *Drosophila melanogaster* with a 67% orthologs genes (Jones A.M. *et. al*. 2008) and *Saccharomyces cerevisiae* 41% orthologs genes(Jones A.M. *et. al*. 2008), one can argue that *A. thaliana* is even a better model system in this field of study. Despite advantages with the use of *A. thaliana* as a model organism for studying PD, it is also important to work with other model systems and also in combination with medical research to get the full picture of the disorder.

1.2.2 *DJ-1 from Arabidopsis thaliana*

Arabidopsis thaliana have tree different DJ-1 homolog genes, called AtDJ-1a (AT3G14990), AtDJ-1b (AT1G53280) and AtDJ-1c (AT4G34020). All of the homolog genes seem to be connected to ThiJ/PfpI super family and DJ-1 super family. In this thesis AtDJ-1a was studied (interaction study with AtCat2), this because it showed the most homology to DJ-1 and because it was localized in the cytosol. For detailed information on AtDJ-1a, AtDJ-1b and AtDJ-1c see table 1.2.

Table 1.2: Detailed information on the tree DJ-1 isoform in *A. thaliana*[15].

Name	Locus	Length (bp)	Length (aa)	EST	Contains domain	Molecular weight	isoelectric point
AtDJ-1a	AT3G14990	1179	392	580	ThiJ/PfpI and DJ-1	41856.9	5.0829
AtDJ-1b	AT1G53280	1317	438	100	ThiJ/PfpI and DJ-1	46990,1	7,9546
AtDJ-1c	AT4G34020	1419	472	32	ThiJ/PfpI and DJ-1	50986,1	8,9889

AtDJ-1a has three different splicing variants, AtDJ-1b has one and AtDJ-1c has two. This can be seen in figure 1.5, 1.6 and 1.7.

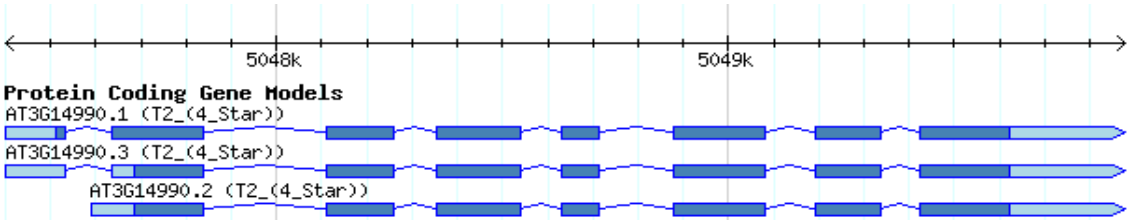


Figure 1.5; Protein coding gene model of AtDJ-1a (AT3G14990). AtDJ-1a has three protein coding gene models. AT3G14990.1 is unspliced version. AT3G14990.2 and AT3G14990.3 are the spliced version of AtDJ-1a[16].

[15] http://www.arabidopsis.org/index.jsp
[16] http://www.arabidopsis.org/servlets/TairObject?id=37700&type=locus (viewed : 22/3-12)

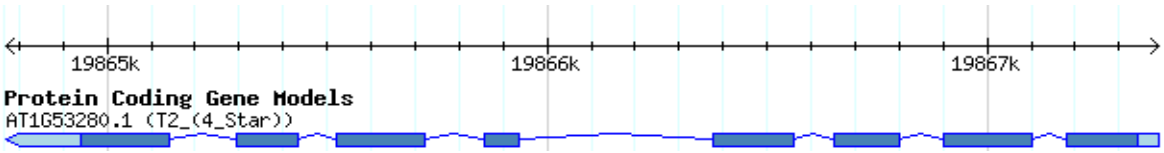


Figure 1.6; Protein coding gene model of AtDJ-1b (AT1G53280) [17].

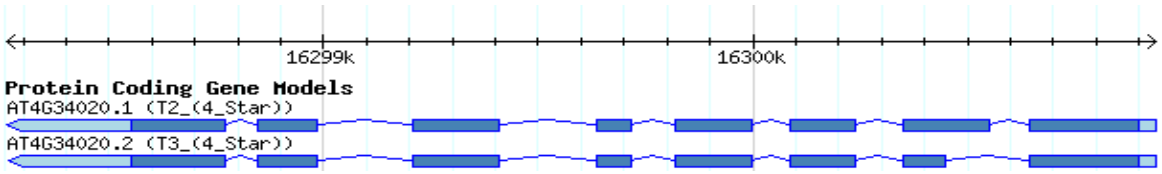


Figure 1.7: Protein coding gene model for AtDJ-1c (AT4G34020)[18]. AtDJ-1c has two protein coding gene models. AT4G34020.1 is unspliced and AT4G34020.2 is spiced.

Two of these genes are located in the chloroplast (AtDJ-1b and AtDJ-1c) and AtDJ-1a is localized to the cytosol and the nucleus. This is the one of the three variants that has been connected to the human DJ-1 (Xu M.X. *et. al.* 2010). When DJ-1 from human is not correctly expressed, neuronal cell death occur in mammalian cells. In the case of AtDJ-1a loss of function leads to apoptosis (Xu M.X. *et. al.* 2010). Studies of AtDJ-1have shown that AtDJ-1a (like DJ-1) response to stress treatment (Xu X. M. *et. al.* 2010). Transgenic plants with elevated levels of AtDJ-1a had higher protection against environmental stress than WT (Xu X. M. *et. al.* 2010). This indicates that DJ-1 and AtDJ-1a have similar functions. The same study also found that DJ-1a and DJ-1 have an interaction with superoxide dismutase 1 (SOD 1) and glutathione peroxidase 2 (GPX 2) and that this interaction result in AtDJ-1a- and DJ-1 mediated cytosolic SOD 1 activation in copper- dependent fashion (Xu X . M. *et. al.* 2010 and Xiang Ming Xu and Simon G.Møller 2010).

[17] http://www.arabidopsis.org/servlets/TairObject?id=27092&type=locus (viewed : 22/3-12)
[18] http://www.arabidopsis.org/servlets/TairObject?id=127912&type=locus (viewed : 22/3-12)

1.2.3 *ROS and ROS removal by DJ-1in A .thaliana and in human*

ROS (reactive oxygen species) result in serious damage and is toxic to aerobe cells. ROS can be generated in the body in different ways. Figure 1.8 shows a simplified explanation of how ROS function in the human body. Her it is shown that ROS can be introduced to the cells by an oxidative burst. This is a rapid release of ROS and plays a part in the immune system. Under an oxidative burst for example neutrophils and monocytes are released. Neutrophils and monocytes protect the cell from an intruder, like for example bacteria. ROS can also come from ordinary cellular respiration. Oxygen is an important component that is necessary for aerobe organisms. When oxygen changes and forms O_2^-, the oxygen molecule becomes dangerous for the cell and ROS can induce cell death. Another way for ROS to get inside the cell is through environmental factors for example pollution. Different molecules in the body have the property of making ROS harmless. For example the complex SOD (superoxide dismutase) plays a part in converting O_2^- to H_2O_2 and further to water with the help of CAT (catalase) and GPX (glutathion peroxidase). The main function of GPX is to reduce lipid hydroperoxidase and to convert free hydrogen peroxide to water. Catalase also plays a part in protecting the cell from ROS by converting hydrogen peroxide to water (see fig. 1.8). Damage inflicted by ROS is; DNA damage, lipide peroxidation and protein peroxidation. This is also shown on figure 1.8.

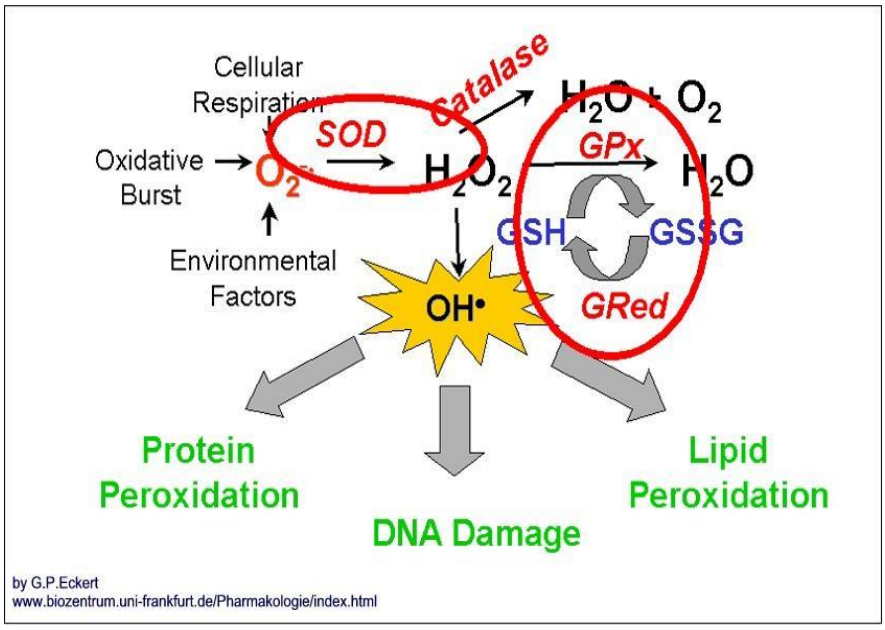


Figure 1.8: An overview of ROS pathway. ROS can be generated in the cells in different ways, such as cellular respiration, oxidative burst and by environmental factors. The outcome of ROS (if not handled by the cell) can lead to several types of damage to the cell.

Research has shown that ROS component (for example; H_2O_2) can act as a signaling molecule in plants (Neill *et. al.* 2001). Cellular response to H_2O_2 are complex, with a possible crass- talk between response to several stimuli (Neill *et. al.* 2001). A close relationship exists between intracellular H_2O_2 and cytosolic calcium, this in response to both biotic and abiotic stress. Studies suggest that an increase in cytosolic calcium boost the generation of H_2O_2 (Yang T. and Poovaiah B.W. 2002). This study also report binding and activation of calmodulin (CaM) to catalase (Yang T. and Poovaiah B.W. 2002). This document that calcium/CaM can down regulate H_2O_2 levels in plant through activation of plant catalase (Yang T. and Poovaiah B.W. 2002).

The scavenger pathway is a pathway where reactive oxygen species are broken down to water and other harmless components. Here SOD, GPX and CAT are important components in the breakdown ROS. These proteins are found in mitochondria, cytosol, chloroplast and microbody.

Resent research have shown that DJ-1 (for human) and AtDJ-1a (for *A. thaliana*) play a part in the removal of ROS from the cell, indicating that the process of ROS removal is similar in plant and in humans. Figure 1.9 shows a overview scheme of ROS removal in *A. thaliana* and

in humans. The only different is that *A. thaliana* have an extra ascorbate peroxidase (APX) and that instead of CSD human have SOD.

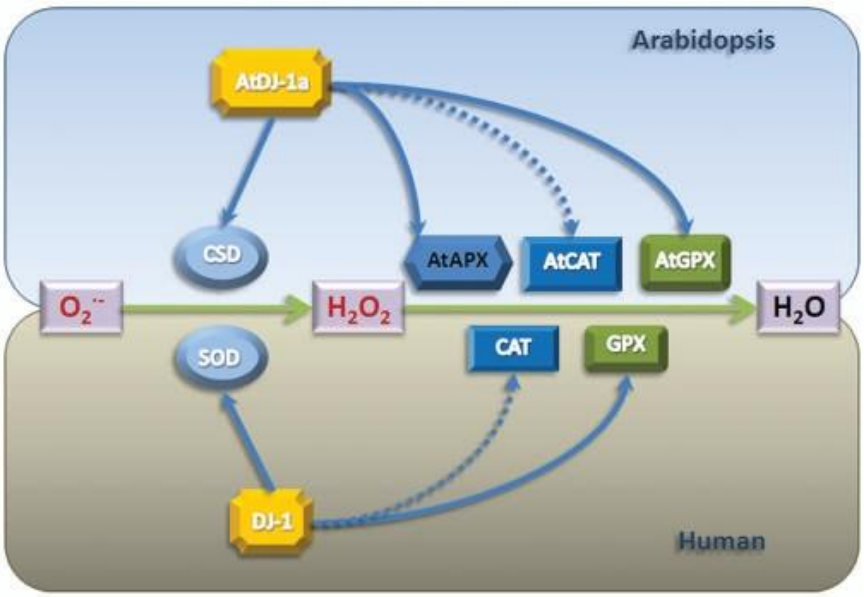


Figure 1.9: The involvement of DJ-1 (for human) and AtDJ-1 (for *A. thaliana*) in the ROS scavenger pathway (Xiang Ming Xu and Simon G. Møller 2010). Superoxide anion is converted to H_2O_2 by the protein CSD in *A. thaliana* and by SOD in human. H_2O_2 is further converted to H_2O by APX, CAT and GPX in *A. thaliana* and by CAT and GPX in human. The broken arrows indicate the there might be an interaction with the DJ-1 like proteins and CAT.

Studies done with BiFC and ITC have shown that AtDJ-1a and CSD1 have an interaction and that DJ-1 has an interaction with SOD1 (Xiang Ming Xu and Simon G. Møller 2010). Similar analyses were also done and the interaction between AtDJ-1a and DJ-1 with GPX was found in both mammalian cells and in plant cells (Xiang Ming Xu and Simon G. Møller 2010). AtDJ-1a and DJ-1 stimulate CSD1/SOD activity, but only DJ-1/ AtDJ-1a containing copper activated CSD1/SOD suggesting that CSD1/SOD is provides with copper from DJ-1/ AtDJ-1a (Xu X. M. *et. al* 2010 and Xiang Ming Xu and Simon G. Møller 2010).). See figure 1.10 for working model of AtDJ-1a and DJ-1.

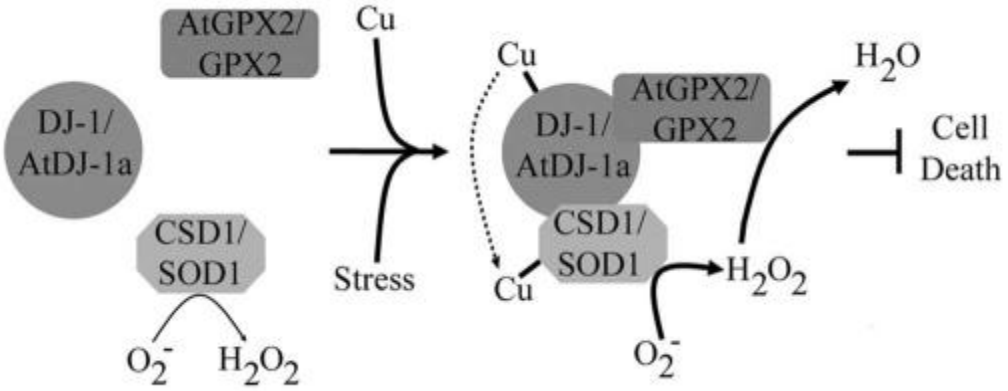


Figure: 1.10: A working model of how AtDJ-1a and DJ-1 work in action (Xiang Ming Xu and Simon G. Møller 2010). AtDJa-1 and DJ-1 interacts with GPX2 and SOD, this leads to the activation of SOD1 by copper. This copper is thought to come from AtDJ-1a/DJ-1 and is thought to raise the activity of SOD. GPX2 is thought to be anchored to AtDJ-1 and DJ-1 to ensure convention of the SOD- generated H_2O_2 to H_2O.

1.2.4 *Catalase in Arabidopsis thaliana*

The three different catalase isoforms of *A. thaliana* are predominantly located in peroxisomes (Mhamdi *et. al.* 2010). Sequence analyses show similarity between the three different catalases and the carboxy terminal regions that have been implicated to be imported into the peroxisomes (Mhamdi *et al* 2010), this can be seen in figure 1.14 a sequence alignment done with BioEdit (Mhamdi *et al* 2010). The three different catalases of *A. thaliana* are named AtCat1, AtCat2 and AtCat3. See table 1.3 for detailed information on the different isoforms.

Table 1.3: Detailed information on the three catalase isoforms in *A. thaliana.*

Name	Locus	Length (bp)	Length (aa)	EST	Contains domain	Molecular weight	isoelectric point
AtCat1	AT1G20630	1479	492	104	IPR018028 Catalase, mono-functional, haem-containing	56761.7	7.4411
AtCat2	AT4G35090	1479	492	772	IPR018028 Catalase, mono-functional, haem-containing	56930,8	7,1209
AtCat3	AT1G20620	1479	492	2553	IPR018028 Catalase, mono-functional, haem-containing	56694,9	7,6937

The three different catalases are similar to each other in length (table 1.3) and sequence (fig. 1.14), but the nucleotide sequence is sufficiently different, allowing gene specific primers to be designed for each isoform. Figure 1.11, 1.12 and 1.13 show the protein coding gene model for AtCat1, AtCat2 and AtCat3. AtCat1 have one variant, AtCat2 have two variants and AtCat3 have four different variants.

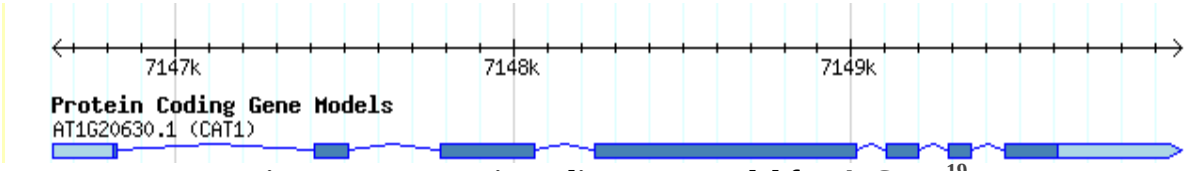


Figure 1.11: Protein coding gene model for AtCat1.[19]

[19] http://www.arabidopsis.org/servlets/TairObject?id=30587&type=locus

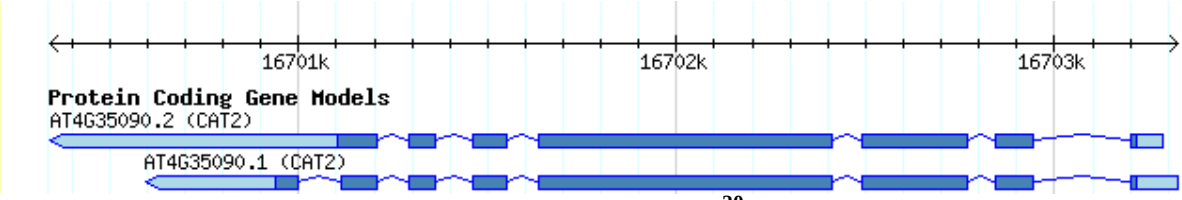


Figure 1.12: Protein coding gene model for AtCat2.[20] AT4G35090.1 is the original and AT4G35090.2 is the spliced version.

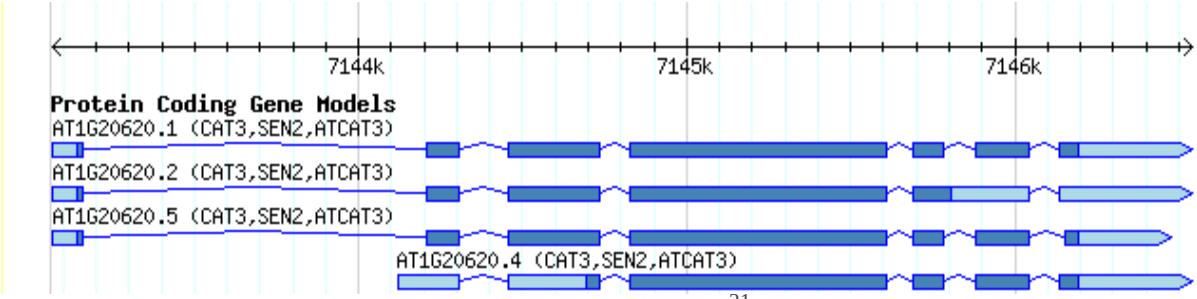


Figure 1.13: Protein coding gene model for AtCat3.[21] AT1G20620.1 is the original and AT1G20620.2, AT1G20620.4 and AT1G20620.5 are the spiced version.

Two pathways have been identified for entering proteins into peroxisomes. The most common one is called Peroxisomal Targeting Sequence 1 (PTS 1) and the other less common one is called Peroxisomal Targeting Sequence 2 (PTS 2) (Kaur *et. al.* 2009, Rauman *et. al.* 2007).The mechanism of which catalase is imported into peroxisomes still remains unclear, but experiments have shown that peroxisomes have a high catalase activity (Mullen *et. al.* 1997). PTS 1 are governed by a non-cleaved tripeptide sequence at the extreme c- terminus of the polypeptide (Kaur *et. al.* 2009, Rauman *et. al.* 2007). Figure 1.14 show PTS 1 and possible PTS 1 boxed in box 1, 2 and 3. The tripeptide S-R-L is a classic PTS 1 motif (Rauman *et. al.* 2007) and is found in AtCat2 and AtCat3. This can be seen in figure 1.14, box 1. In AtCat1 a similar tripeptide sequence can be seen, T-R-L. Although similar to the S-R-L tripeptide, T-R-L is not identified and confirmed to be a PTS1. These tripeptides are not localized at the extreme c – terminus, but at -7 to -9 upstream. The tripeptides in box 2 in figure 1.12 have not been confirmed to be PTS 1. Box 3, figure 1.14 show the sequence Q-K-L, this is not a PTS 1 tripeptide, but a study done on catalase from pumpkin suggest that Cat1 and the PTS 1 receptor protein Pex5p have an interaction. However this interaction was not confirmed to be a direct interaction (Kamigaki *et. al.* 2003).

[20] http://www.arabidopsis.org/servlets/TairObject?id=129011&type=locus
[21] http://www.arabidopsis.org/servlets/TairObject?id=30574&type=locus

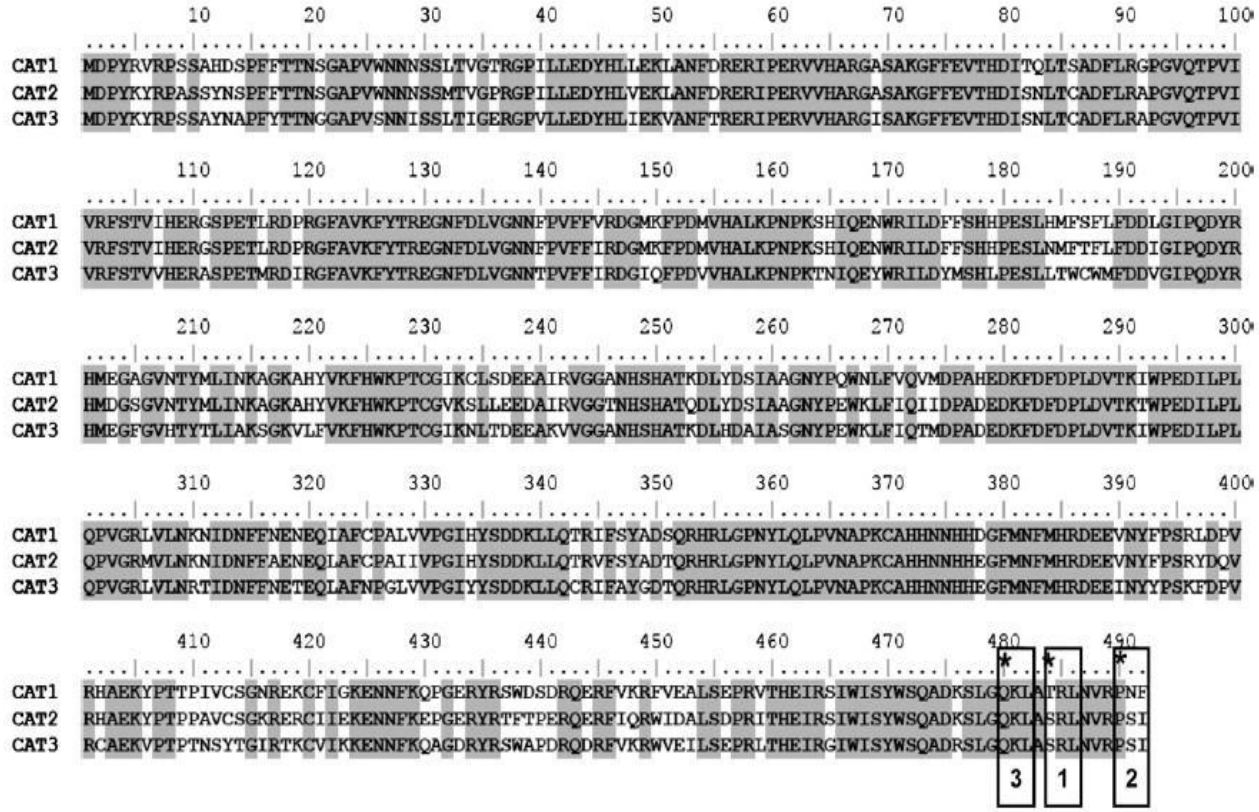


Figure 1.14: Alignment of AtCat1, AtCAt2 and AtCat3. (Mhamdi *et al* 2010**).** Possible proxisomal target sequences are boxed inn in box 1, 2 and 3.

Plant catalases have also been reported to be localized in cytosol and mitochondria (Mhamdi *et. al.* 2010). Highly purified mitochondria have been extracted from *Arabidopsis thaliana* and proteomic analyses have been used to identify AtCat2 and AtCat3 peptide sequence (Heazlewood *et. al.* 2004). Some studies have also suggested that catalase can be found in cytosol. Interaction between SOS2 (a class 3 sucrose – nonfermenting 1- related kinase) and AtCat2 and AtCat3 have been found (Verslues *et. al.* 2007). This suggesting that AtCat2 and AtCat3 function in the cytosol in addition to the H_2O_2 detoxification role in peroxisomes. This interaction was confirmed by TAP- tagged SOS2 – containing protein complex and yeast two-hybrid assays (Verslues *et. al.* 2007). Also interaction between NDK and AtCat1, AtCat2 and AtCat3 raise suspicion to believe that catalase can be found in cytosol (Fukamatsu *et. al.* 2003). Whether some cytosolic catalase activity is a result of incomplete import of catalase into peroxisomes is still unclear. Analyses have shown that some peroxisomal proteins can enter peroxisomes as performed oligomers or as monomers following oligomer disassembly (Kamigaki *et. al.* 2003). It might be possible that cytosolic active catalase might come from

assembled tetramers waiting to be imported to the peroxisomes. In this thesis localization and interaction analyses was done with AtCat2 and other different cytoslic proteins to try proving that catalase could be found active in the cytocol.

According to a classification made from the naming of tobacco genes (Willekens H. *et. al.* 1995) catalase can be divided into three classes. Class 1 is strongly expressed in photosynthetic tissue, class 2 in vascular tissue and class 3 is expressed in seeds and reproductive tissue. The three catalases in *A. thaliana* have all been placed in the different classes (Mhamdi *et. al.* 2010). AtCat1 is placed in class 3, expressed mainly in pollen and seeds. AtCat2 correspond to class 1 and are expressed in photosynthetic tissue, but also in roots and seeds. AtCat3 corresponds to class 2 and are associated with vascular tissue and also leafs. Catalase gene regulation and catalase activity control are a complex system that is not fully understood. AtCat2 is found to be under the circadian clock, with highest expression during light period (photosynthetic- type rhythm) (Zhong H. H. and McClung C. R. 1996). This correlates with the classification of AtCat2 in class 1 (expressed in photosynthetic tissue). AtCat3 is also found to be under the circadian clock, but in an opposite manner that AtCat2, AtCat3 expression is highest in the dark period (Zhong H. H. and McClung C. R. 1996).Research have shown that AtCat2 and AtCat3 activity per leaf decrease with the progression of senescence (Zimmermann P. *et. al.* 2006). Senescence is an orderly loss off normal cell function which is controlled by the nucleus. The decrease in AtCat2 and AtCat3 result in lowering the antioxidative capacity, and this might create a signal for the cells to promote senescence (Zimmermann P. *et. al.* 2006). The same study reveled that AtCat2 activity decreases at a very early stage in blotting time, but AtCat3 activity increased with plant age (Zimmermann P. *et. al.* 2006). In this thesis AtCat1, AtCat2 and AtCat3 levels was measured in plants of different age and also in different plant tissue, this to look at catalase levels.

1.2.5 *Catalase and ROS*

Although oxygen is needed for aerobe respiration, it can also be harmful for the organism. If oxygen is not controlled in the cell it can cause damage. Oxygen can easily convert to other reactive compounds, called reactive oxygen species (ROS). Among these species are H_2O_2, super oxide radicals, hydroxyl radicals and singlet oxygen. When the normal protection against ROS is not enough to protect the cell and ROS levels elevates, a major enzymatic defense kicks inn. Among this enzymatic defense are proteins called superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalas (CAT) (Imlay J.A. *et. al.* 2003, McKee T. and McKee J.R). Catalase is mainly attaching H_2O_2 and convert it into oxygen and water. This is shown in equation 1. Figure 1.8 shows how catalase is involved in the removal of ROS from the cell and figure 1.9 shows how catalase is believed to have a connection with DJ-1 and PD.

(eq.1) $$2H_2O_2 \longrightarrow 2\,H_2O + O_2$$

Three classes of proteins (unrelated on basis of sequence and structure) exhibit significant catalase activity. The most widespread group in nature is the heme- containing enzyme. This group of enzymes are subdivided based on having large (> 75 kD) or small (<60 kD) subunit (Klotz M.G. *et. al.* 1997). The second group is composed of bifunctional heme- containing catalase – peroxidase (Nicholls P. *et. al.* 2001). This group is closely related to plant catalase in both structure and function. The third class includes the non- heme or Mn- containing catalase (Nicholls P. *et. al.* 2001).

Bacteria can for example produce one or more catalases that usually respond to oxidative stress, either directly to the level of H_2O_2 or to the presence of other active oxygen species.

Monofunctional, heme –containing catalase have all in common a tow stage mechanism for degradation of H_2O_2. This can be seen in equation 2 and equation 3.

(eq.2) $$Enz(pro\text{-}Fe^{3}) + H_2O_2 \longrightarrow Cpd\ I\ (pro^{+}\text{-}\ Fe^{4}\text{-}O) + H_2O$$

(eq.3) $Cpd\ I\ (pro^{+}\text{-}\ Fe^{4}\text{-}O) + H_2O_2 \longrightarrow Enz(pro\text{-}Fe^{3}) + H_2O + O_2$

In the first step of the degradation, H_2O_2 oxidize the heme- group to an oxyferryl species in which one oxidation equivalent is removed from the iron and one from the porphyrin ring to generate a prophyrine cation reaction (Chelikani P. *et. al.* 2001). In step two, H_2O_2 is used to regenerate the enzyme to its original state, water and oxygen are produced.

In general the enzyme catalase does not follow the Michaelis - Menten kinetic (Chelikani P. *et. al.* 2001), except in very low substrate conditions. At high substrate levels different catalases are affected differently. This makes catalase to a relatively stable enzyme. Human catalase is divided into four different sub units. Each having their own active site buried deep within the subunit. A iron- ion is placed in the center of the dick shaped enzyme[22,23].

[22] http://www.ncbi.nlm.nih.gov/protein/NP_001743.1(viewed : 05/3-12)
[23] http://www.pdb.org/pdb/101/motm.do?momID=57 (viewed : 05/3-12)

2.0 Materials and methods

Research flow scheme of molecular cloning

Overview of molecular cloning and sub- cloning of DNA fragment from cDNA

Extract vector (plasmid with wanted properties) from glycerol stock and amplify vector.

↓

Digestion test (on vector) to verify that the right vector is amplified.

↓

Amplify DNA fragment of interest with PCR (with gene sp. primers) from cDNA.

↓

Run PCR product on agarose gel to verify fragment size and PCR specificity.

↓

Cut DNA fragment of interest from agarose gel and extract the fragment from gel.

↓

Digest DNA fragment and vector with compatible restriction enzymes. Clean up digestion system by running on an agarose gel and extracting from the agarose gel.

↓

Ligate DNA fragment and plasmid using DNA ligase.

↓

Transform the ligation into competent *E.coli* (DHα) cells and spread on LB plates containing appropriate antibiotic. Incubate plates on 37^{o}C.

↓

Look for positive colonies with PCR (vector sp. primers). Run PCR product on an agarose gel to verify fragment size and positive transformed colonies.

Amplify bacteria cells with positive transformed colony by making an overnight culture.

Extract plasmid from overnight culture.

Test extracted plasmid with PCR and digestion test, followed by run on agarose gel to verify size (PCR test) and size pattern (digestion test).

For final confirmation that the vector contains the DNA fragment of interest is done by sending it for sequencing.

Use plasmid with inserted DNA fragment of interest in intended experiment.

The gene can now also be sub- cloned into different vectors.

2.1 From RNA to DNA fragment of interest

2.1.1 *RNA extraction*

Wild type (WT) seeds was planted and grown, RNA was extracted from plans of different age and different plant tissue to measured AtCat2 levels in the different plant materials.

Total RNA purification from tissue with NucleoSpin® RNA XS

Plant tissue from WT *A.thaliana* was collected and about 50 mg seedlings was grinded with N_2 (l) into a fine powder using a mortar and pestle. The liquid nitrogen evaporated and the tissue powder was transferred to a microsentrifuge tube. The tubes with sample were put on ice. Cell lysis and further homogenization was done by adding RA1 buffer (200µl) and TCEP (4 µl). The sample was mixed by pipetting up and down. Carrier RNA working solution (5 µl, equals 20 ng) was added to the sample and mixed by vortexing for 10 sec. The tube was briefly centrifuged to clear the lid. Sample was added to a NucleoSpin filter column (violet ring) and placed in a collection tube. The sample was centrifuged for 30 sec. at 11 000 rpm. Ethanol (200 µl, 70% EtOH) was added to the sample by pipetting up and down. This was done to adjust the binding conditions of the RNA. The sample was added to a NucleoSpin RNA XS column (light blue ring) and centrifuged for 30 sec. at 11 000rpm. Membrane desalting buffer (MDB 100µl) was added and centrifuged again for 30 sec. at 11 000 rpm. To digest DNA that might be in the sample a DNA reaction mixture was prepared by adding rDNase (3 µl) to reaction buffer of rDNase (27 µl). DNase reaction mixture (25µl) was applied to the center of the silica membrane and incubated at room T°C for 15 min. to digest DNA in the sample. First washing step was done by adding buffer RA2 (200 µl), incubated for 2 min and centrifuged. Second washing step was done by adding buffer RA3 (400 µl) to the column and centrifuged for 30 sec. at 11 000 rpm. Third washing step was done by adding buffer RA3 (200 µl) to the column and centrifuged for 2 min. at 11 000 rpm. RNA was eluted with RNase- free H_2O (10 µl).

2.1.2 RNA quantification

RNA quantification was a procedure done to determine the concentration of the RNA. This was done with northern blot and spectrophotometry. For northern blot the RNA sample was run on a gel made of 1xMOPS buffer. Before loading the RNA sample, it was treated with DL buffer (5 µl RNA sample and 5 µl DL buffer) and put on 65^0C for 15 min. After heating the sample it was put on ice. Running buffer used for RNA was 1xMOPS.
For spectrophotometry RNA concentration is measured at 260 nm and at 280 nm. By calculating the ratio between these wavelengths the RNA concentration can be measured. When A_{260}/A_{280} is calculated to bee 1,8 – 2 the quality of the RNA is good.

2.1.3 Reverse transcription

Reverse transcription was conducted to convert RNA into cDNA. cDNA was then used further in the polymerase chain reaction. Figure 2.1 show the principle of RT-PCR. An oligoprimer binds to the single stranded mRNA and through RT-PCR polymerase binds to the primer and amplifies a single stranded DNA. This is made double stranded by a new amplification. This is shown in figure 2.1. The result is a double stranded DNA molecule that can be used further in the PCR reaction.

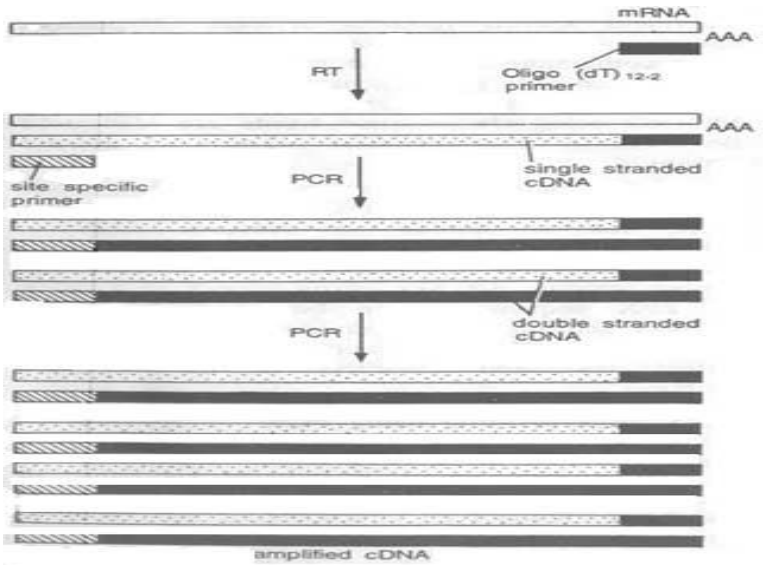


Figure 2.1: RT-PCR technique.[24] Also called cDNA synthesis. Converts mRNA to cDNA with the help of RT-PCR and oligoprimers.

[24] http://www.studentsguide.in/biotechnology-genomics/polymers-chain-reaction-PCR-gene-amplification/images/different-steps-involved-in-rt-pcr.jpg

To make cDNA intended for cloning, a greater volume of RNA was used (because the RNA concentration was lower than expected.). For RT-PCR on RNA extracted from plant of different age and different plant tissue, calculations was done (see section 3.7) to make cDNA of a final concentration of 5 µg/ml (ideally for real- time PCR). RNA (2µl) and random olgonucleotid primers (0,5 µl) was mixed. The PCR machine was used to heat the RNA/primer mix to 70 ^{0}C for 5- 10 min. The RNA mix was than putted on ice and after 5 min M-MLV 5xbuffer (5 µl), dNTP 10 mM (5 µl) and 200 units M-MLVRT (1µl =200 units) was added. The sample was heated again for 2 hr on 42^{o}C, resulting in RNA converted into cDNA.

2.1.4 Real time PCR

Primer for ubiquitine, AtCat1, AtCat2 and AtCat3 was ordered from Primerdesign[25]. These primers was pretested and optimized and mixed in a primer mix. A 97 well real-time PCR plate was used. A master mix was made for all of the four primer sets. The master mix consisted of primer mix (1,25), SYBRgreen (12,5), water (10,25) per reaction. Master mix was added into each well (24µl) and cDNA (1µl of 5 µl/ml cDNA). 25 cycles was used in the real time PCR run.

2.1.5 DNA extraction

DNA isolation was performed on mutant plants to determine through PCR correct tDNA insertion. For DNA isolation E.Z.N.A ^{TM} Plant DNA Mini Kit was used.

Plant DNA Mini Kit Procedure.

Plant tissue from WT *A. thaliana* was collected (3-4 week old leaf, 100 mg) and grinded with N_2 (l) into a fine powder using a mortar and pestle. Buffer P1 (600µl) was immediately added. The tube was incubated at 65^{o}C for 10 min. The sample was mixed twice during incubation. Buffer P2 (140µl) was added, mixed by vortexing and centrifuged at 10 000 for 10 min. The flow through was added to a new tube, isopropanol (0,7 volume) was added and mixed by vortexing. The sample was then centrifuged for 2 min. at 14 000 rpm to pallet the DNA. The supernatant was thrown away and sterile deionized water (300 µl), pre-heated to 65°C was added and mixed by vortexing. Buffer P3 (150 µl) and absolute EtOH (300µl) was added to

[25] PrimerDesign Ltd, The Miller Yard,Rowahams; Southampton; SO160AJ, UK

adjust the binding conditions and mixed by vortexing. The sample was transferred to a HiBind DNA column and centrifuged for 1 min. at 10 000rpm. The column was then transferred to a new collection tube and washed with wash buffer (650 µl) and centrifuged for 1 min. at 10 000 rpm. Wash step was repeated. To dry the silica membrane the column was centrifuged at max speed in 2 min. The column was transferred to a clean tube and elution buffer (50- 100 µl, pre-heated to 65°) was added and centrifuged for1 min at 10 000 rpm. Elution step was repeated in a new clean tube. This was preformed to maintain a higher DNA concentration than the first elution.

DNA concentration was measured on spectrophotometer and on NanoDrop 2000. (See section 3.9 for DNA calculations).

2.1.6 Polymerase chain reaction (PCR)

Through the PCR process the DNA fragment of interest was amplified and copied up in large amounts and used further in the cloning procedure. The PCR product was run on a 1% agarose gel to visually see the fragment and to determine the fragment size. Table 2.1 show a typically PCR system for both pow polymerase and taq polymerase.

Table 2.1: Polymerase chain reaction (PCR) system. This was a standard PCR system and individual alterations were sometimes made for cretin PCR runes.

	Pow- poly. system	**Taq-poly. system**
Primer- L	1 µl	1 µl
Primer- R	1 µl	1 µl
PCR buffer	3 µl (Pow buff. incl. dNTP)	2 µl(buffer Nx10)
$MgCl_2$	1 µl	1 µl
dNTP	-	1 µl
PCR enzyme (pow or taq polymerase)	Pow 0,2 µl (Taq 0,8 µl)	Taq 0,5 -1 µl
Template	cDNA 1-2 µl	1 µl
dH_2O	Up till 20 µl	Up till 20 µl

For colony PCR a taq polymerase system was used with 1 µl taq polymerase. Bacteria were used as template.
Simple PCR to confirm insertion of gene into vector was also done with taq polymerase system. Simple PCR was PCR done on overnight culture and PCR on purified plasmid.

For gene amplification PCR a pow polymerase system was used. cDNA was used as template (usually 1 µl cDNA was used.)

Custom PCR program was designed with respect to the different genes and different TM values of the primers. For amplification of gene fragment a PCR program with 20 cycles was used. For colony PCR or standard PCR 25 – 30 cycles was used. Table 2.2 show a standard PCR program.

Table 2.2: Standard PCR program. This standard PCR program was in some PCR runes altered to better suit the individual runes.

Temp (°C)	**Time (min)**
95	5:00
95	0:30
55	0:30
72	2:30 – 0:30
Go to step 2 for 20-30 times	
72	4:00
4	Forever

2.1.6.1 Pow DNA Polymerase

Pow is a type of DNA polymerase that was used for high fidelity amplification. Pow DNA polymerase was originally isolated from hyperthermophilic archaebacterium, *Pyrococcus woesei*[26]. Pow polymerase had a very effective 5` → 3` polymerase reading and it also had a effective 3`→ 5` exonuclease activity/proofreading activity. This was the reason this polymerase was chosen for gene amplification. Pow polymerase was easily degraded and sensitive for temperature and therefore while working with this polymerase conditions was always cool (on ice, 0^0C.).

2.1.6.2 Taq DNA polymerase

Taq DNA polymerase catalyzes the incorporation of dNTPs into DNA. It requires a DNA template, primer, and the divalent cation Mg^{2+}. *Taq* Polymerase contains a polymerization dependent 5'→3' activity. It does not have a 3'→5' exonuclease activity. In this thesis homemade taq polymerase was used.

2.1.7 Agarose gel and gel electrophoresis

After PCR amplification the PCR product was checked by running on a 1% agarose gel. Loading dye containing gel red was mixed with the PCR product and loaded in the wells. Agarose gel was made by mixing agarose powder with 1xTAE buffer in a 1 g: 100 ml ratio. The mix was heated in the microwave oven until the agarose powder was completely dissolved. The liquid gel was poured into a mould and left to stiffening. The gel was pleased in a chamber containing 1xTAE buffer. The sample (containing loading dye and gel red) was pipetted into the wells and electricity was applied to the chamber. After gel electrophoresis the gel was placed under UV-light and bands could be seen. A ladder was run along the PCR samples to keep track of the size of the PCR product. In this thesis the ladder GeneRuler1 kb was used. See figure 2.2 for GeneRuler map. Using the marker together with the sample it was possible to determine the size and also the concentration of the DNA sample.

[26] https://www.roche-applied-science.com/pack-insert/4340868a.pdf

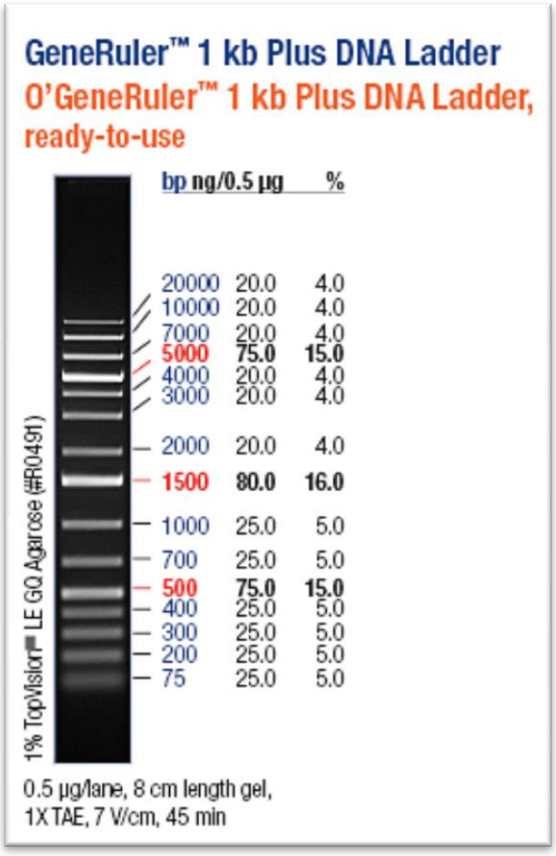


Figure 2.2: Map of GeneRuler 1 kb plus DNA ladder.
This was the DNA marker of choice in this thesis Normally 2 -5 µl DNA ladder was used for each electrophoresis run.

2.2 Molecular cloning

Extracted fragments of cDNA were ligated into different vectors containing different properties. The vector containing the insert was then transformed into bacteria cells and with the use of antibiotic selective media successfully transformed cells was localized. Colony PCR, standard PCR on overnight cultures and PCR on purified plasmid, as well as digestion tests were done to make sure that the vector contained the gene of interest. Final proof of correct insertion was done by sending a sample of purified plasmid containing the gene for sequencing. In this thesis AtCat1, AtCat2 and AtCat3 was cloned into different vectors using different properties. (See table 2.3 for details information on vector properties.)

Table 2.3: Vectors with different properties. Show the different R.E. for the different genes, different cofactors, buffer and appropriate antibiotic for the different vectors that was used in the thesis.

	Vector property	**Restriction sites for AtCat1**	**Restriction sites for AtCat2**	**Restriction sites for AtCat3**	**Cofactor**	**Buffer**	**Antibiotic resistance**
pPCR-script	Storage	SmaI	SmaI	EcorV	-	-	Cp
pWEN-18	Fluorescent	XhoI / KpnI	XhoI / KpnI	XhoI / KpnI	BSA	Buffer 1	Amp
pWEN-18-NY	Fluorescent (half YFP)	XhoI / KpnI	XhoI / KpnI	XhoI / KpnI	BSA	Buffer 1	Amp
pWEN-18-CY	Fluorescent (half YFP)	XhoI / KpnI	XhoI / KpnI	XhoI / KpnI	BSA	Buffer 1	Amp
pWEN-25	Fluorescent	SalI / KpnI	SalI / KpnI	SalI / KpnI	BSA	Dobble digestion	Amp
pBA002	Over expression	PacI / AscI	PacI / AscI	PacI / AscI			Bact:Spec Plant:Basta

Different restriction sites were used for the different vectors. A restriction enzyme (R.E) is an enzyme that was used to locate a DNA sequence either double or single and cut the sequence. The sequence that was cut was called the restriction site (R.S.). The different vectors had different multiple cloning sites (M.C.S.) (see appendix for vector maps.) and therefore different R.E had to be used for different vectors (see table 2.3). It is also important to select R.S. for cloning that does not cut the gene itself. Information on R.E was found on the web cutter page[27] or on the web site restriction-mapper[28]. For cloning into pPCR– script blunt end ligation was used. For the other vectors different techniques was used. Information on cofactors, buffer and reaction time was found on New England Biolabs[29](see table 2.3). For cloning of AtCat1 into the pWEN vectors some problems came about. The R.E XhoI cut inside of the gene and therefore XhoI could not be used for this gene. The only problem was that the only two restriction sites possible to use for pWEN 18, pWEN 18-NY and pWEN 18- CY is XhoI and KpnI (pluss Acc65I). Two different approaches was attempted to see which one gave a positive insertion of AtCat1 into these vectors. One approach was to digest the gene and the vector with KpnI and after SAP treatment of the vector do ligation. SAP was an enzyme that dephosphorylated the ends of the vector DNA, making it difficult for relegation to happen. The other approach was to digest the gene with SalI and KpnI and the vector with XhoI and KpnI. This was a better suggestion since SalI and XhoI was compatible R.E and therefore could be ligated together. Information on R.E and their cutting site can be seen in table 2.4.

[27] http://bio.lundberg.gu.se/cutter2/
[28] http://www.restrictionmapper.org/
[29] http://www.neb.com/nebecomm/tech_reference/default.asp

Table 2.4: Different restriction enzymes used and their cutting site.[30]Cutting site is marked with an arrow above and below the sequence, leaving an overhang. R.E. SmaI did not leave an overhang.

Restriction enzyme(R.S)	Sequence	Restriction enzyme(R.S)	Sequence
XbaI	T T A A T T A A A A T T A A T T	**KpnI**	G G T A C C C C A T G G
AscI	G G C G C G C C C C G C G C G G	**XhoI**	C T C G A G G A G C T C
SalI	G T C G A C C A G C T C	**SmaI**	C C C G G G G G G C C C

2.2.1 Blunt end cloning

Blunt end cloning was done for cloning into pPCR script cam Sk+[31]. Blunt end mean that the double stranded DNA is cut right off with no overhang. In this thesis SmaI and EcorV was used for cloning AtCat1, AtCat2 and AtCat3 into pPCR script. When blunt end R.E was used, the reaction cannot go back. When the blunt end restriction site was cut and the gene was inserted, the R.S was destroyed and therefore R.E in the ligation could not cut the site again. Se figure 2.3 for illustration on blunt end cloning.

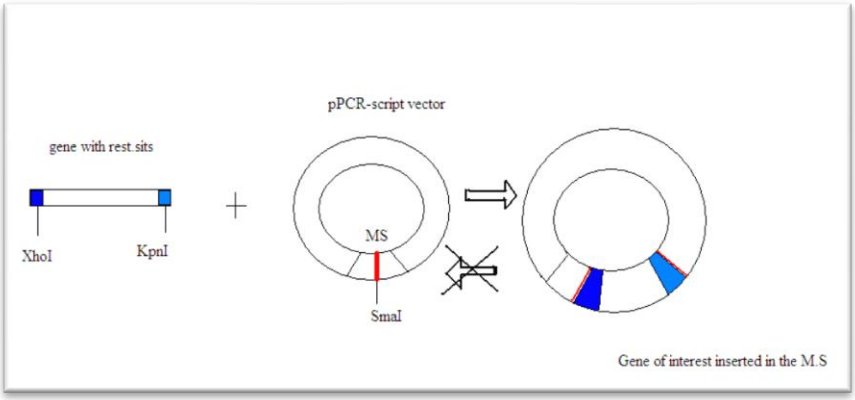


Figure 2.3: The figure show a simplified version of blunt end cloning. R.E .was destroyed in the cloning process, preventing the relegation of the vector.

[30] http://www.neb.com/nebecomm/ (http://www.neb.com/nebecomm/products/category1.asp?#2)
[31] PCR-script Cam Cloning Kit. Instruction Manual, catalog #211192 (25 reaction) revisjon B. Agilent Technologies

2.2.1.1 Procedure for cloning into pPCR- script

The gene of interest was amplified with pow polymerase, cDNA was used as template (1-2µl) and 20 cycles was used in PCR. All the PCR product was then run on a 1% agarose gel and the bands were cut out and purified (see section 2.2.2.1). The gene was ligated with pPCR script (see table 2.5) and left on the bench over night. All of the ligation was transformed into competent bacteria cells (see section 2.2.2.4) and spread on LB plates containing 40 µl of Xgal and antibiotic chlorophenicol (cp). The plates were incubated at 37° C over night. Positive colonies (white colonies) were confirmed to be positive with colony PCR done with vector specific primers (T7and T3 TM for these primers was 50- 55 °C and 25 cycles was used). An overnight culture was made (10µl cp+ 10 ml liquid LB media +some positive colony) and incubated on shacking bed at 37 °C over night. The following day mini-prep (see section 2.2.2.6) was done on the culture to get the plasmids out of the bacteria cells. A digestion test was done to be sure of insertion of gene into the vector. See table 2.5 for blunt end ligation system.

Table 2.5 : Blunt end ligation system (10µl system).

pPCR-script cam- cloning vector (10 ng/µl)	**1 µl**
pPCR-script 10xreaction buffer	1 µl
10nM aATP	0,5 µl
Blunt end PCR product	2-6 µl
Blunt end reaction enzyme (5 U/µl)	0,5 µl
T4 DNA ligase (4 U/µl)	1 µl
dH_2O up till 10 µl	

2.2.2 Cloning with vector and DNA fragment containing overhang.

Cloning of fragments/vector with overhang was preformed when cloning into pWEN vectors (see table 2.3.) The sequence of the overhang depends on the R.E used (see table 2.4). Fist the DNA fragment of interest was amplified, purified and then both of the vector and the DNA fragment were digested with compatible restriction enzymes. Then they were ligated

together and transformed into competent cells. From positive colonies an overnight culture was made and plasmids were extracted from the bacteria cells.

2.2.2.1 Amplification of DNA fragment of interest and clean up of PCR product from gel extraction.

Amplification was done with pow polymerase PCR system (Described in section 2.1.6 and 2.1.6.1). Clean up of PCR product was conducted by running all of the product (20µl) on a 1% agarose gel and extracting it from the gel using the kit NucleoSpin® Extract II [32]. The agarose gel was placed under a UV- light and the bonds were cut out of the gel. The kit was not good with small volumes and if the weight (of the bond that was cut out) was less than 100 mg, water was added. Buffer NT (200 µl for every 100 mg of gel) was added. The microsentrifuge tube was put on heating block until the gel was completely dissolved. Every 2 -3 min. the tube was vortexed to mix the gel and the buffer. The melted gel was transferred to a NucleoSpin column and centrifuge for 1 min. at 41000rpm. The flow through was thrown away and buffer NT3 (600 µl) was added and centrifuge at 41000rpm, the flow through was thrown away. The tubes was centrifuge again for 2 min., this to make the filter dry. Buffer NE (15 -50 µl) was added to free the DNA from the filter and the DNA was collected in a microsentrifuge tube.

2.2.2.2 Digestion

In this step the gene or vector were digested with the R.E chosen. Digestion left the gene and the vector with sticky ends (an overhang). Digestion was done in water bath at 37°C for 2-3 hr, depending on the R.E. After digestion the R.E was removed from the digestion solution by running on a 1 % agarose gel. The bands for the vector and the gene were cut out and purified (described in section 2.2.2.1). Table 2.6 show the digestion system used.

[32] http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/DNA%20clean-up/UM_PCRcleanup_Gelex_NSExII.pdf NucleoSpin® Extract II, User Manual, Protocol for DNA extraction from agarose gels p. 15-16.

Table 2.6: Digestion system for vector and DNA fragment. This system was sometimes altered to make each individual digestion optimal.

	50µl system	**100µl system**
Vector or gene	30 µl	50 µl
dH_2O	13µl	35 µl
Buffer	4.5 µl	10 µl
BSA	0.5 µl	1 µl
Restriction enzyme A	1 µl	2 µl
Restriction enzyme B	1 µl	2 µl

2.2.2.3 Ligation

In the ligation step the gene fragment and the vector came together and DNA ligase ligated the fragments together. In this thesis T4 ligase was used. This relation between volume gene and volume vector has to be calculated by quantify the concentration of both the gene and the vector, this was done with help of equation 4. The ligation was stored at room temperature overnight. Table 2.7 show an example of a ligation system.

Table 2.7: Ligation system. This was just an example of a ligation system. The concentration of the gene and the vector had to be taken in consideration and calculation had to be conducted to know how much of the gene and how much of the vector that would make the optimal ligation.

Vector	**1µl**
Gene	6µl
T4 ligase buffer	2µl
T4 ligase	1µl

(eq. 4) $$\frac{(\mu l\ of\ vector)\times(bp\ of\ insert)}{bp\ of\ vector} = \mu g\ of\ insert$$

Equation 4 gave the 1:1 ratio between vector and insert. In this thesis 1:3/ 1:6 / 1:8 ratio was normally used.

2.2.2.4 Transformation

In the transformation the ligation was mixed with competent cells in the hope that some of the bacteria cells take up the vector carrying the gene. Colony PCR was done to confirm positive colonies. This colony was further used for up cultivation.

Transformation procedure.

In the transformation process the vector containing the DNA fragment of interest was transported into the competent bacteria cells. In this thesis both commercial made competent cells and homemade cells was used. The comp. cells that was homemade was of lower quality then the commercial comp. cells. This meant that the procedure was a little bit different. With regard to the homemade competent cells not being of super quality, longer time was used on some steps in the transformation.

The competent cells were defrosted on ice (10 min.) and the ligation was also put on ice to obtain the same temperature as the comp. cells. Ligation (5 µl) was mixed with comp. cells (50 µl) and left on ice (10-20 min.). After resting on ice the transformation mix was put in water –bath at 42 °C for 1-1,30min. After heat shock it was put directly on ice for (5-10 min). LB media was added (800µl) and the tube was placed on shacking at 37°C for 1-2 hours. To collect all the bacteria cells after shacking the tube was centrifuged for 5 min at 5000rpm. The supernatant was thrown away and the pallet was resuspended in 100 µl LB medium and plated on petri dishes containing LB media (with appropriate antibiotic).

2.2.2.5 Overnight cultures

Overnight cultures were made with LB medium and the antibiotic specific for the vector. They were incubated at 37°C until the following day. For appropriate antibiotic for the different vectors see table 2.3.

2.2.2.6 Plasmid extraction and preparation

Plasmid was extracted from overnight culture with the help of the kit NucleoSpin Plasmid[33]. For extracting plasmid the overnight culture was centrifuged to recover the bacteria cells.

[33] http://www.mn-et.com/Portals/8/attachments/Redakteure_Bio/Protocols/Plasmid%20DNA%20Purification/UM_pDNA_NS.pdf

From the pellet of 1,5 ml bacteria culture, buffer A1(250 µl) was added and mixed by pipetteing in and out. Buffer A2 (250 µl) was added and mixed gently. When the lysate appeared clear buffer A3 (300 µl) was added and mixed gently. The lysate was centrifuged for 5 min at 14`000 rpm. The supernatant was transferred to a NucleoSpin Plasmid column and centrifuged for 1 min. at 14`000 rpm. This step ensures binding of the DNA (plasmid). Washing was done by adding buffer A4 (600 µl) and centrifuged for 1 min. at 14`000 rpm. The flow through was thrown away and the column was centrifuged in 2 min. at 14`000 rpm, to dray the filter. Elution was done by adding elution Buffer AE (50 µl). The purified plasmid was stores at -20 ^{0}C.
Plasmid concentration was measured with NanoDrop 200 and by running on a 1% agarose gel.

NucleoSpin Plasmid kit User Manual: NucleoSpin® Plasmi: Isolation of high-copy plasmid DNA from *E. coli*(p 13-14.)

2.3 Preparation and transformation of component cell using calcium chloride ($CaCl_2$)

When cells were made component the ability to take up extracellular DNA from the environment increased. This was necessary to do with the cells (*E.coli*, DHα strain) that were used in the transformation process.

From an overnight culture (5 ml) of DHα cells ,1 ml was transferred to an 1000 ml – Erlendmeyer- flask containing LB medium (200ml)(the medium is already heated to 37 °C when the culture is added.). This was done in four flasks. All of the four flasks were put in a incubator (shaker at 37°C until the OD_{600} is 0,5 – 0,7). The flasks were put on ice (15 min). To collect all of the bacteria cells the culture was centrifuged (200 ml in each tube) for 10 min at 4000rpm/ 4°C. The supernatant was removed. The pallet was resuspended in ice cold $MgCl_2$ (50 ml, 0,1 M), then incubated on ice (20 min.) and centrifuged for 10 min at 4000 rpm/4°C. Again the supernatant was removed. The pallet was resuspended in ice cold $CaCl_2$ (20 ml, 0,1 M), then incubated on ice for 30 min and centrifuged for 10 min at 4000 rpm/4°C. The supernatant was removed and the pallet was resuspended in MOPS glycerol (4 ml). The resuspended pallet was aliquot in eppendorf tubes (100 µl) and put immediately into liquid nitrogen. The competent cells were stored at – 80°C until use.

2.4 Introduction of DNA/ gold- mix to plant tissue

The DNA was shoot into onion cells and also into tobacco cells. Tobacco plants was grown for 20- 30 days in 1,5% MS containing myo-inositol (See table 2.9). A leaf was cut off and placed on a petri dish containing MS media. The plates were kept dark. For onion one layer was pleased in an empty petri dish and DNA was shoot into the onion layer.

Before the DNA could be shot into the plant tissue the DNA had to be attached to gold particles. Gold (35 mg, 0,6 µm) was first suspended in absolute ethanol (1,5 ml) and vortexed in 2-3 min. The tube was centrifuged at 11`000 rpm. for 5 sec. and the supernatant consisting of ethanol was through away and the gold pellet was washed 3 times with ethanol, between each wash the gold was centrifuged at 2000 rpm. for 5 sec. After washing, the pellet was resuspended in ethanol (1 ml) and stored in the freezer at -20^0C for further use.
Gold (50 µl of gold stock) was wash in ethanol tree times. Each time centrifuge at 6200 rpm in 2 sec. The pallet was then washed in sterile ice cold water tree times. The gold pallet was then resuspended in ice cold sterile water (50 µl). The gold/H_2O stock was aliquot in a 50 µl eppendorf tube and DNA (10µl, 1 µg/µl) was added and mixed by pipetteing. $CaCl_2$ (50µl, 2,5 M) and spermidine free base (20µl, 0,1 M) were added to the DNA/gold – mix and vortexed in 30 min. in cold room to mix them completely. Absolute ethanol (200µl) was added and centrifuged at 6200 rpm. in 2 sec. The supernatant was thrown away and the sample was washed with cold ethanol four times. The DNA/gold pellet was resuspended in absolute ethanol (30µl) and stored on ice until shooting.

For combination shooting (CY and NY - plasmids), the two vectors was mixed good before added to the gold. The combination of the vectors can be seen in table 2.8.

Table 2.8: Combinations of vectors for shooting into plant tissue.

pWEN-18-CY-AtCat2	pWEN-18-NY-CSD1*
pWEN-18-CY-AtCat2	pWEN-18-NY-GPX2*
pWEN-18-CY-AtCat2	pWEN-18-NY-DJ1*
pWEN-18-NY-AtCat2	pWEN-18-CY-AtCat2
pWEN-18-NY-AtCat2	pWEN-18-CY- CSD1*
pWEN-18-NY-AtCat2	pWEN-18-CY- GPX2*
pWEN-18-NY-AtCat2	pWEN-18-CY- DJ1*
pWEN-18-AtCat2	
pWEN-25-AtCat2	

*Vectors provided by Dr. Xiang Ming Xu

2.4.1 Biolistic gun

The biolistic gun (also called a gene gun) was used to shoot gold/DNA mix into plant leaf and onion layer. Figure 2.4 show how the gene gun works and figure 2.5 shows a photo of the gene gun used in this thesis.

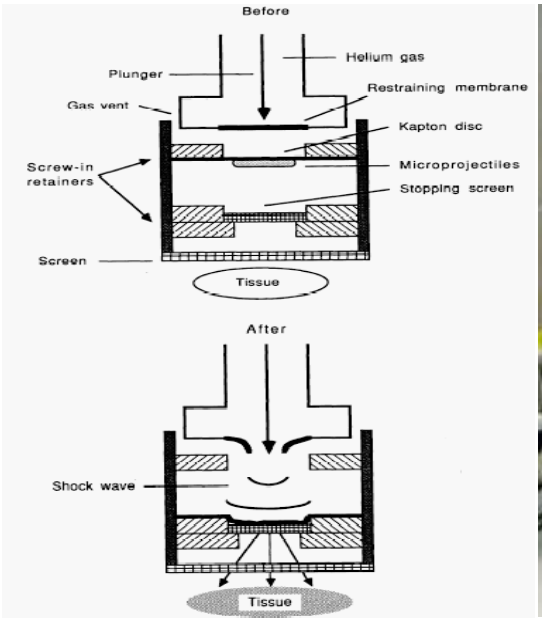


Figure 2.4: The biolistic gun (gene gun)[34]

Figure 2.5: The biolistic gun, (Similar to the one used in this thesis)[35]

[34] http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/McDonald/Gene_gun.html

2.5 Plant transformation

2.5.1 Cloning into vector pBA002

Gene fragment and fluorescent part of pWEN-25 was cut out using restriction enzymes PacI and AscI (see section 2.2 Molecular cloning and section 2.2.2.2 Digestion.). pBA002 was also digested with PacI and AscI. Digestion systems were run on a gel and DNA fragment and vector was cut from the gel and extracted (as described in section 2.2.2.1). Ligation was done according to description in section 2.2.2.3. The ligation was transformed and colony PCR was preformed to identify colonies with successful insertion. From positive colony an overnight culture was made and the following day the plasmids was extracted and prepared (see section 2.2.2.6.) For vector pBA002 transformed into *E. coli* the appropriate antibiotic to use was spectromycine.

2.5.2 Sterilizing and planting seeds

Seeds were sterilized and planted on petri dishes containing MS (see table 2.9 for recipe). After 2 weeks the seedling was transformed to soil and cultivated in a 16hr. / 8 hr. light/dark cycle in soil for 4-5 weeks, followed by plant transformation.

Table 2.9: Murashige and Skoog medium (MS medium).

Chemical	Amount	Optional	
MS salt	4,3 g/L		pH is adjusted till 5,8
Sucrose	10 g/l (MS 1%)	15 g/L (MS15%)	
MicroAgar	8 g/l		Blended into the MS/suc.
Myo-Inositol		1 ml/L	

Seed sterilization procedure

Seeds was placed in an eppendorf tube and washed with 70% EtOH and 0,05% Triton X-100 (vortexed 10 min.). The EtOH was thrown away and replaced with 100% EtOH (vortexed 5min). This step was repeated. The seeds were placed on a per-sterilized (with EtOH) filter in

[35] http://www.absoluteastronomy.com/topics/Gene_gun

the sterile hood. After the EtOH had evaporated the seeds were transferred to a sterile eppendorf tube for storage.

2.5.3 Plant transformation with agro bacteria

Plasmid (1 µl pBA002 containing fragment of interest) was added to agro bacteria (50 µl) and incubated for 5 min at 37°C. LB medium (1ml) was added and incubated for 2 – 4 hr at 28°C. After incubation the tube was centrifuged for 30 sec. to recover the bacteria cells. The cell pallet was resuspended in LB media (0,1ml) and spread on plated containing scectromycine. The plates were incubated at 28°C for 2 days. Colony PCR was conducted to confirm positive insertion of plasmid into bacteria. Several positive colonies was picked and resuspended in LB media (1 ml). Resuspended culture was divides on two flasks containing LB media (150 ml) and incubated overnight at 28°C. The following day the cultures was divided into four centrifuge flasks and centrifuged for 10 min. at 4 °C on 4000-5000 rpm. The cell pallet was suspended in infiltration media (see table 2.10). Plants were dipped in the resuspended cells for 9 min and placed (lying down) in a plastic tray covered with plastic for two days. The plants was transformed to the growth room and grown to seeds could be harvested. The seeds were sown on MS containing the antibiotic basta. A successfully transformed plant would grow on this antibiotic, thereby confirming the positive transformation. Since the YFP part of the plasmid also was ligated into the pBA002 vector it was possible to check positive plant in the microscope.

Table 2.10: Infiltration medium

Chemical	**Amount**
Sucrose	50 g/L
$McCl_2$ (Fw = 95,2g/mol)	2,03 g/L
Syvett	50µl

2.6 Knock out mutant cultivation

Knock out AtCat2 mutant was ordered (see table 2.11) and the seeds was sterilized (like described in section 2.5.2) and planted on MS media containing the appropriate antibiotic.

Table 2.11: Description of mutant seeds ordered.

	NASC code	Mutant name	Antibiotic resista.
Knock out mut. 1	N556998	SALK_076998	Kan
Knock out mut. 2	N557998	SALK_057998	Kan
Knock out mut. 3	N839249	SAIL_872_H06	PPT

Just in case the antibiotic resistance had been lost (can happen in SALK mutant lines) the seeds were also planted on MS plates that did not contain any antibiotic. The seeds were cultivated on the different MS plates for 2 weeks in the green house (16/ 8 hr. light / dark cycle). Then transferred to soil and further cultivated in the green house until no more flower developed. In week 4 one leaf from each plant was collected and DNA tested to see if the plant was a true knock out mutant. The plants were put in a room with normal light for drying. Seeds were collected for further use.

3.0 Results

3.1 Primer design

For designing primers the DNA sequence was first retrieved from NCBI. See figure 3.1 for DNA sequence of AtCat2.

```
ATGGATCCTTACAAGTATCGTCCAGCTAGTTCTTACAACTCTCCCTTCTTCACCACCAACTCTGGTGCTCCTGTATGGAACAACAAC
TCCTCCATGACCGTTGGACCCAGAGGTCCTATCCTTCTTGAGGATTACCATCTCGTTGAGAAGCTTGCCAATTTCGACAGGGAACGG
ATTCCAGAGCGTGTGGTTCATGCCAGAGGAGCCAGTGCTAAAGGTTTCTTTGAGGTCACTCATGATATCTCTAACCTCACTTGTGCT
GACTTTCTCCGAGCTCCCGGTGTTCAGACTCCTGTCATTGTCCGGTTCTCCACCGTTATCCATGAGCGTGGAAGTCCCGAGACCTTG
AGAGACCCTCGTGGTTTTGCAGTCAAGTTCTACACCAGAGAGGGGAACTTTGATCTTGTTGGAAACAACTTTCCTGTTTTCTTCATC
CGCGATGGGATGAAGTTCCCTGACATGGTCCACGCTCTTAAGCCGAACCCAAAATCTCACATCCAAGAGAACTGGAGAATCCTTGAC
TTCTTCTCCCACCACCCTGAAAGTTTGAACATGTTCACTTTCCTCTTCGATGATATCGGTATCCCACAAGATTACAGGCACATGGAT
GGTTCAGGTGTCAATACATACATGTTGATCAACAAAGCTGGCAAAGCTCACTACGTGAAGTTCCATTGGAAACCAACTTGTGGAGTC
AAGTCTCTTTTGGAAGAAGATGCAATTCGTGTTGGAGGAACCAACCACAGTCATGCGACTCAAGACTTGTATGACTCTATAGCTGCT
GGAAACTACCCTGAATGGAAGCTCTTTATCCAAATCATTGATCCTGCTGATGAAGACAAGTTCGACTTTGACCCGCTCGATGTGACC
AAGACCTGGCCTGAGGATATCTTGCCTCTTCAACCTGTTGGACGTATGGTGTTGAACAAGAACATTGACAACTTCTTTGCAGAGAAT
GAGCAACTTGCTTTCTGTCCTGCAATTATTGTCCCAGGGATACATTACTCAGATGATAAGCTGCTTCAAACCCGTGTCTTCTCCTAT
GCCGATACTCAGAGACACCGTCTTGGACCAAACTACCTTCAGCTGCCAGTCAATGCTCCAAAATGTGCTCACCACAACAACCACCAT
GAGGGATTCATGAATTTCATGCACAGGGACGAGGAGGTTAACTACTTCCCGTCGAGGTATGACCAGGTTCGTCATGCTGAGAAGTAT
CCAACTCCGCCTGCTGTCTGTTCTGGAAAACGTGAGAGGTGCATTATTGAGAAAGAGAACAACTTCAAGGAGCCTGGAGAGAGATAC
CGTACCTTTACACCAGAGAGGCAAGAACGATTCATCCAGAGATGGATTGATGCCCTATCCGACCCACGCATCACGCATGAAATCCGC
AGTATCTGGATCTCTTACTGGTCTCAGGTCTGA
```

Figure 3.1: DNA sequence of AtCat2[36].

How many primers needed depended on the length of the DNA fragment. In this thesis the gene sequence cloned was about 1.5 kB and therefore two primers were used. In this thesis the primers for the gene was made with ~ 20 bp. DNA sequence was obtained, ends (or where the primers are supposed to anneal to the DNA) were made complimentary. Figure 3.2 show the first 20 and the last 20 bp of AtCat2. Figure 3.2 show how the DNA sequence was made compatible. This was the base of the primers.

[36] http://www.ncbi.nlm.nih.gov/nuccore/240256162?from=72&to=1496&report=fasta

5` ATGGATCCTTACAAGTATCG-- --TGAACGTGAGACCAAGCATCTAA- 3`
3` TACCTAGGAATGTTCATAGC-- --ACTTGCACTCTGGTTCGTAGATT -5`

Figure 3.2: Making the DNA sequence complimentary.

Forward and the reveres primer were made, like illustrated on figure 3.3. Figure 3.3 show that the stop codon was not a part of the reverse primer. This was because the fluorescent part of the vector was behind the M.C.S. If the stop codon was a part of the primer the translation would stop at the gene end and not translate the fluorescent part of the vector. If the fluorescent part was in front of the M.C.S. the reverse primer must have the stop codon intact. This to stop the translating after the gene is coded.

5` ATGGATCCTTACAAGTATCG
5` ATGGATCCTTACAAGTATCG-- --TGAACGTGAGACCAAGCATCTAA- 3`
3` TACCTAGGAATGTTCATAGC-- --ACTTGCACTCTGGTTCGTAGATT -5`
ACTTGCACTCTGGTTCGTAG-5`

Figure 3.3: How to make the forward (red) and the reveres (green) primer.

Figure 3.3 show the base of the primer design. Restriction sites were pleased in front of the primer and also a buffer codon was added in the front of the primer. The buffer codon used was ATT. In this case of AtCat2 primers (for amplification and insertion into the vector of pWEN-18, pWE-NY and pWEN-CY) the restriction sites XhoI and KpnI was chosen. For cloning into pWEN-25 restriction sites KpnI and SalI was chosen. pWEN- 25 had the fluorescent properties in front of the MCS and therefore the stop codon in the primer should not have been taken away. But however this was done. The vector pWEN-25 had a stop codon right after the MCS and therefore the primer was not designed with the stop codon. It was however important to take this in consideration when interpreting the results from the microscope. Primers were tested on the computer in the program BioEdit and in the lab with PCR. Table 3.1 show the different primers with different restriction sits used for cloning into the different vectors. Primers for pWEN-18 were also used for vector pWEN-18-NY and pWEN-18-CY.

Table 3.1: The different primers used for the different vectors and gene.

Vector	AtCat1-L	AtCat1-R	AtCat2-L	AtCat2-R	AtCat3-L	AtCat3-R
pPCR-scripr-cam (+)	AtCat1-L	AtCat1-R	AtCat2-L	AtCat2-R	AtCat3-L	AtCat3-R
pWEN-18	AtCat1-KpnI-L	AtCat1-KpnI-R	AtCat2-XhoI-L	AtCat2-KpnI-R	AtCat3-XhoI-L	AtCat3-KpnI-R
pWEN-25	AtCat1-SalI-L	AtCat1-KpnI-R	AtCat2-SalI-L	AtCat2-KpnI-R	AtCat3-SalI-L	AtCat3-KpnI-R

From the three catalases found in *Arabidopsis thaliana*, catalase 2 was selected for further studies. Table 3.2 shows the primers used. See appendix 3 for information on the other primers uses in the thesis.

Table 3.2: The primers used to amplify AtCat2. The table shows both primers with and without restriction sites.

Primer	Sequence
AtCat2-L	5’ - ATGGATCCTTACAAGTATCGTC
AtCat2-R	5’ - TTAGATGCTTGGTCTCACGTTC
AtCat2-XhoI-L	5’ - ATTCTCGAGATGGATCCTTACAAGTATCGTC
AtCat2-KpnI-R	5’ – ATTGGTACCGATGCTTGGTCTCACGTTC
AtCat2-SalI-L	5’ - ATTTCGACATGGATCCTTACAAGTATCGTC

3.2 Sequencing results

Catalase 2 was cloned into the vector pWEN-18-CY. The sequence was confirmed by sending the plasmid for sequencing to Genome Enterprise[37]. See appendix 1 for sequencing results. Catalase 2 was cut out from this vector and relegated into the other vectors. One expects no change in the gene bp when only cutting and relegation. When positive colonies was obtained these was confirmed by PCR and digestion test. They were also sent for sequencing to confirm insertion of catalase into the vector. (Data not shown.)

[37] Genome Enterprise Ltd. BBSRC Genome Analysis Centre. Norwich Research Park. Norwich. NR4 7UH. UK

3.3 In silico analysis of catalase for *Arabidopsis thaliana*, *Homo sapiens* and *E.coli*

The program BioEdit was used to compare HsCat, KatE, AtCat1, AtCat2 and AtCat3. The information used in BioEdit was found on NCBI. The amino acids that are market with black (fig.3.4) are amino acids that are identical in all of the genes in the different organisms. Amino acids that are marked with gray (fig.3.4) are sequences that are similar between the different organisms.

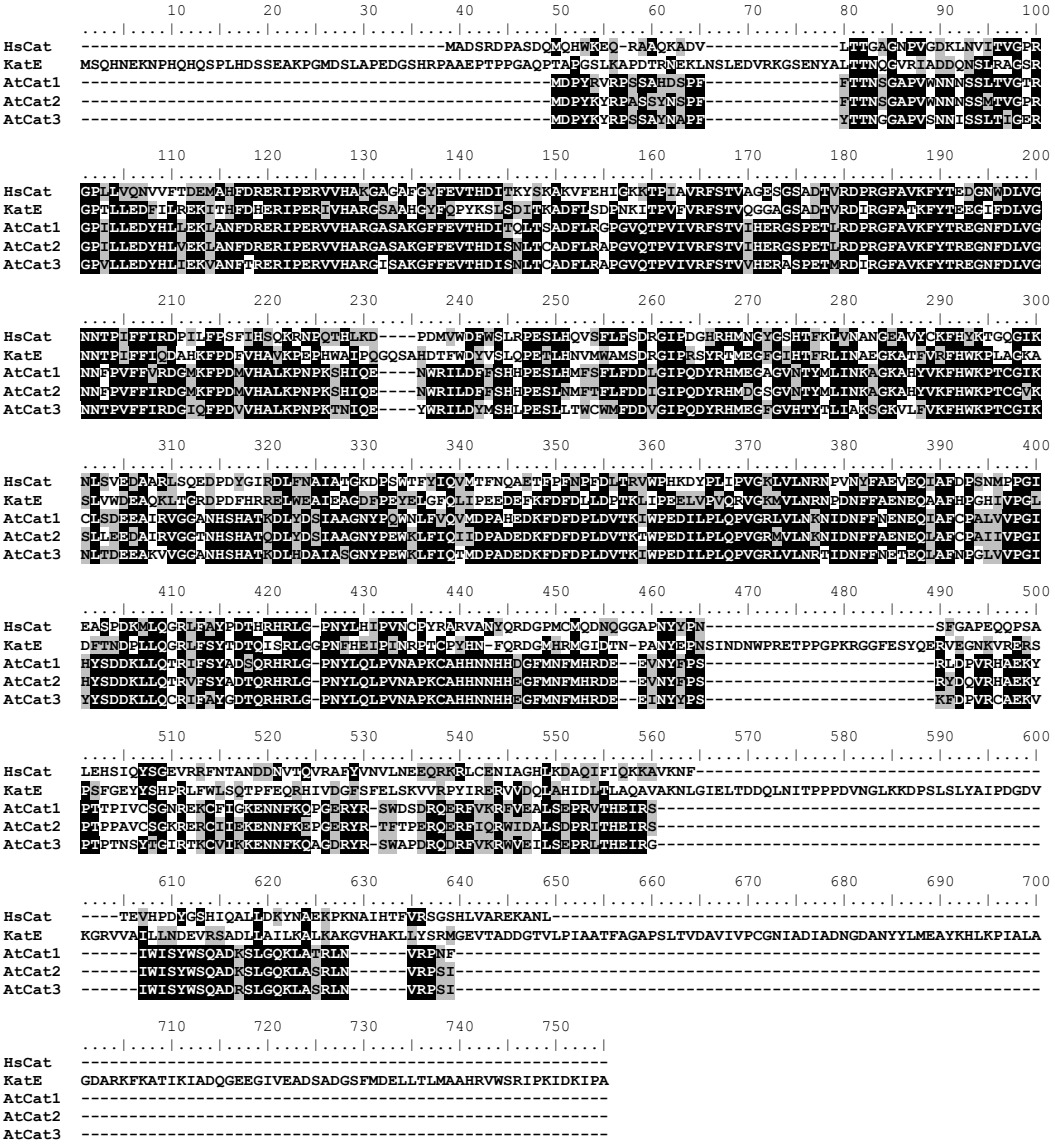


Figure 3.4: In silico analyses of catalase from human, *E. coli* and *A. thaliana.*

On the program BioEdit it was also possible to put up this figure (fig. 3.5) to show the relationship between catalase from the different species. Figure 3.5 show the relationship between the different catalase genes in human, *E. coli* and *A. thaliana*.

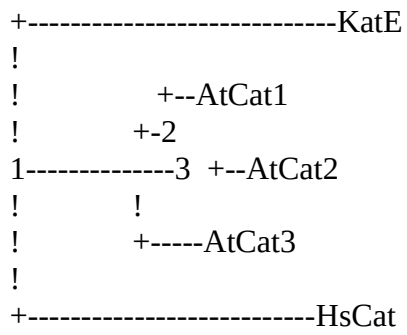


Figure 3.5: The relationship between KatE, AtCat1, AtCat2, AtCat3 and HsCat.

3.4 Cloning AtCat2 from *Arabidopsis thaliana* into vectors selected for interaction and localization analyses.

The illustration below (Figure 3.6) shows the cloning procedure.

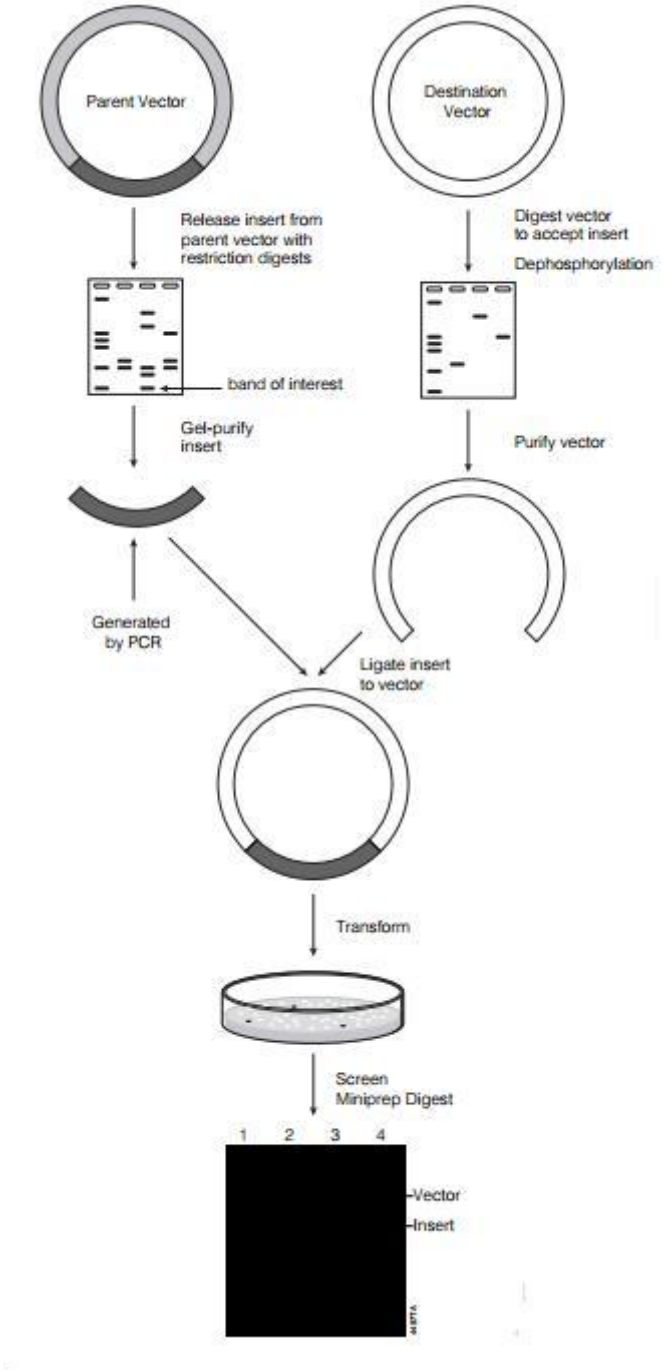


Figure 3.6: Flow scheme of the cloning process.

3.4.1 cDNA amplification of AtCat2 and molecular cloning

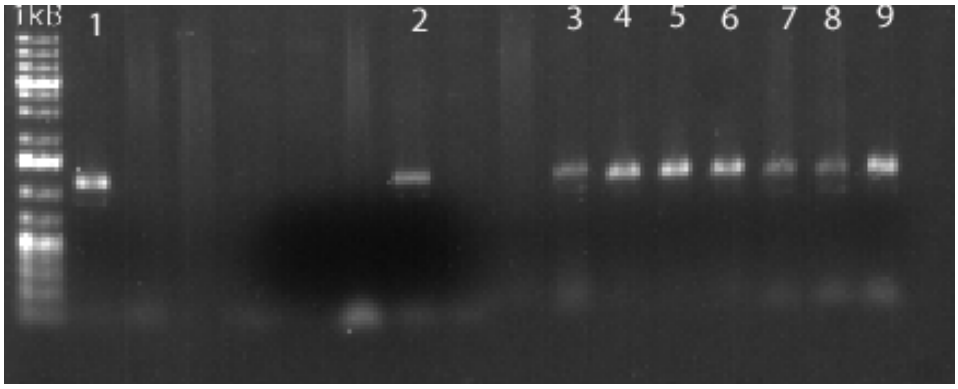


Figure: 3.7: Amplification of AtCat2 from cDNA using pow polymerase PCR system. 20 µl from pow polymerase PCR product (with gene specific primers) was loaded on the gel. 1kb GeneRuler (3 µl) was used as ladder. Line marked 1- 9 show where AtCat2 have been amplified.

The bands representing AtCat2 was cut and extracted from the gel. A 50 μl digestion system was used both for gene and vector. R.E. KpnI and XhoI were used and the digestion was incubated at water bath at 37^{o}C for 3hr. The digestion reaction itself takes place in less time, but to be sure that all DNA fragments were digested the reaction was incubated for longer time. After the incubation the digestion reaction was loaded on to a 1% agarose gel to clean out the other fragments in the reaction mix. Figure 3.8 show the digestion of AtCat2 purified by running on a 1% agarose gel.

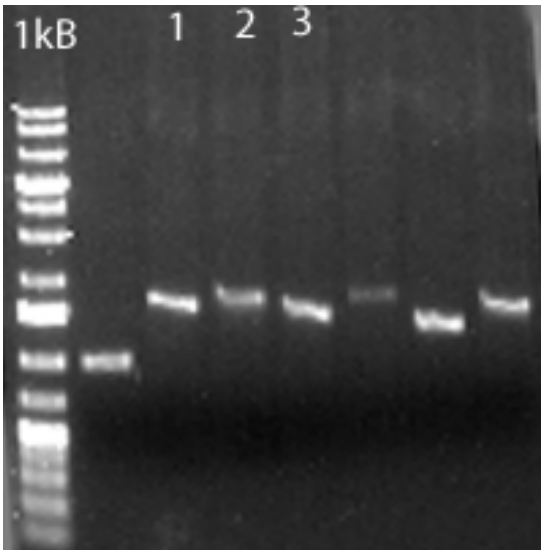


Figure 3.8: KpnI/ XhoI digestion reaction of AtCat2 run on a 1% agarose gel. A digestion system of 50 µl was used and loaded on the gel. Line 1- 3 represent AtCat2 after digestion. Ladder 1 kB GeneRuler (4 µl) was used as ladder.

The digested gene fragments was cut from the gel and purified. A ligation was made with plasmid pWEN-18, pWEN-25, pWEN-18-CY and pWEN-18-NY. The ligation was stored at room temperature till the next day and transformation was conducted. Plates containing amphicilin (antibiotic) were used. All four plasmids contain resistance against this antibiotic and therefore in theory only transformed cells could grow on these plates. PCR with vector specific primers was used to identify positive colonies. Only colonies from pWEN-18-CY gave positive results. This can be seen in figure 3.9.

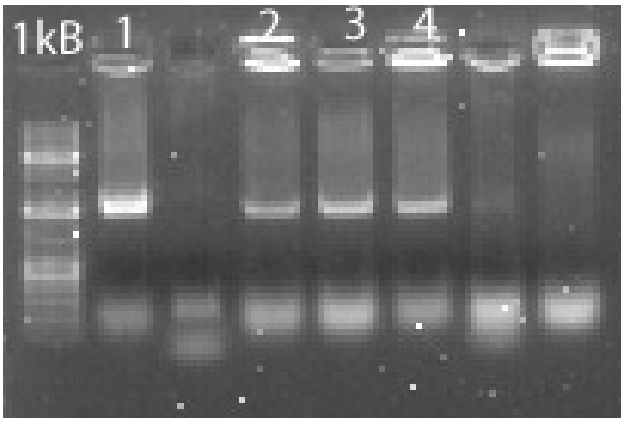


Figure 3.9: Colony PCR for pWEN-18-CY-AtCat2. Taq polymerase system with vector specific primers was used to screen for positive colonies. 15 µl of PCR product was loaded on the gel. Line marked 1- 4 represent positive colonies. Ladder 1 kB GeneRuler (2 µl) was used as ladder

From colonies represented by line 1 and 3 an overnight culture was made with 10 ml LB medium and 10 µl amphicilin. The cultures were incubated on shaker (37^{o}C) over night. The following day PCR was conducted with vector specific primers to confirm positive culture. In figure 3.10 line1 represent colony nr.3 and line 2 represent colony nr1. Clearly colony nr.1 (represented in figure 3.10 in line 2) was used further. Plasmid was extracted from the overnight colony and a sample was sent for sequencing. (Se section 3.2 for more information on sequencing.)

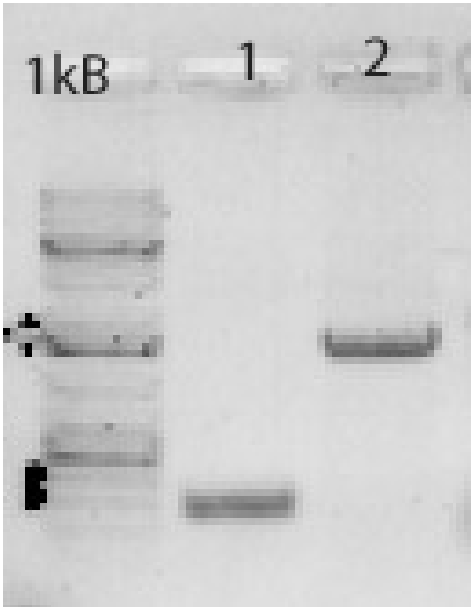


Figure 3.10: PCR check on overnight cultures of pWEN-18-CY-AtCat2. Taq polymerase system with 35s /YFP-seq primers was used. 15 µl PCR product was loaded on the gel and ladder 1 kB GeneRuler (3 µl) was used as ladder.

3.4.2 Subcloning of AtCAt2 into pWEN-18, pWEN-18-NY and pWEN-25

Vector pWEN-18-CY-AtCat2 was digested with KpnI and XhoI in a 50 µl digestion system to cut the gene out of the vector. Since the fragment had been confirmed by sequencing, it was possible to cut it out and relegate the fragment into the other vectors. This digestion was run on a 1% agarose gel to clean out the other components of the digestion system. This can be seen in figure 3.11, line 1 and 2. Figure 3.11 line 3 and 4 represent another digestion made with pWEN-18-CY-AtCat2 and the R.E. SacI. SacI cut the vector one time and

cut AtCat2 one time. This cutting site is unique for AtCat2 (non cutter for AtCAt1 and AtCat3). Therefore digestion with SacI was a good test to confirm that AtCat2 had been inserted.

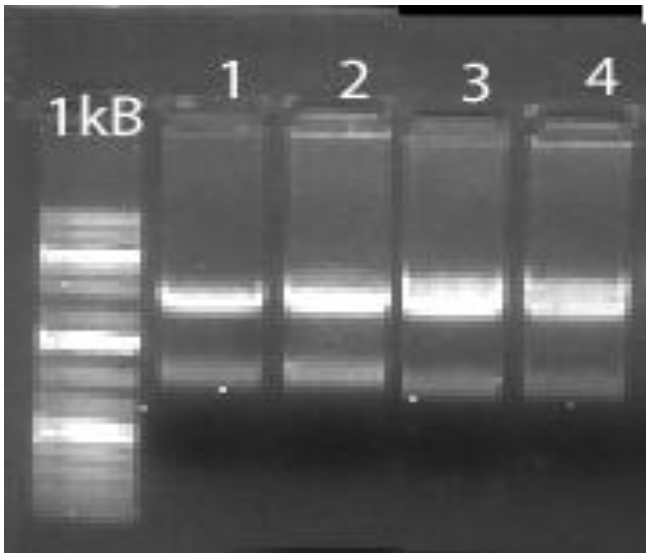


Figure 3.11: Digestion test of pWEN-18-CY-AtCat2 and clean up of digestion system with KpnI/XhoI.
Line 1 and 2 represent digestion of pWEN-18-CY-AtCat2 with KpnI/XhoI. Line 3 and 4 represent digestion test with SacI. Ladder 1 kB GeneRuler (3 µl) was used as ladder. Each line represents 25 µl of digestion system.

The gene fragment in line 1 and 2 was cut out and extracted from the gel. The fragment was relegated into the vector pWEN-18, pWEN-25 and pWEN-18-NY. Figure 3.12 a, b and c show colony PCR done on colonies from pWEN-18-NY, pWEN-25 and pWEN-18. See figure for description on primer used and also colonies selected for overnight cultures.

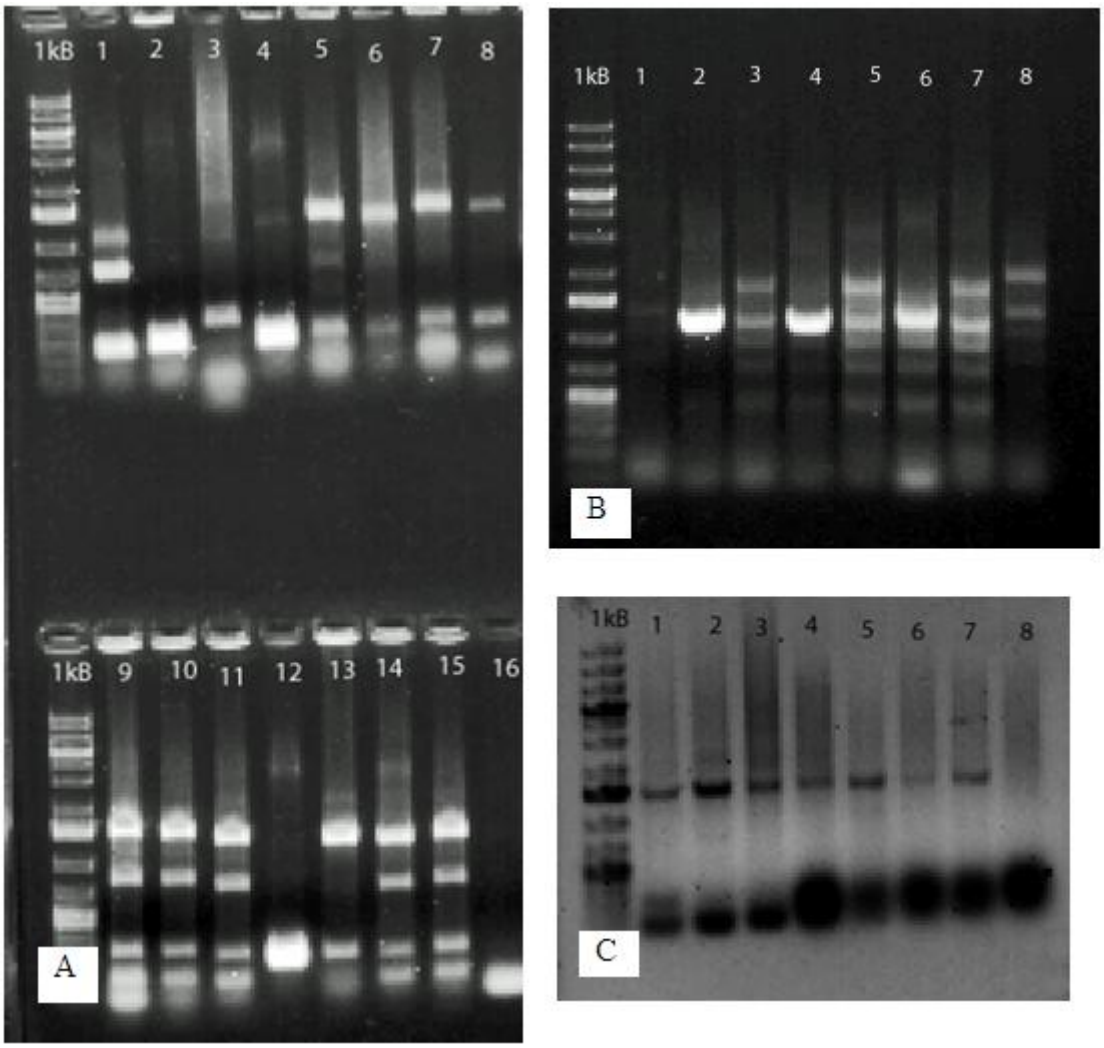


Figure 3.12: Colony PCR of pWEN-18-NY-AtCat2, pWEN-18-AtCat2 and pWEN-25-AtCat2. (taq poly. PCR system)

A: Colony PCR done on pWEN-18-NY-AtCat2. Vector sp. primers (35s/YFP-seq.) were used in a taq poly. PCR. 15µl of PCR product was loaded on the gel. Colony represented by line 6 and 13 was selected for making overnight culture. Ladder 1 kB GeneRuler (3 µl)

B: Colony PCR done on pWEN-25-AtCat2. One vector sp. and one gene sp. primer (L-gen sp./NOS) were used in a taq poly. PCR. 15µl of PCR product was loaded on the gel. Colony represented by line 2 and 4 was selected for further use. Ladder 1 kB GeneRuler (3 µl) was used as ladder.

C: Colony PCR done on pWEN-18- AtCat2. Vector sp. primers (35s/YFP-seq.) were used in a taq poly. PCR. 15µl PCR product was loaded on the gel. Colony represented with line 2,3,4 and 5 was selected for making overnight culture. Ladder 1 kB GeneRuler (3 µl) was used as ladder.

Overnight cultures was made from selected positive colonies (LB media (10 ml) and amphicilin (10 µl)). The cultures was incubated over night at shacker (37^{o}C). The overnight cultures was tested the following day by taq poly. PCR. See figure 3.13, 3.14 and 3.15 for description of primers and colonies chosen to work with futher.

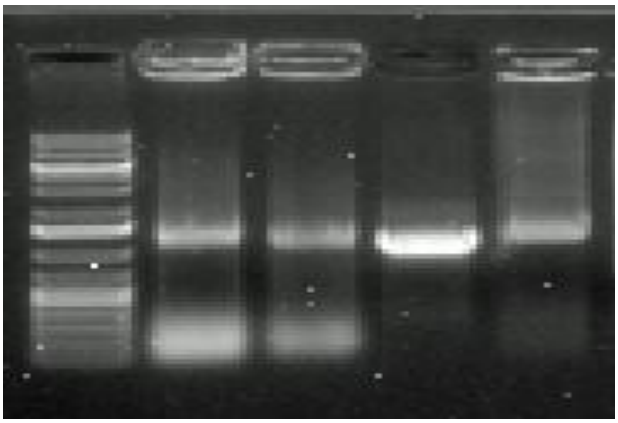


Figure 3.13: PCR on overnight cultures from pWEN-18-AtCat2. Line 1,2,3 and 4 represent the colonies 2,3,4 and 5 (from fig. 3.11 c). 35s vector specific primer and R-gene specific primer was uses in this taq PCR. Ladder 1 kB GeneRuler (3 µl) was used as ladder. Colony represented by 3 (colony nr. 4 on figure 3.11.c) was used further.

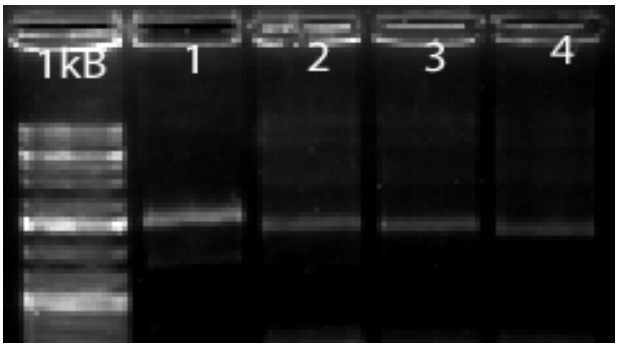


Figure 3.14: PCR on overnight cultures from pWEN-18-NY-AtCat2. . 35s vector specific primer and R-gene specific primer was uses in this taq PCR. Line 1 and 2 represent taq PCR with 2µl overnight culture as template. Line 3 and 4 represent taq PCR with 1µl overnight culture as template. This means line 1 and 3 is overnight culture from the same colony and line 2 and 4 is overnight culture from the same colony. Culture representing line 1 and 3 was selected for further use. Ladder 1 kB GeneRuler (3 µl) was used as ladder.

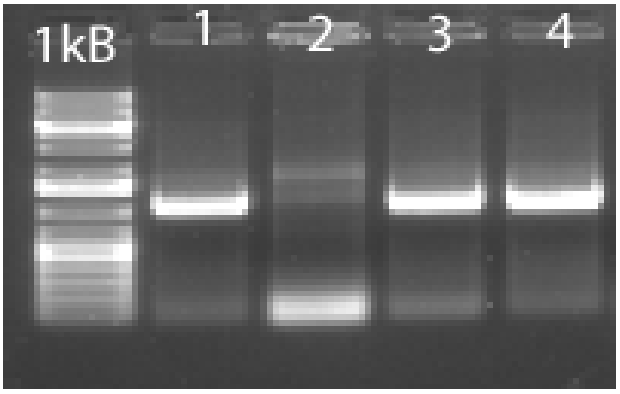


Figure 3.15: PCR on overnight cultures from pWEN-25-AtCat2. . NOS vector specific primer and L-gene specific primer was uses in a taq PCR. Line 1 and 2 represent taq PCR with 2µl overnight culture as template. Line 3 and 4 represent taq PCR with 1µl overnight culture as template. This means line 1 and 3 is overnight culture from the same colony and line 2 and 4 is overnight culture from the same colony. Colony representing 1 and 3 was selected for further use. Ladder 1 kB GeneRuler (3 µl) was used as ladder.

Table 3.3: The vector concentration measured by Nan drop 2000.

Vector	Concentration Mini prep	Concentration Midi prep
pWEN-18-AtCat2	211,6 µg/µl	5436,2 µg/µl
pWEN-25-AtCat2	652,4 µg/µl	4873,2 µg/µl
pWEN-18-NY-AtCat2	628,4 µg/µl	
pWEN-18-CY-AtCat2	199,6µg/µl	

The vectors in this section of the result part were used for shooting into plant tissue. DNA intended for shooting should have a final concentration of 1 µg/µl.

3.4.3 Subcloning of AtCAt2+YFP into pBA002.

The vector pWEN-25 was digested with PacI and AscI in a 25 µl digestion system. This cut out YFP-AtCat2 from pWEN-25. This fragment was then ligated into the pBA002 vector. This is a vector capable of over expressing the gene of interest in plants. The digestion was loaded on a gel to separate fragment of interest from vector backbone. This is shown in figure

3.15 line two. Line one in figure 3.15 represent a digestion of the vector pBA002 with PacI and AscI. Digested fragment and digested vector was cut out of the gel and extracted from the gel.

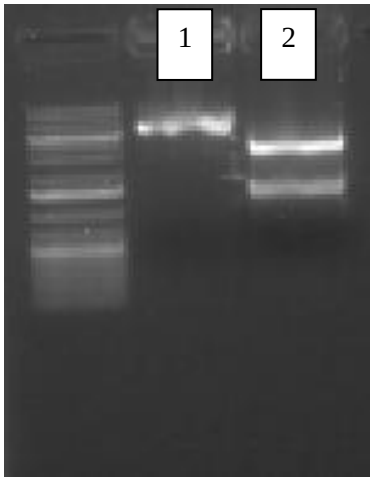


Figure 3.16: Digestion of pBA002 and of pWEN-25-AtCat2. Digestion of pBA002 was done with PacI and AscI., this represented in line 1. Digestion of pWEN-25-AtCat2 done with PacI and AscI, represented in line 2. 1kB GeneRuler (2 µl) was used as ladder.

Fragment and vector was ligated together and left on the bench until the following day. Transformation into competent cells was conducted and the cells were spread on LB plates containing the antibiotic spectinomycine. pBA002 contain resistance against this antibiotic and therefore transformed bacteria are able to grow on media containing spec. Positive colonies was obtained by colony PCR. Se figure 3.17 for primers used and information of colonies selected for further use.

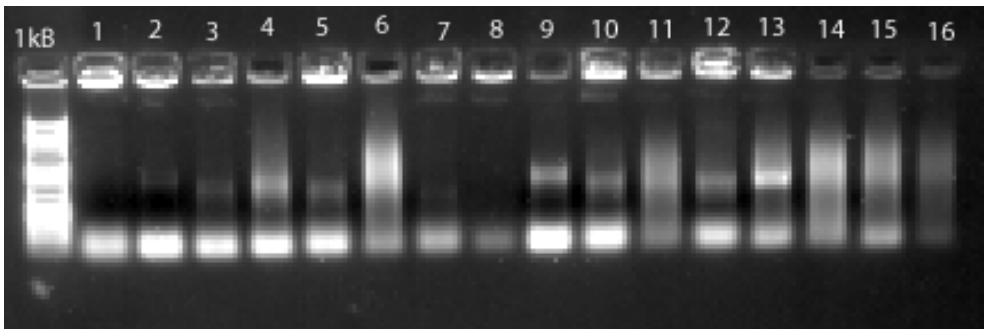


Figure 3.17: Colony PCR on pBA002-YFP-AtCat2. L-genesp. primer and NOS vector sp. primer was used in a taq PCR. 15µl was loaded on the gel and 4 µl 1kB GeneRuler was used as ladder. Colony represented by line 9,12 and 13 was selected for overnight cultures.

Overnight cultures were made and the following day tested again with PCR. Se figure 3.18 for this PCR result. Overnight culture represented by line 2 in figure 3.18 was used further for plant transformation.

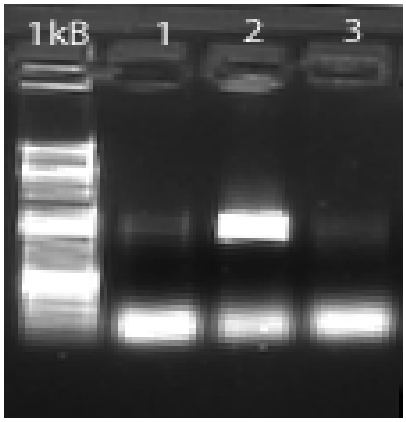


Figure 3.18: PCR on overnight cultures from pBA002-YFP-AtCat2. Taq PCR with one gene sp.primer and one vector specific primer (L-gene sp.primer/ NOS) was used. 15µl PCR product was loaded on the gel and 3µl 1kB GeneRuler was used. Line 1,2,3 represent colony 9,13 and 12 from figure 3.17.

Plasmid was extracted (cons.114, 4 µg/ µl) from the culture represented by line 2 in figure 3.18, followed by transformation into agro bacteria and further cultivated in the green room. See section 3.8 for further results on plants transformation.

3.4.4. Amplification of additional plasmids used for interaction analyses

Additional vectors were provided by Dr. Xiang Ming Xu. The vectors were amplified by transformation into comp. cells and extraction of plasmids from overnight cultures made from positive colonies. The purpose was to test the interaction of these genes with AtCat2. See table 3.4 for gene description.

Table 3.4: The gene, the gene name and gene size of the genes tested for interaction with AtCat2.

Gene	Gene name	Gene size
CDS1	AT1G08830.1	454 bp
DJ1-a	AT3G14990	1179 bp
GPX2	AT2G3157	510 bp
AtCat2	AT4G35090	1479 bp

Figure 3.19 show the length and gene name from the different vectors. Table 3.5 shows the concentration of the different vectors and the short name for them.

Table 3.5: The name, short name and the concentration of the vectors after purification.

Name	Short name	Concentration (µg/ µl)
pWEN-18-CY-DJ1	CY-DJ1	91,9
pWEN-18-NY-DJ1	NY-DJ1	74,7
pWEN-18-CY-CSD1	CY-CSD1	466,8
pWEN-18-NY-CSD1	NY-CSD1	362,4
pWEN-18-CY-GPX2	CY-GPX2	285,2
pWEN-18-NY-GPX2	NY-GPX2	290,6

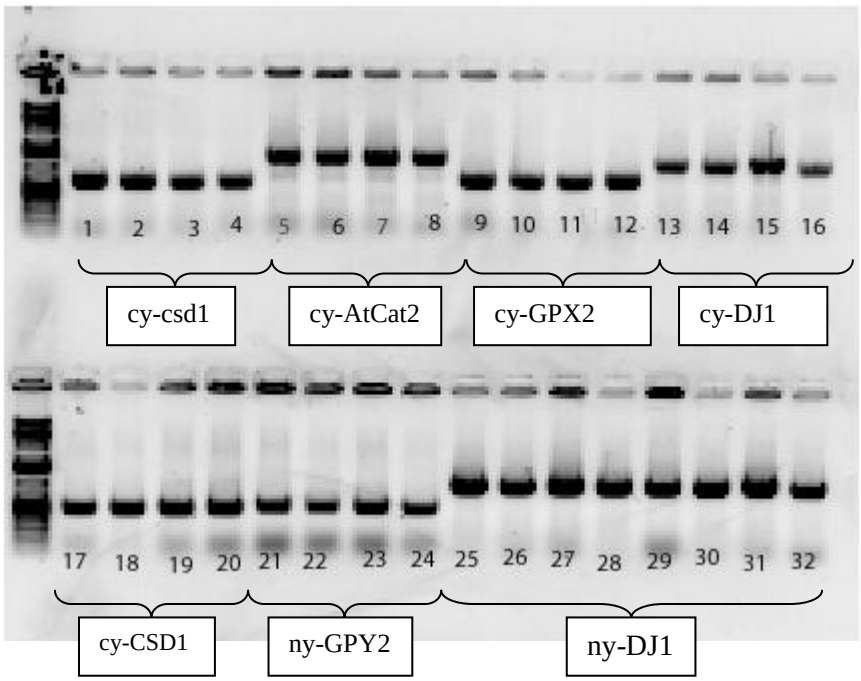


Figure 3.19: PCR amplification on the different vectors AtCat2 was tested for interaction against. PCR product loaded on the gel was 15µl and GeneRuler was used as ladder (5 µl). PCR was done with vector sp. primers. Template was colony transformed using vectors already containing the genes of interest.

3.5 Localization studies of AtCat2

AtCat2 cloned into vector pWEN-18 and pWEN-25 was shoot into *Nicotiana tabacum* leafs. pWEN-25-AtCat2 would give the localization N-terminal and pWEN-18-AtCat2 would give the localization C-terminal. Normally pWEN-18 was used, but in case of peroxysomal localization pWEN-25 was also used. First empty vectors was shoot into leafs. This to practice and see how empty vector would give fluorescent, as a control.

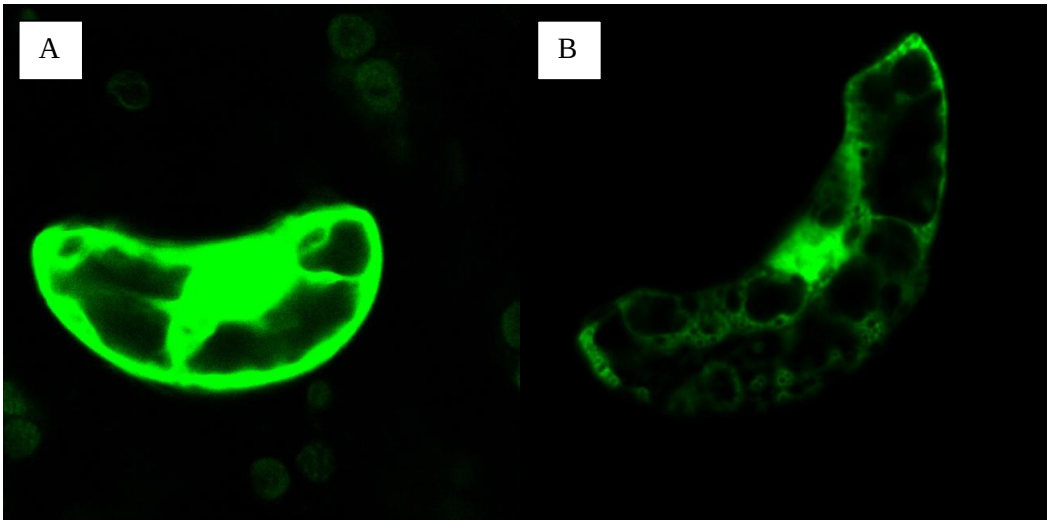


Figure 3.20: Empty vector shoot into *Nicotiana tabacum* leafs. Figure 3.20.A show the empty vector pWEN-18. Figure 3.20.B show the empty vector pWEN-25.

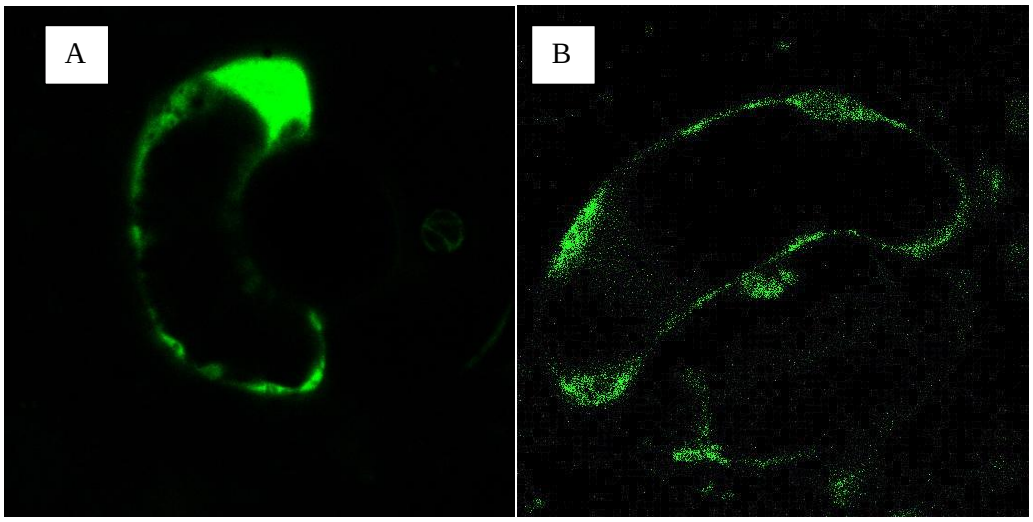


Figure 3.21: AtCat2 cloned into the vector pWEN-25. Shoot into *Nicotiana tabacum* leafs. Figure 3.21 A and B both show two different cells that have taken up pWEN-25-AtCat2. (However no localization can be determined from these photos.)

3.6 Interaction studies of AtCat2

Interaction vectors were named pWEN-18-NY and pWEN-18-CY was used. These vectors contained half part of YFP. (Short name: CY and NY.) Combinations of vectors containing one CY and one NY plasmid were shoot into *Nicotiana tabacum* leafs together. The shooting combinations can be seen in table 2.8 in materials and methods. However time and other complications made it possible to only do combination shooting twice and no interaction could be found in these attempts.

3.7 Catalase level in different plant tissue and in plants of different age.

This experiment was conducted to see where the tree different catalases in *A. thaliana* were expressed. Two different approaches was used, one testing catalase level in plants with different age and the other one testing different tissue of the plant to see were AtCat1, AtCat2 and AtCat3 was expressed.

3.7.1 Catalase expression in plants of different age

Wild type *Arabidopsis thaliana* was cultivated on MS media for two weeks and then transferred to soil and further cultivated in soil for one, two and four weeks. RNA was isolated and quality/ quantity were measured with spectrophotometer. The results can be seen in table 3.6. RT-PCR was conducted on the RNA samples. Wanted concentration of the cDNA was 5 µg/ml. This was the concentration that was optimal for running real-time PCR. Calculations was done to find out how much RNA was needed in the RT-PCR for obtaining a final concentration of cDNA 5 µg/ml. This can be seen in table 3.7. Two parallels were obtained for each sample.

Table 3.6: Quantity and quality of isolated RNA samples from plants of different age.

Sample	Cons. (µg/ml)	A260	A280	A260/A280	A260/A230
Week 1.1	162,4	4,060	1,773	2,29	1,50
Week1.2	210,2	5,256	2,265	2,32	1,04
Week 2.1	47,6	1,189	0,525	2,27	0,15
Week2.2	95,7	2,392	1,092	2,19	0,17
Week 4.1	8,9	0,153	0,057	2,69	0,06
Week4.2	10,2	0,223	0,087	2,58	0,26

Table 3.7: Calculations of RNA concentration (for making 5 µg/ml).

Week	RNA cons.(µg/ml)	Final volume	RNA	dH_2O
Week 1	186,3	50 µl	3 µl	22 µl
Week 2	71,7	50 µl	8 µl	16 µl
Week 4	~ 10,0	50 µl	25 µl	0 µl

A standard PCR was conducted to see if it was possible to visually see any difference in the level of AtCat1, AtCat2 and AtCat3 at different time in the plant life. This can be seen in figure 3.20

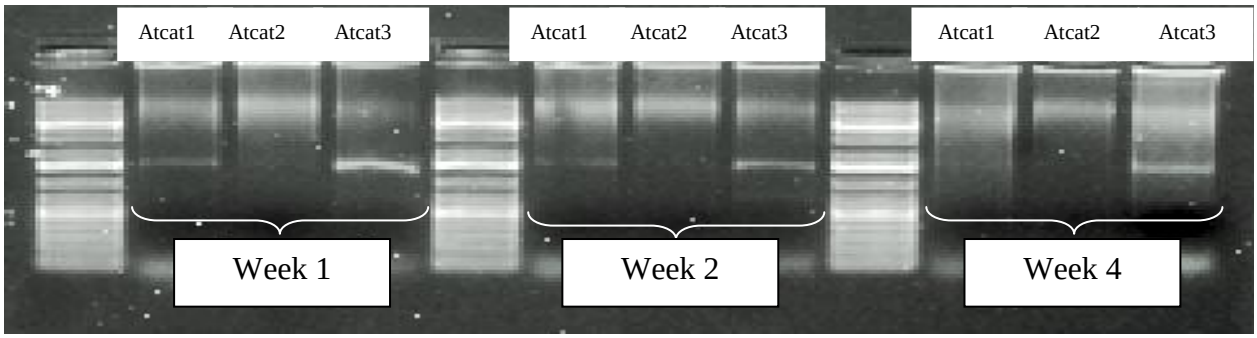
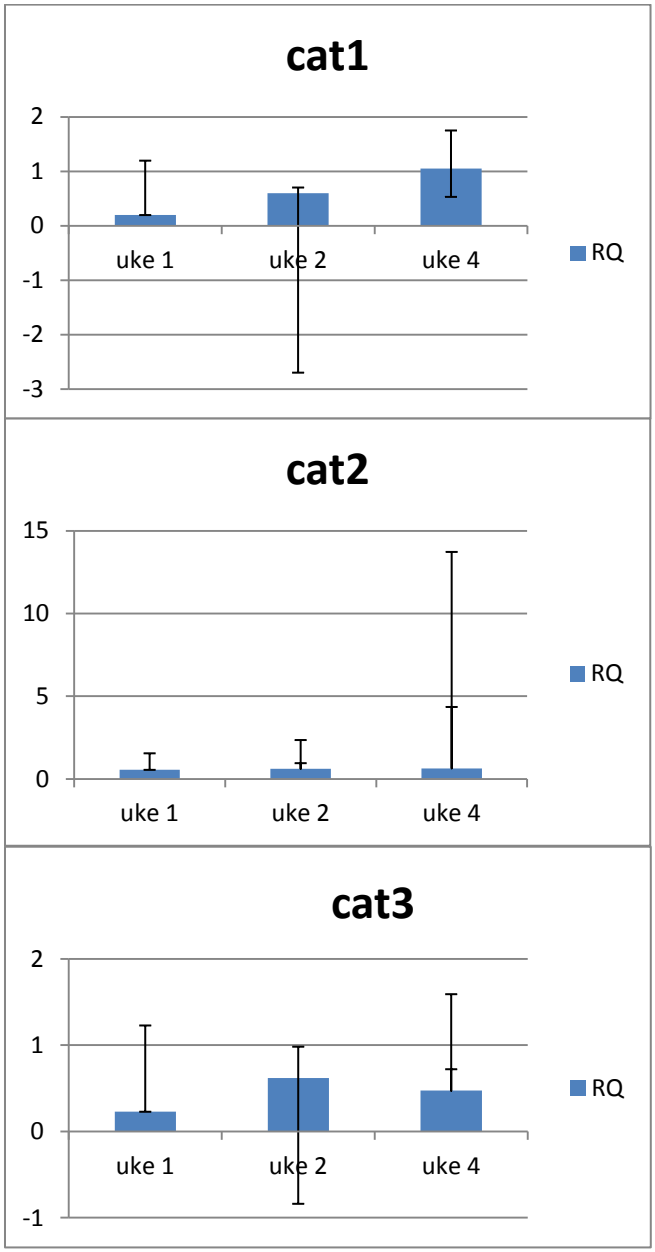


Figure 3.22: Show expression of AtCat1, AtCat2 and AtCat3 after week 1, 2 and 4 in soil. 5 µl PCR product was loaded on the gel and 1 µl loading dye containing GelRed was added. Ladder used was GeneRuler (4 µl).



Graph 3.1: Real- time analyses of AtCat1, AtCat2 and AtCat3 after 1,2 and 4 weeks of growth on soil. It seems like AtCat1 increase with time, AtCat2 have a relative low expression and AtCat3 have highest expression in week 3.

The standard aberration for samples from week 4 was too high to be able to trust the RQ values from this week. Strangely week 2 had negative standard aberration, this seemed strange since it is not possible to have a negative amount of gene expression. This experiment should have been repeated to be able to trust the results given in graphs 3.1. The real time experiment also gave some different results from the PCR test seen in figure 3.22.

3.7.2 Catalase expression in different plant tissue of 4 weeks old plants.

WT *Arabidopsis thaliana* was cultivated in soil for four weeks. Leaf, stem, flower and seed capsule was collected. RNA was extracted and quality/ quantity were measured with spectrophotometer. The results can be seen in table 3.8. Calculations were also done here for making 5 µg/ml cDNA. These calculations can be seen in table 3.9. Two parallels for leaf and stem were obtained. For further use in RT-PCR leaf 2, stem 1, flower and seed capsule was used (see table 3.8.) These RNA extractions had the highest concentration and were therefore selected.

Table 3.8: Quantity and quality of isolated RNA samples from different plant tissue.

Sample	Cons. (µg/ml)	A260	A280	A260/A280	A260/A230
Leaf 1	1,2	0,029	0,001	22,03	
Leaf 2	105,2	2,631	1,164	2,26	0,01
Stem 1	73,5	1,838	0,884	2,03	0,28
Stem 2	29,0	0,726	0,336	2,16	0,90
Flower	199,7	4,993	2,594	1,93	0,17
Seed capsule	61,6	1,541	0,692	2,23	0,60

Table 3.9: Calculations of RNA concentration from different plant tissue.

Week	RNA cons.(µg/ml)	Final volume	RNA	dH_2O
Leaf	105,2	50 µl	4,75 µl	20,25 µl
Stem	73,5	50 µl	6,80 µl	18,20 µl
Flower	199,7	50 µl	2,50 µl	22,50 µl
Seed capsule	61,6	50 µl	8,12 µl	16,88 µl

Figure 3.23 show standard PCR done on cDNA. This was conducted to see if any differences in catalase levels could be seen visually in the different plant tissue.

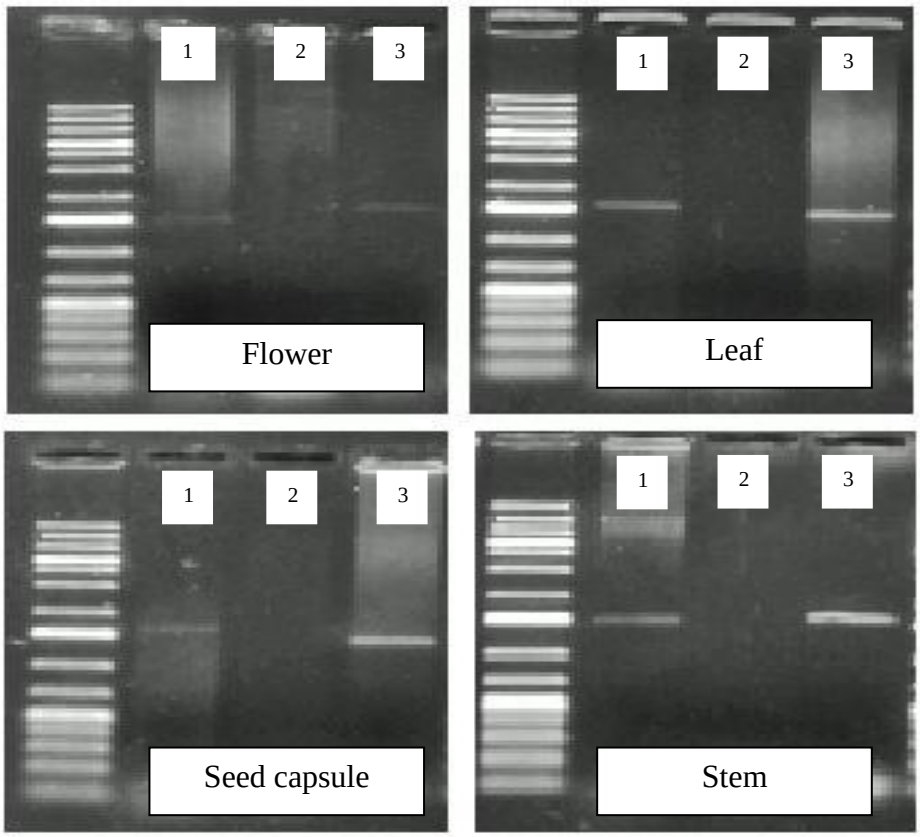


Figure 3.23: Show expression of AtCat1, AtCat2 and AtCat3 in flower, leaf, seed capsule and stem. 5 µl PCR product was loaded on the gel and 1 µl loading dye containing GelRed was added. Ladder used was GeneRuler (4 µl).

3.8 Cultivating of over expression plants

Seeds from plants, dipped in infiltration medium (containing pBA002-YFP-AtCat2) was harvested and plants on MS+ ppt plates. pBA002 had resistance to this antibiotic and plants that were successfully transformed could grow on this media. This growth can be seen in figure 3.24. Some green plants can be seen, indicating a positive plant.

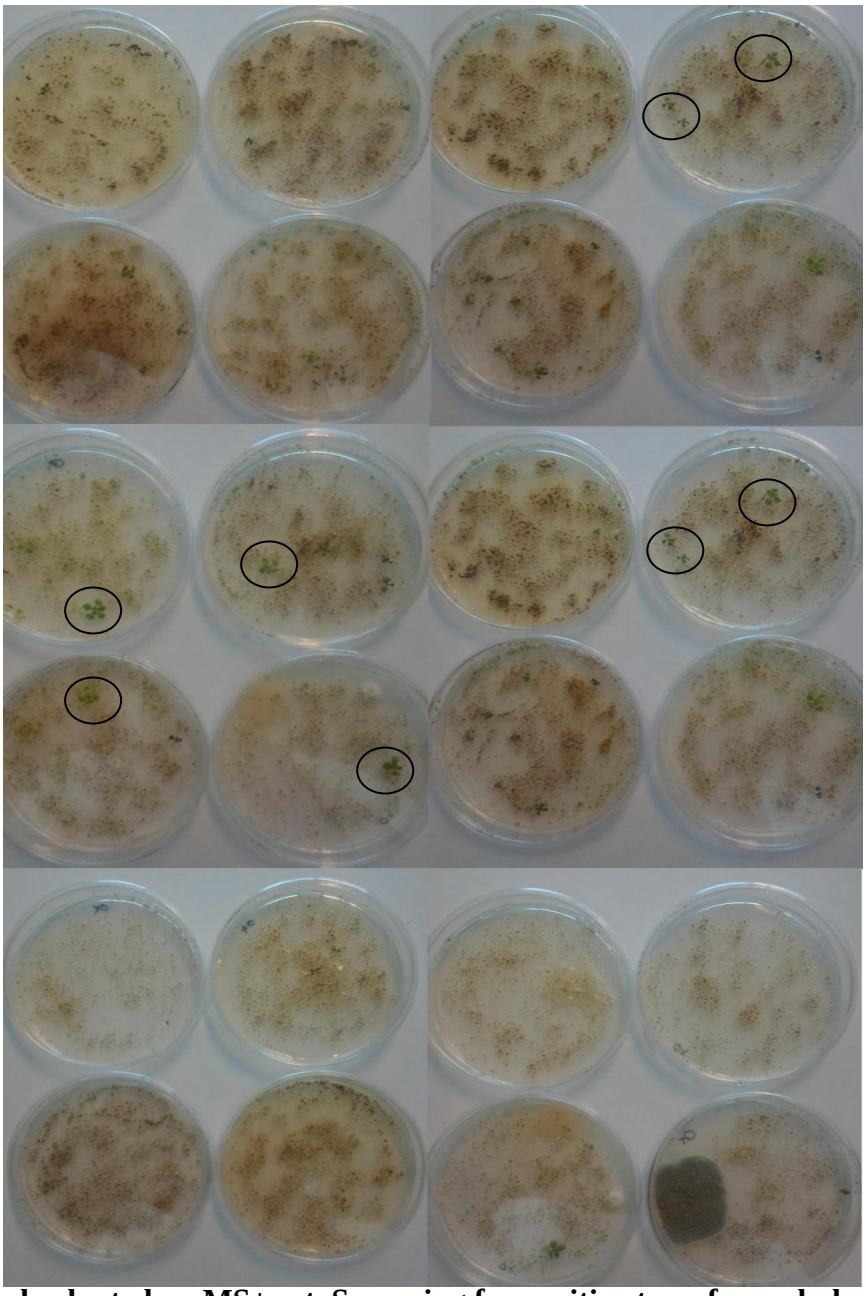


Figure 3.24: Seeds planted on MS+ppt. Screening for positive transformed plants with pBA002-YFP-AtCat2 inside.

See appendix for detailed information on the vector pBA002. As seen in figure 3.24 only a few seeds were successfully transformed. Positive transformed plant was transferred to soil and further cultivated in the growth room for 3- 4 weeks. At this age it is possible to harvest one small leaf from each plant and study the leaf under the microscope. If the plant is a true positive transformed plant the YFP protein co transferred into the pBA002 vector will show fluorescent under the microscope and in this way confirming positive transformed plant. However time did not allow for this experiment to be completed.

3.9 Cultivation of mutant plant

AtCat2 knock out mutants was ordered and cultivated on MS medium and on MS medium containing the appropriate antibiotic (kan or ppt). NASC code for figure 3.25 A and B was N556998 and the name of this mutant was SALK_076998. NASC code for figure 3.25 C and D was N557998 and the name of this mutant was SALK_057998. NASC code for figure 3.25 E and F was N839249 and the name of this mutant was SAIL_872_H06. The plates with seeds were cultivated in a growth room with light/dark 16 hr/ 8 hr cycle.

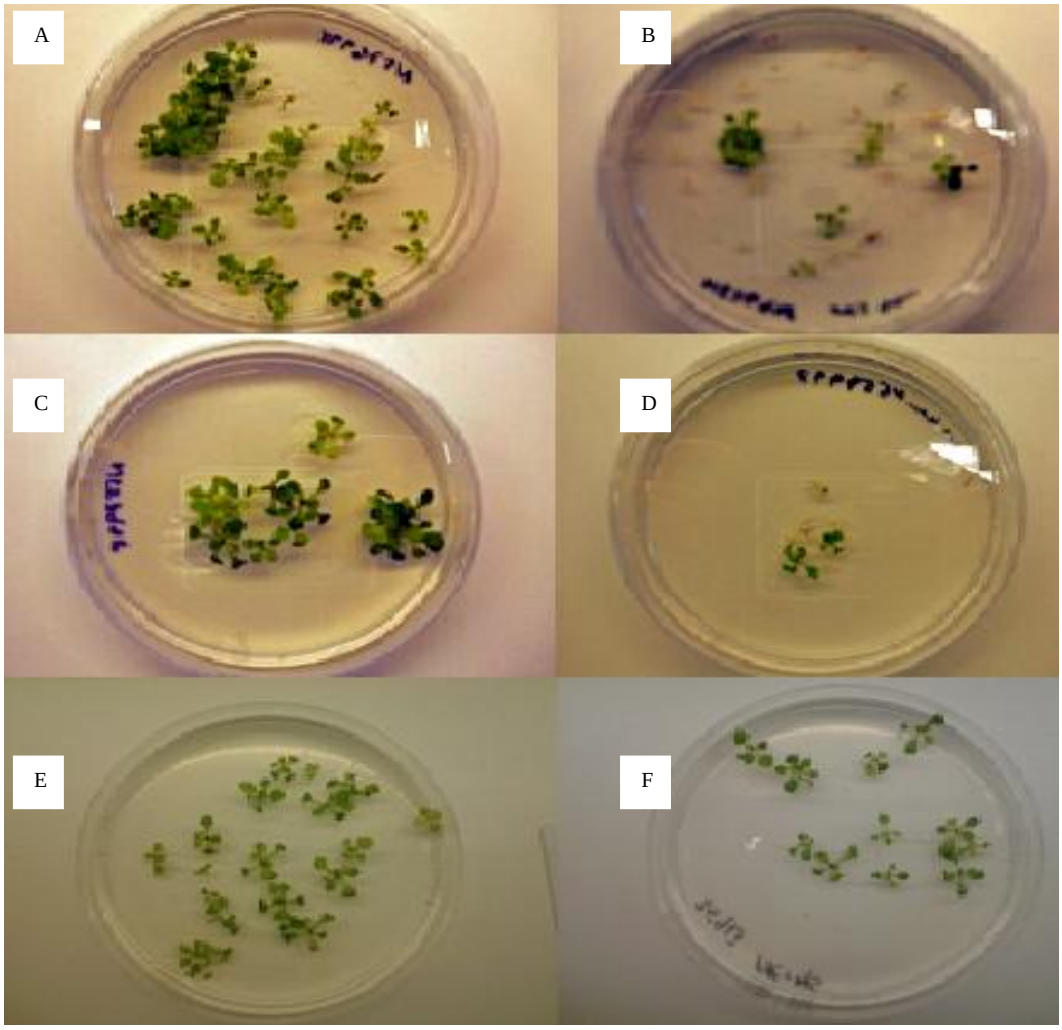


Figure 3.25: Seeds from Atcat2 knockout mutants.
A and B represent the knock out mutant N556998. A show seeds grown on MS medium and B show seeds grown on MS+kan medium.
C and D represent the knock out mutant N557998. C show seeds grown on MS medium and D show seeds grown on MS+kan medium.
E and F represent the knock out mutant N839249. E show seeds grown on MS medium and F show seeds grown on MS+ppt medium.

Photos in figure 3.25 were taken after 2 weeks of growth in the plant room. At this stage the plants were transferred to soil and cultivated father. After 3-4 weeks of cultivation in soil one leaf was collected from each of the plants grown on MS containing appropriate antibiotic. From one leaf DNA was extracted and PCR was done with one gene specific primer and one tDNA insertion primer. This was done to identify a true AtCat2 knock out plant. The concentration of extracted DNA and the PCR amplification can be seen in table 3.10 and figure 3.26.

Table 3.10: The concentration of extracted DNA from mutant plants.

Sample	Concentration (µg/µl)	A 260	A 280	A 260/280
Plant 1	81,5	1,630	1,082	1,51
Plant 2	8,0	0,161	0,125	1,28
Plant 3	37,1	0,741	0,436	1,70
Plant 4	18,9	0,378	0,243	1,56
Plant 5	29,9	0,598	0,354	1,69
Plant 6	29,6	0,593	0,348	1,70

The concentration of extracted DNA was not that great, but since the DNA only was intended for PCR the concentration is tolerable. For each PCR 1 µl of extracted DNA was used. For each sample two PCR reactions was conducted, one with L-gene specific primer and tDNA specific primer (LB1) and the other one with R-gene specific and tDNA specific primer (LB1). This can be seen in figure 3.26, one line represent L-gene spes. + LB1 and the other one represent R-spes. + LB1.

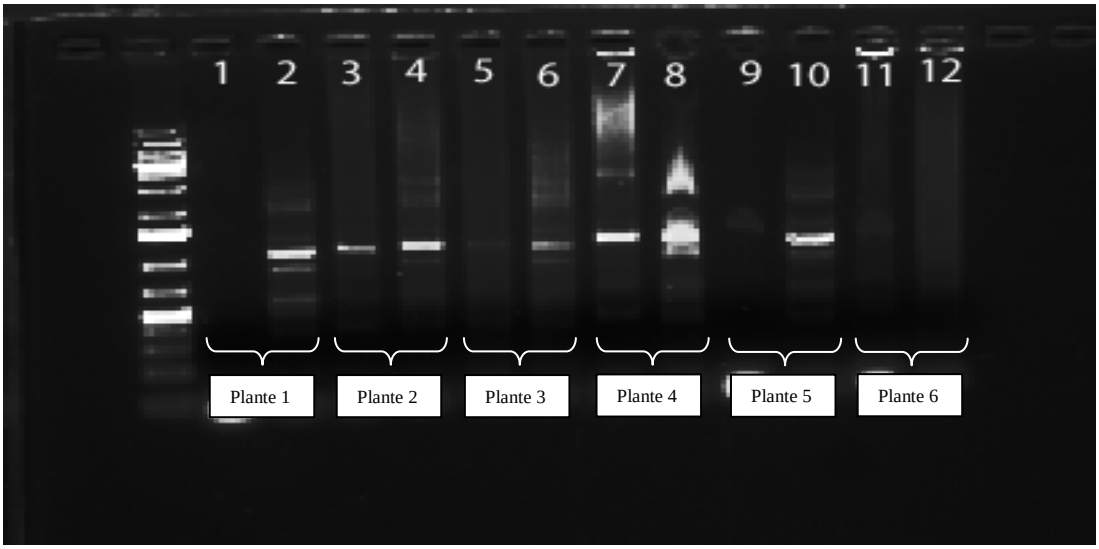


Figure 3.26: PCR test of mutant plant DNA to locate a true AtCat2 knock out mutant. Line 1 and 2 represent plant 1. Line 1 represents L-gene + LB1 and line 2 R-gene+LB1. Line 3 and 4 represent plant 2. Line 3 represents L-gene + LB1 and line 4 R-gene+LB1. Line 5 and 6 represent plant 3. Line 5 represents L-gene + LB1 and line 6 R-gene+LB1. Line 7 and 8 represent plant 4. Line 7 represents L-gene + LB1 and line 8 R-gene+LB1. Line 9 and 10 represent plant 5. Line 9 represents L-gene + LB1 and line 10 R-gene+LB1. Line 11 and 12 represent plant 6. Line 11 represents L-gene + LB1 and line 12 R-gene+LB1.

Plant 2 was selected for further use. This because both set of primers could amplify the gene/tDNA insertion fragment. This indicate that plant 2 have homozygote AtCat2 knock out. Ideally both amplified gene/tDNA insertion fragment from plant 2 should be extracted from the gel and send for sequencing. This would verify the tDNA insertion and show were the insertion has occurred.

Plant 2 was grown to mature size (until no more development of flower) and put on in the drying room. When the plant was completely dry seeds could be collected and used for stress treatment experiment. However time did not allow for this to be done.

4.0 Discussion

The goal of this thesis was to identify the function of the three variants of catalase *in A. Thaliana.* Work was done to try to identify the localization and also interaction partners of the three variants. Work was also done to look at the phenotype of knock out mutant plants and catalase over- expressed plants. Catalase levels in plants of different age and of different plant tissue was also studied.

Localization analyses were done by cloning the gene into vectors containing fluorescent properties. With the help of the vector pWEN-18 and the vector pWEN-25 the localization (both C-terminal and N-terminal) could be looked at. From this localization could be found and it could be determined if the different catalase variants in *A. thaliana* had different localization. If any of the three variants could be localized to the cytocol it would open up the possible that the gene had an interaction with the gene DJ-1.
For interaction analyses the technique, BiFC, was used. Catalase was tested for interaction against the three proteins, DJ-1a, GPX 2 and CSD1. In these analyses genes was put into vectors called pWEN-18-NY and pWEN-18-CY containing half YFP- protein. CY vector and NY vectors was shoot into plant cells and if the protein encoded by the gene in the vector came in close precipitation to on another interaction between the genes could be found with YFP- protein giving fluorescent, proving an interaction(at last an indirect interaction).
Through the studies of knock out mutants and catalase over expression plants phenotype characterisation would be done. Stress treatment was also intended to be done on WT, mutant and O.X. plants. This to see the behaviour of the plants with normal catalase level (WT), no catalase (knock out mutant) and over expressed levels of catalase. Unfortunately time was an issue in this experiment and therefore the experiment was not finished.
Through PCR and real time PCR analyses the level of catalase in plants of different age and in different plant tissue was looked at. This to see if the three different catalases had different expression level.

4.1 Localization analyses of AtCat2

From the three different variants of catalase found in *A. thaliana*, AtCat2 gave the best results in the cloning work and was therefore chosen for further work. For localization analyses the gene was cloned into vectors containing the YFP-gene. When translated to protein, AtCat2 would be attached to the YFP protein. After the DNA (vectors containing AtCat2) was shoot into leafs of *N. tabacum* and onion layers, microscopy was used to see where in the cell the protein of AtCat2 was localized. This experiment (together with interaction analyses) was the most time consuming experiment conducted. Some problems came about when cloning the gene into the vector. The gene was first attempted to clone direct into the different vectors. If cloning work were to be done again in a more efficient manner, the best way of cloning the gene successfully into different vectors must probably be through the vector pPCR-script and relegate into vectors with YFP.

Studies done on catalases from *A. thaliana* show that catalase proteins could be detected in the cytocol (Mhamdi *et. al.* 2010). Whether or not the proteins were active in the cytocol or not is yet to be discovered. Through this experiment I wanted to proof this. If catalase had a cytosolic localization it would also be possible that the protein was active in cytocol and able to interact with other cytosolic proteins, for example DJ-1. However only the vector pWEN-25, with AtCat2 inside, showed fluorescent in the microscope. No signal from the vector pWEN-18 carrying AtCat2 could be seen. PCR tests and sequencing were done on this vector to determine what the problem was. However no faults could be found in either of the tests. This indicated that something was wrong with the vector and not the gene inserted. Time did not allow for trying to clone the gene inside new vector of pWEN-18 extracted from the glycerol stock. Therefore only microscopy photos from the vector pWEN-25 can be seen in the result part (section 3.5 Localization of AtCat2). Both sequencing compartment (Figure 1.12: Alignment of AtCat1, AtCat2 and AtCat3) and laboratory work (Kamigiaki *et. al.* 2003) indicate a paroxysmal localization for AtCat2. However in this experiment no paroxysmal AtCat2 fluorescent signal could be detected in the microscope. Import of proteins into the peroxisomes should show in the microscope with use of the pWEN-25 vector, since this vector had the fluorescent part in front of the gene.

Based on findings in this experiment it is not possible to find the localization for AtCat2. More time would have been needed for recloning the gene inside new pWEN-18 and for studying the result this vector would give. This experiment (microscopy localization analyses) alone is not sufficient by itself to prove the localization of the gene. At least two different types of experiments must be done and only if both of the experiments show consistent localization for the protein, localization can be determined for the protein.

4.2 Interaction analyses done with BiFC technique for AtCat2

Interaction study was done with the help of the technique called bimolecular fluorescent complementation (BiFC). In this technique the genes of interest was placed inside vectors containing half YFP (shown in figure 4.1). If the two proteins come in close precipitation to one another fluorescent light can be detected in the microscope. An experiment like this does not proof a direct interaction, since the two hales of the YFP protein only need to be in close precipitation to one another. This kind of analyses is often done to test and see if interaction can be found and if it can, other experiments are needed to see how the proteins interact.

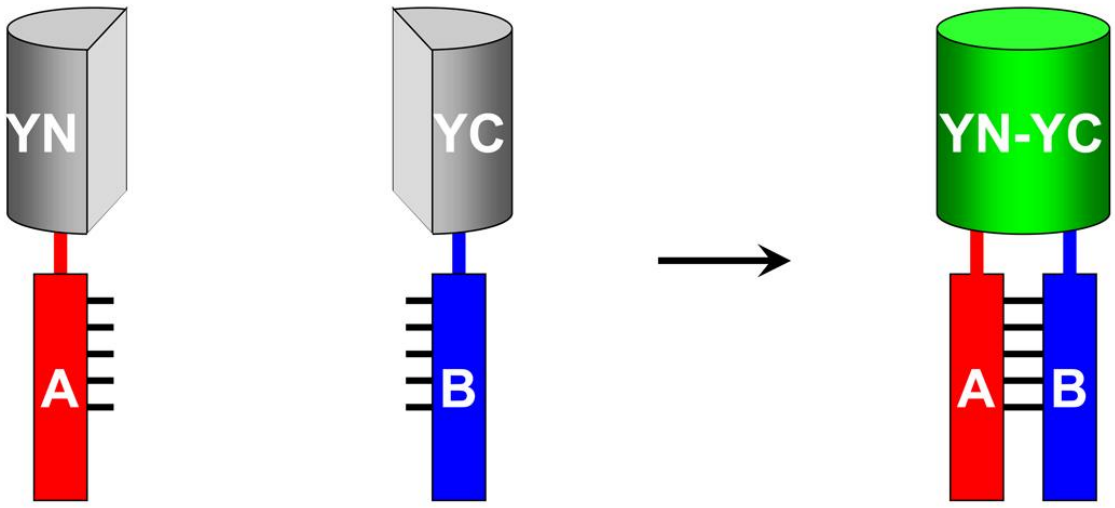


Figure 4.1: Show how the BiFC analyses work.(Tom K. Kerppola 2008)

In this thesis AtCat2 was tested for interaction against the cytosolic protein DJ-1a, GPX2 and CSD1. (See shooting combinations in table 2.8) It was reasonable to believe that AtCat2 could have an interaction with one or more of these proteins since other studies have shown AtCat2 and AtCat3 to interact with SOS2 (Verslues *et. al.* 2007) and with NDK (Fukamatsu *et. al.* 2003). Both SOS2 and NDK were cytosolic proteins. In this thesis no fluorescent could be detected from the combination shootings, indicating that AtCat2 had no interaction with the proteins mentioned. Shooting with combinations (table 2.8) was done two times, but non fluorescent could be detected under the microscope. However this was not sufficient evidence that AtCat2 did not interact with the proteins mentioned. If there was more time, first the recloning of pWEN-18 would have been done, to see if the protein AtCat2 had a cytosolic localization. If cytosolic localization could be determined for AtCat2 it would also be worth doing interaction analyses again. If the protein of AtCat2 did not have cytosolic localization, there would be no point of retesting the interaction with the proteins mentioned above. This since all of them was localized in the cytocol.
It could be possible that one of the other catalase types (AtCat1 or AtCat3) could have an interaction with DJ-1 or the other genes mentioned.

4.3 Catalase expression levels in different plant tissue and in plants of different age.

In this thesis WT plants seeds was sterilized and planted on MS medium plates. The seeds was cultivated in the green house (16/8 hr light/dark cycle) for two weeks and then transferred to soil. After further cultivation in soil, after 1 week, 2 weeks and 4 weeks, plants was harvested and frozen at -80^{o}C. At week 4, leaf, flower, stem and seed capsules were also collected and frozen. RNA was extracted from the samples and calculations were done to make 5 µg/ml cDNA from each sample. Gene specific primers were used in PCR and in real time PCR to investigate the levels of AtCat1, AtCat2 and AtCAt3.
The result that was found in the PCR analyses was different than expected. It looked like AtCat3 increased with the plant age and that AtCat1 decreased with the plant age, this is consistent with other studies that have been done (Zimmermann P. *et. al.* 2006). AtCat3 had higher expression than AtCat1 in all of the samples of plants with different age. Strangely

AtCat2 could not be detected in any of the samples. This was some strange since previous studies suggest the AtCat2 is the catalase variant that have the highest expression level in leaf and photorespiration tissue (Hu *et. al.* 2010). Mutant work has shown that AtCat2 mutant has a reduced photorespiration phenotype, but no reduction was noticed in AtCat1 and AtCat3 mutant plants (Hu *et. al.* 2010). This also confirms the photorespiration role of AtCat2. Result from the samples collected from different plant tissue at week 4 also showed no AtCAt2 expression. AtCat3 had the highest expression and AtCat1 had the lowest expression level in all of the samples. In leaf tissue and in stem tissue both AtCat1 and AtCat3 had high expression levels. AtCat3 had high levels in seed capsules, AtCat1 had low levels in seed capsules. In samples from flower, low expression of both AtCat1 and AtCat3 could be seen. The plant tissue was collected from the green house in the morning, right after dark – light shift. Maybe this could be one reason why AtCat2 could not be detected in the samples with PCR analyses. AtCat2 have been found to be under the circadian clock and are up- regulated during light periods (Zhong H.H and McClung C.R. 1996). This corresponds to the class 1 classification of AtCat2, to be in photosynthetic tissue (Ni and Trelase 1991, Willkens *et. al.* 1994). Promoter analyses (done with GUS-staining) show that AtCat2 promoter has an important regulatory role and that the photo respiratory role of AtCat2 is determined mainly by its own promoter (Hu *et. al.* 2010). This was proven with experiments done where AtCat2 was expressed under the control of AtCat1 promoter. This showed that AtCat2 activity in AtCat1-expression regions was detected only in senescent leaves (Hu *et. al.* 2010). AtCat2 under the control of AtCat3 promoter showed AtCat2 activity in AtCat3- expressed regions (Hu *et. al.* 2010). However, although catalase activity increased by approximately 50% the accumulation of H_2O_2 did not markedly decrease.

Analyses done during senescence of *A. thaliana* show that AtCat2 activity decreased with blotting time, this in parallel with APX1 and the H_2O_2 content increased (Zimmermann *et. al.*2006). Subsequently the AtCat3 was activated, APX1 activity was recovered and H_2O_2 levels decreased (Zimmermann *et. al.* 2006). This can be seen in figure 4.2. AtCat2 expression is down regulated during leaf senescence and AtCat3 expression is induced with plant age.

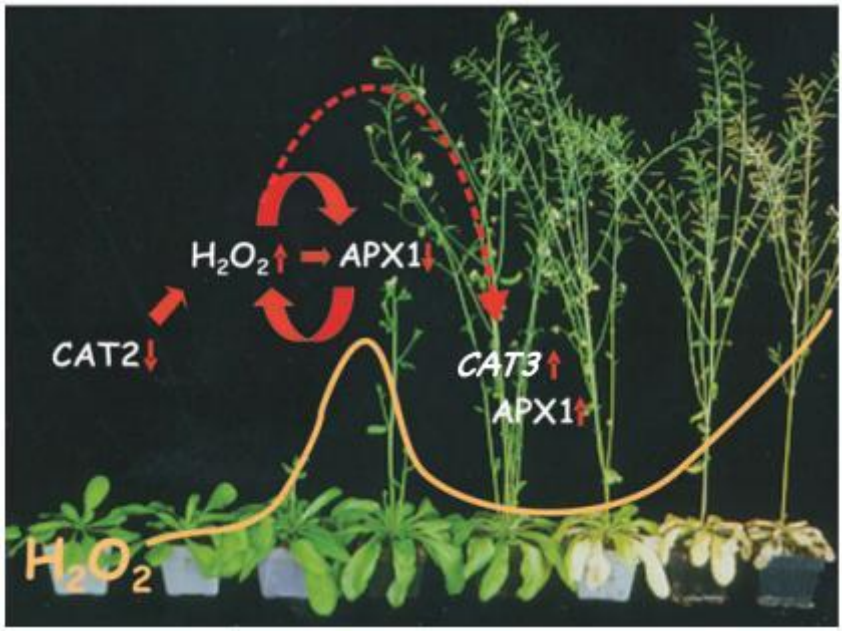


Figure 4.2: Model of the formation of H_2O_2 peak during blotting time in *A. thaliana.* (Zimmermann *et. al.* 2006). AtCat2 is down regulated as an initial step in elevating levels of H_2O_2. This might lead to the inactivation of APX1 activity that increase with H_2O_2 level. This might function as feedback amplification regulation by the post transcriptional inhibition of APX1 activity of H_2O_2. Levels of H_2O_2 raise and this increased the AtCat3 expression and activity. This raise in AtCat3 lowers the H_2O_2 level. (Zimmermann *et. al.* 2006).

The reason why AtCat2 was not detected by PCR analyses maybe since the samples was collected right after the shift in dark to light and the gene was not "turned on" yet. This is just a hypothesis. It could also be the RNA extraction, RT-PCR or the PCR analyses that is the reason for no detectable AtCat2 level. However it was strange that all of the samples did not show any expression of AtCat2. This suggests that the reason was not the extraction, RT-PCR of the PCR. The only variant that could affect all of the samples was the gene specific primers used in the PCR analyses. These primers was tested multiple times with positive control, where the positive control came up in all of the PCR runs but not the PCR done with sample as template. This confirms the suspicion that the reason for no expression of AtCat2 was not due to the PCR analyses.

4.4 Plant cultivation for making knock out mutant plants and plants with over-expressed AtCat2

For over- expression AtCat2 plants, plant transformation was done. AtCat2 (+ YFP) was cloned into the over-expression vector pBA002 and transformed into agro bacteria. The bacteria were up- cultivated, extracted from the media and used for making an infiltration media. Plants was dipped into the media and when the time was due, seeds was collected from the plants and spread on MS + ppt plates. Positive transformed plants grew on the media and were spouse to be used further. Seeds were intended to be collected from the positive plants and used further in stress treatment analyses. Due to time issues it was not possible to collect seeds from positive transformed plants and therefore stress treatment experiments could not be conducted.

Mutant seeds were also ordered. Seeds was sterilized and spread on MS+ kan and MS+ ppt plates. Mutant plants that contained the resistance against the antibiotic grew on the plates and were further transformed to soil. The mutant plants (lacking AtCat2) was cultivated in the green house (16/8 hr. light/dark cycle) for 3 weeks and then a leaf was used for DNA testing to see if the plant was a true mutant with no AtCat2. When time was due, seeds were collected and these seeds could be used for the stress treatment. To be absolutely sure that the plant was a knock out mutant the gel bands from the DNA testing should have been sent for sequencing. This was not done.

The intention with this experiment was to characterise the AtCat2 knock out mutant phenotype and to look at the phenotype of AtCat2 over- expression plant. It was also the intention to do stress treatment (perhaps with H_2O_2, high light and low light, high and low CO_2 levels) on mutant and OX plant and compare the result with those of WT. However as already told time did not allow for this experiment to be finish.

As far as analysing mutants and WT affected by stress, studies have shown that ABA-induced H_2O_2 lead to an clear increase in AtCat1 transcription levels and isoform activity (Du *et. al.* 2008, Xing *et. al.* 2008). Expression of AtCat3 activity enhanced under oxidative stress at the development stage, and also by a clear increase in AtCat3 activity (Orendi *et. al.* 2001).

AtCat2 activity however did not always correlate with AtCat2 activity expression (Orendi *et. al.* 2001, Queval *et. al.* 2007). Increasing the rate of photorespiration causes a significant increase in catalase activity by an increase in AtCat2 expression, but not an increase in AtCat2 transcription (Queval *et. al.* 2007). This suggests a mechanism that adjusts the catalase activity of AtCat2 at a post- transcriptional level (Queval *et. al.* 2007, Hu *et. al.* 2010).

Other studies show that plants grown under non- stress conditions (low levels of irradiation and high levels of CO_2) have AtCat2 protein levels that remain low, resulting in a optimal level of H_2O_2 signalling (Aple and Hirt 2004). When the plants were transferred to stress conditions (high irradiation and air) the protein level would increase (Queval *et. al.* 2007). This would increase AtCat2 by a twofold, that would protect the cell from H_2O_2 toxicity and also it would modulate the level of H_2O_2 to maintain the optimal redox state of antioxidants (Vanacker *et. al.* 2008).

4.5 Gene expression methods and analyses

4.5.*1 RNA extraction*

For RNA extraction it is important with high quality RNA for real time PCR and cDNA synthesis (reverse transcription PCR). It is important to always handle the sample with care and keep it under cool conditions (on ice). Before extracting the RNA the sample is kept at -80^{o}C and before extraction the samples were rapidly and completely grind in liquid nitrogen, to prevent degradation. A high quality RNA extraction kit also ensures high quality. After extraction the RNA is stored in RNase free water and the quality and quantity was measured.

RNA yields can be measured at absorbance at A260. This gives an easy and fast measurement of RNA. However other components can also give absorbance at A260. It is therefore important to measure the ratio A260/A280. This ratio is around 2 for pure RNA. This measurement tells that the RNA is of good integrity. If A260/A280 is < 2 this is an indication of protein impurity in the sample. The disadvantage with having protein in the RNA sample is that the protein can inhibit the cDNA synthesis and also PCR analyses. Another problem can

be DNA impurity in the RNA sample. In PCR genomic DNA in the sample also can be amplified.
RNA integrity can also be determined by gel electrophoresis.

4.5.2 *Reverse transcription (RT-PCR)*

Reverse transcription is a process where RNA is converted to cDNA, also called the cDNA synthesis. It is important to calculate the concentration of RNA to be used in the synthesis for making optimal cDNA concentration for real time PCR. To make 50 µg/ml cDNA (for use in real time PCR) 100µg/ml RNA was used in the synthesis. Random primers were used in the synthesis.

4.5.3 *Real time PCR*

In this thesis the non- specific fluorescent dye called SYBR green was used. This dye binds to double stranded DNA, and is therefore less specific than the other types of real –time PCR (for example TaqMan). The method of relative quantification was used in this thesis. This method of analysing gene expression allow for quantifying differences in the expression level of a specific gene between different samples. In this method an endogenous control gene had to be chosen to be able to normalize input amounts. The endogenous control chosen was the gene ubiquitin. The data input was expressed as a fold- change of expression level. In this thesis the gene expression after one week was set to be the reference time point and RQ (relative transcription levels) of gene expression at week two and four was represented as a fold- change of expression level from week one.
Measurements at week four gave very high standard aberration and therefore these results could not be fully trusted. Pour running of the endogenous control was the reason for the large standard aberration. At week two some of the measurements gave a negative standard aberration, this was also somehow strange because gene expression cannot be negative. To fully trust these results several new runs of real- time PCR have to be conducted.

4.5.4 *Molecular cloning*

Figure 4.3 show a flow scheme of the cloning process. Often the experiment does not go as planned and this has to be taken in consideration when working with molecular cloning. Figure 4.3 asks many important questions that one have to ask for getting a successful cloning result.

In this thesis this flow scheme was used for solving some of the problems that came about. In the lab work one problem was due to the digestion of the vector. This problem was solved by doing sequential digestion. Another problem was with AtCat1. For the vector XhoI and KpnI had to be used, but XhoI cut AtCat1 and this created some problem. Two approaches were attempted. The first one was to digest only with KpnI and treat the vector with SAP enzyme (dephosphorylation enzyme), making it difficult for the two ends to relegate. The other approach was to use compatible R.E. Both of the approaches should work, in theory, but the last one gave best results in the lab.

Another problem that was experienced in the thesis was that the PCR amplification was not always as strong. This was due to the home made taq- polymerase. In some PCR runes a higher volume of polymerase was needed for amplification. Using higher volume of polymerase than recommended was however a little bit risky, because if too much is used this could also inhibit the PCR.

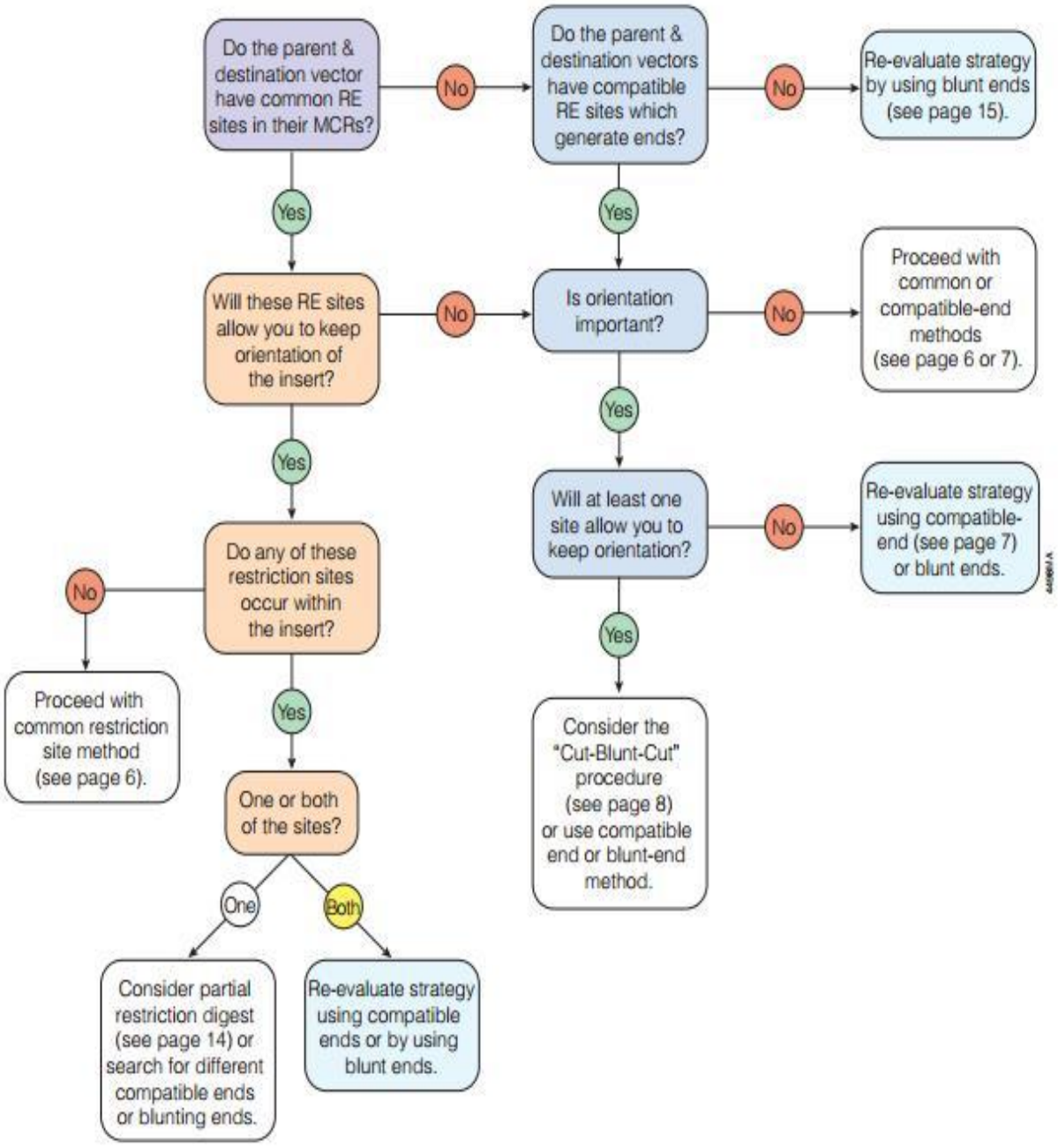


Figure 4.3: Molecular cloning flow scheme. While doing molecular cloning problems can come about. This flow scheme is a good guide to have. It asks important questions and gives a good reply to what the best way to go about is. [38]

[38] Promega notbook, Classic subcloning. http://www.promega.com/~/media/files/resources/product%20guides/subcloning%20notebook/classic_subcloning_row.pdf?la=en

4.5.5 *Plant cultivation*

Plants were cultivated in the growth room under UV-light in a 16/8 hr. light / dark cycle. All the plants used in this thesis were cultivated in this manner. Seeds were sterilized and spread on MS media containing the appropriate antibiotic. Before putting the MS plates with seeds in the growth room the plates were kept in the cold room for two days. After two weeks of cultivation on MS plates the seedlings was transformed to soil for further cultivation.

4.6 Further prospects

Further work on all the three variants of catalase in *A. thaliana* consist to find the precise localization to all of the genes. If one or more is found to be in cytocol it would be interesting to see if an interaction with cytosolic proteins could be found. If time allowed it, it would have been interesting to do cloning of AtCat2 into pWEN-18 again and see what results this gave in the microscope. If a cytosolic localization was found, it would also be interesting to see if the gene was actually active in the cytocol or just a result of pre- folded protein on its way to the peroxisomes. Work could also have been done on catalase for human, to see localization and to look at interaction pattern for this gene.
Any interaction found could be confirmed with the technique ITC and maybe with immune – precipitation (IP, a technique where antigen- antibody technology is used to identify a protein).

If more time was available the experiments with over expressed AtCat2 and knock out mutant would have been completed. Comparing phenotypes of WT, OX and mutant plants would have been interesting to look at. Also stress treatment would have been interesting to do. This would reveal how plants with normal (WT), high (OX- plants) and no (knockout mutant) AtCat2 behaved. This work could also be conducted for AtCat1 and AtCat3.

The three variants of catalase can also be cloned into the vector pET-28-a. This vector can be used for making protein. Purified protein could have been send for crystallization and this would have made it possible to conduct an assay for catalase. This would help to understand the function of the genes and could also be used for checking whether DJ-1 affect catalase function. (If an interaction was found.)

The proteins from catalase could also be analyzed by mass spectrometry (MS) to determine the element composition of the protein. Mass spectrometry (MS) can also explain the chemical structure of the peptide.

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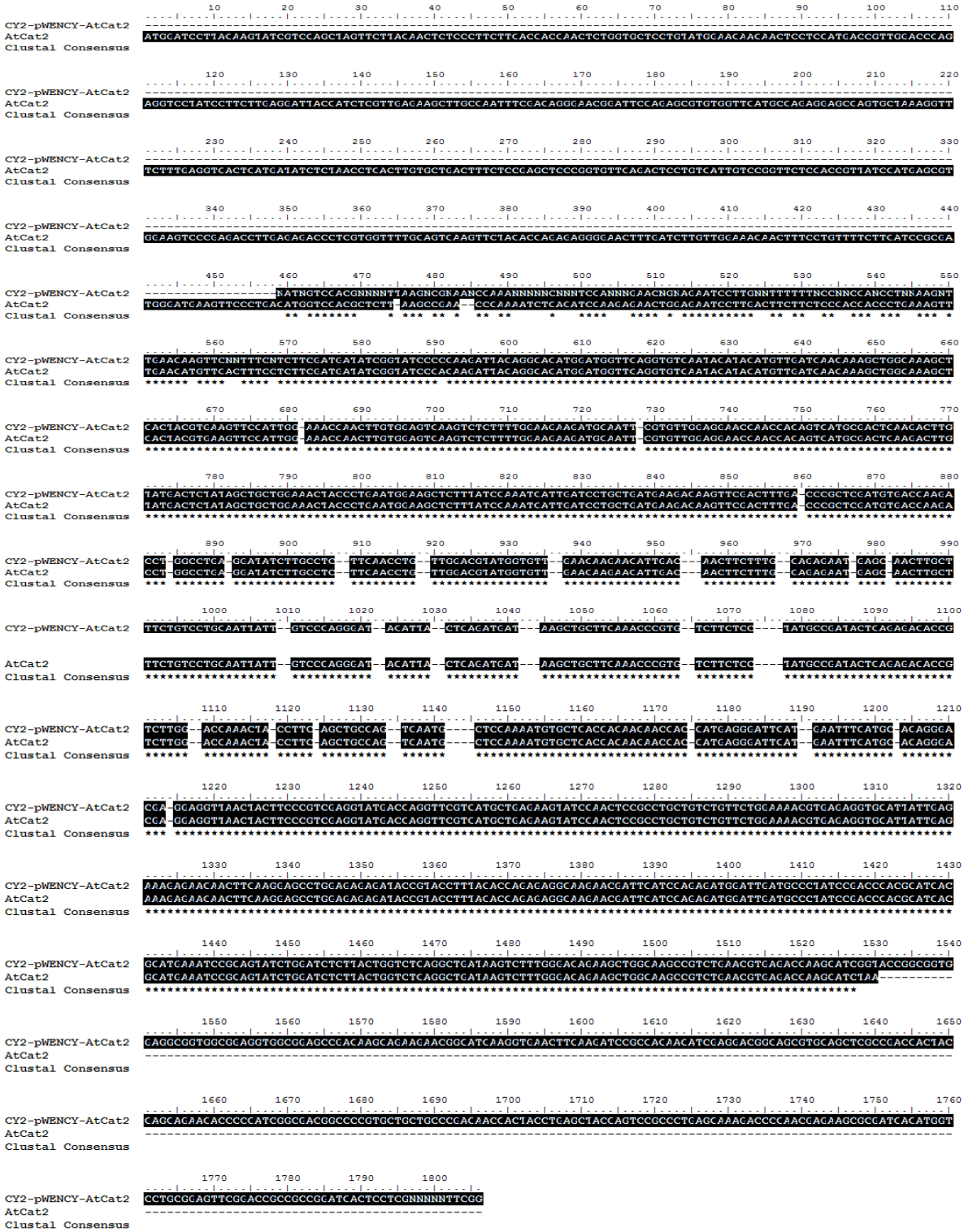
Appendix 1- sequencing results



Figur app.I : The figur show sequencing result from the vector pWEN-18-CY with AtCAt2 inserted. Primer used for sequencing was the vector specific primer CY2.

Figure app. II : The figur show sequencing result from the vector pWEN-18-CY with AtCAt2 inserted. Primer used for sequencing was the vector specific primer 35s.

Appendix 2 – Vector maps

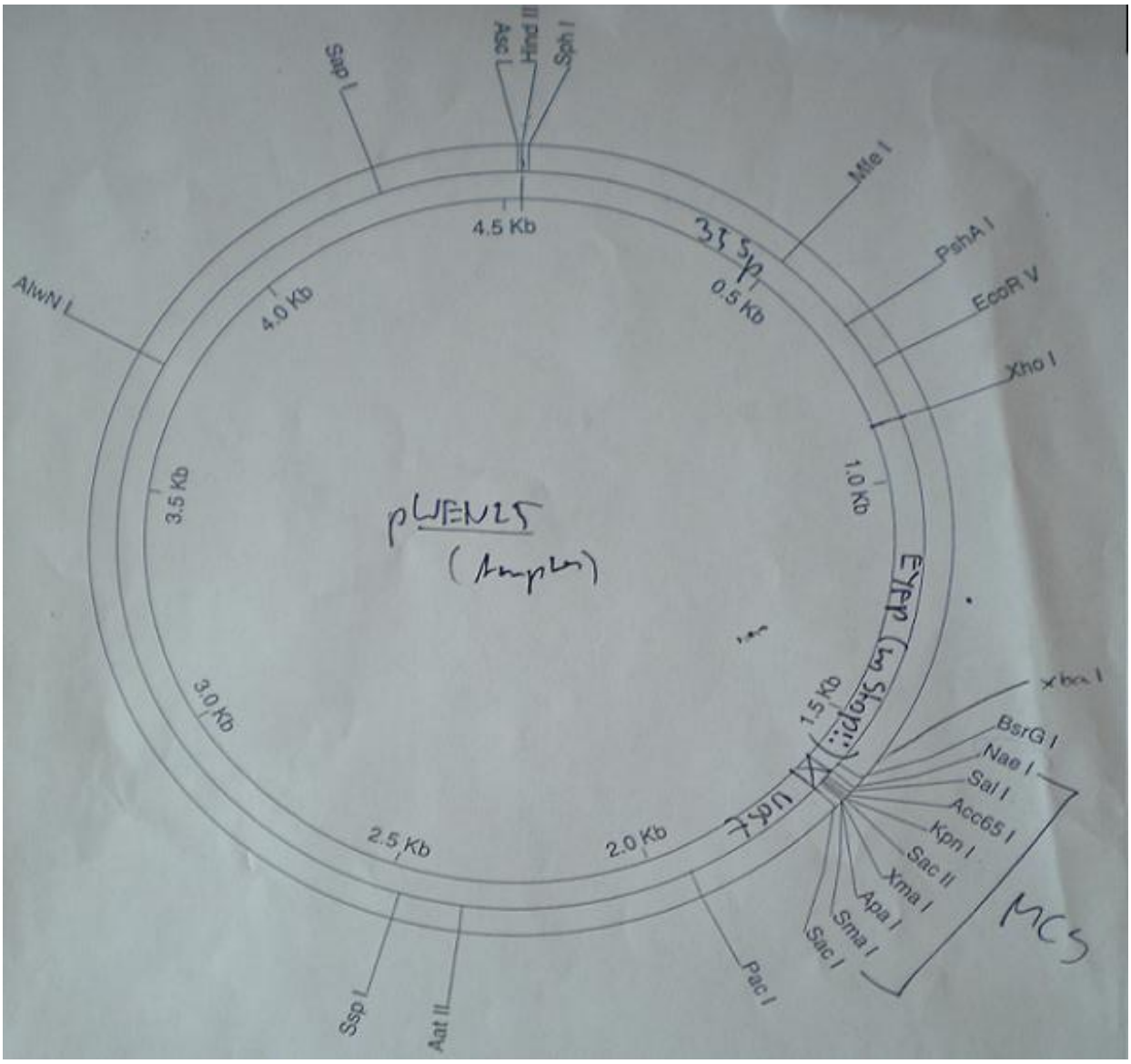


Figure app. III: Vector map for pWEN-25

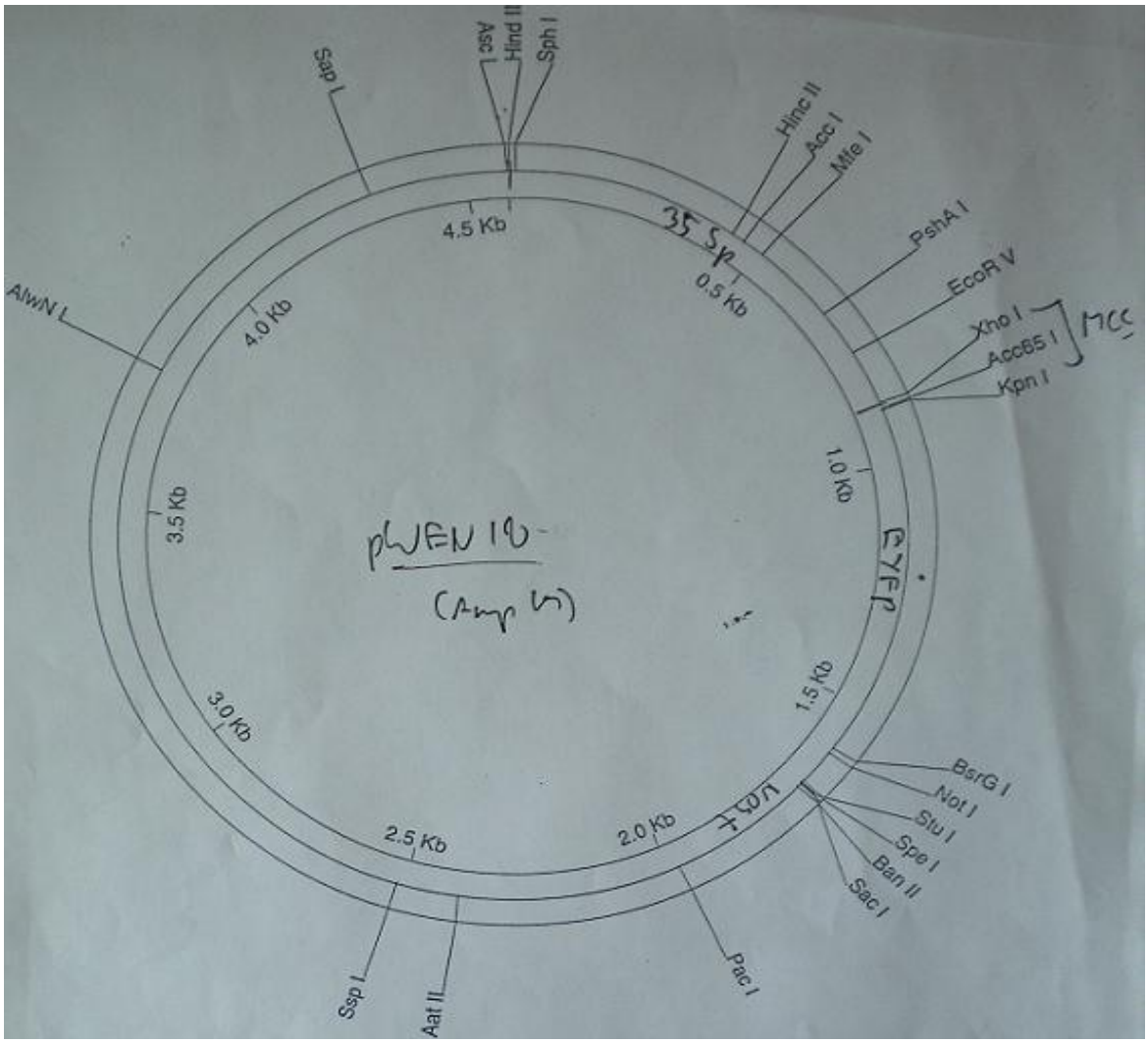


Figure app. IV Vector map of pWEN-18. The same map was used for pWEN-18-NY and for pWEN-18-CY. The only difference was that pWEN-18 had full YFP gene code, but pWEN-18-NY and pWEN-18-CY had half YFP gene code.

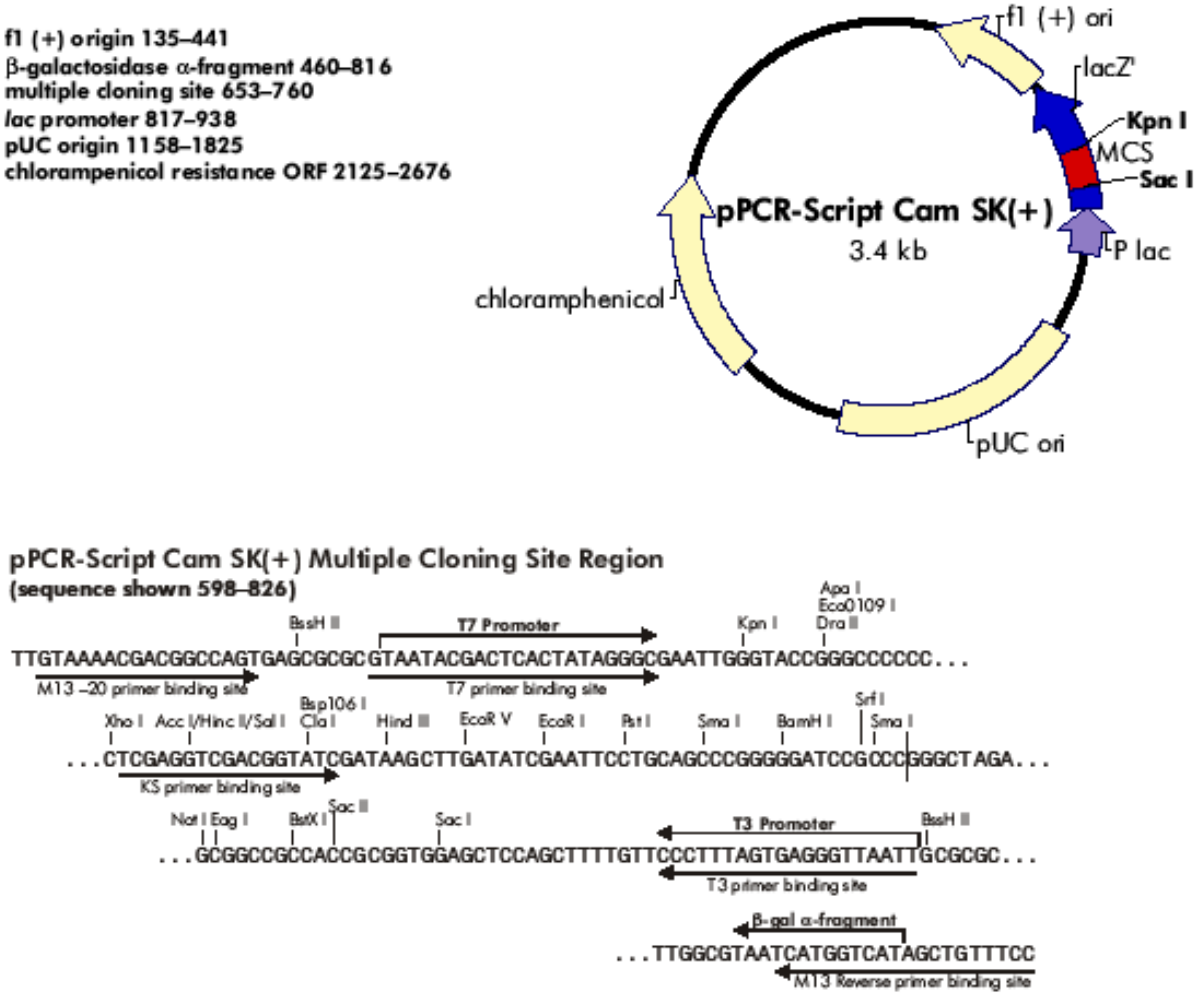


Figure app. V : Vector map for pPCR-script.

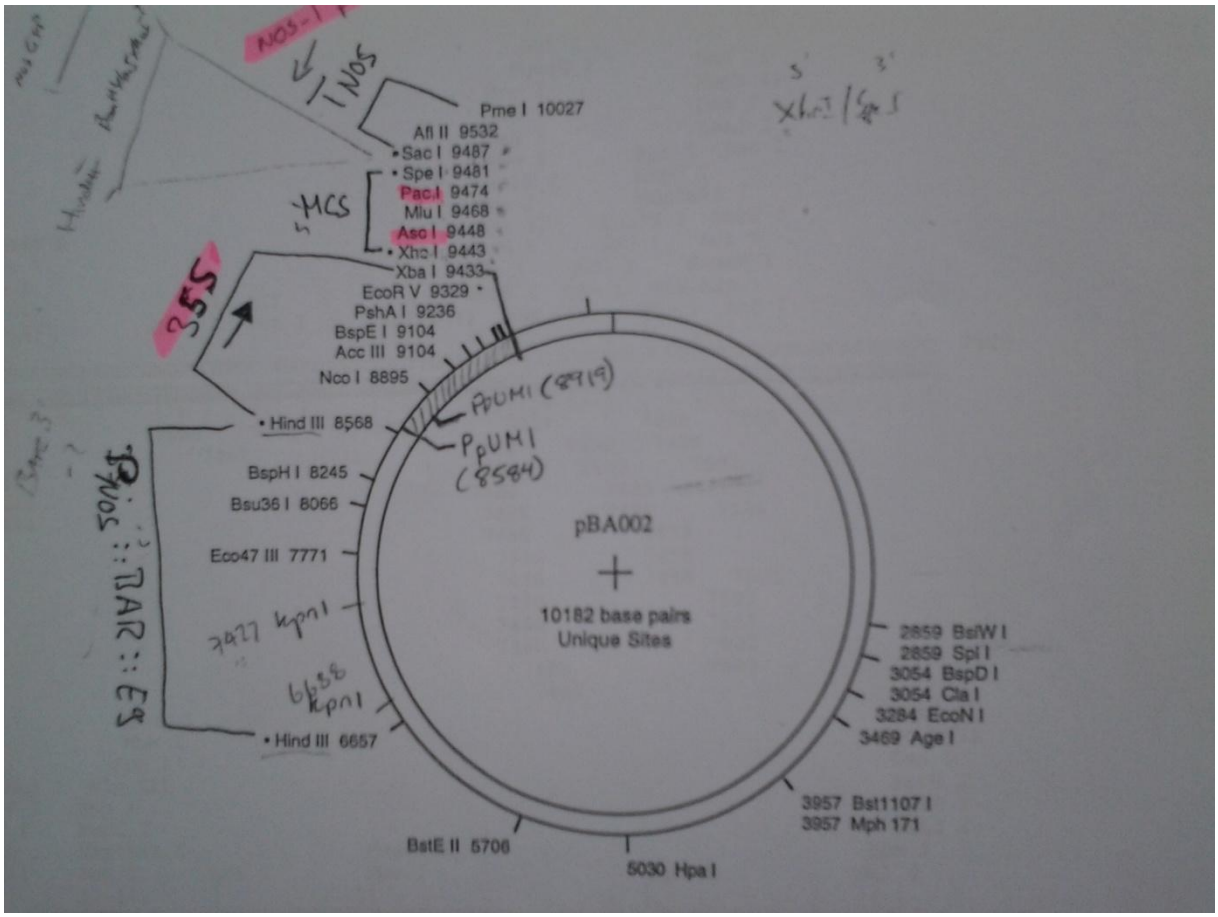


Figure app. VI: Vector map of pBA002.

Appendix 3: Primers used in the thesis.

Table I: Overview of primer psesifications.

Oligo name	Pur	MW	Tm^{o}	µg	GC%	µl for 100 µM
AtCat1-L	DST	6160	63.5	303	50	492
AtCat1-R	DST	6111	64,8	368	50	603
AtCat2-L	DST	6713	58,5	451	40,9	672
AtCat2-R	DST	6710	63,1	334	45,4	498
AtCat3-L	DST	7306	62,6	465	41,6	637
AtCat3-R	DST	5783	63,6	379	57,8	656
AtCat1-M-L	DST	6009	64,0	334	50	555
AtCat1-M-R	DST	6231	64,0	349	50	560
AtCat2-M-L	DST	6058	64,6	330	50	545
AtCat2-M-R	DST	6182	64,6	391	50	632
AtCat3-M-L	DST	6040	64,4	290	50	480
AtCat3-M-R	DST	6200	64,4	374	50	604
AtCat1-KpnI-L	DST	8937	74,1	552		
AtCat1-KpnI-R	DST	7981	73,1	519	50	650
AtCat1-SalI-L	DST	8937	75,4	417,4	48,2	467
AtCat2-KpnI-R	DST	8565	74,2	419	50	489
AtCat2-XhoI-L	DST	9489	69,9	607		
AtCAt2-SalI-L	DST	9489	71,2	463,1	41,9	488
AtCat3-KpnI-R	DST	8863	78,7	504,8	51,7	569
AtCat3-SalI-L	DST	8896	69,8	442,9	41,3	497

Table II: Primer sequence overview.

Oligo name	Primer sequence (5` → 3`)
AtCat1-L	ATGGATCCATACAGGGTTCG
AtCat1-R	TCAGAAGTTTGGCCTCACGT
AtCat2-L	ATGGATCCTTACAAGTATCGTC
AtCat2-R	TTAGATGCTTGGTCTCACGTTC
AtCat3-L	ATGGATCCTTACAAGTTATCGTCCT
AtCat3-R	CTAGATGCTTGGCCTCACG
AtCat1-M-L	AGAGATCTTTGGTGGCATGG
AtCat1-M-R	AGAGATCTTTGGTGGCATGG
AtCat2-M-L	AACCACAGTCATGCGACTCA
AtCat2-M-R	TGAGTCGCATGACTGTGGTT
AtCat3-M-L	TCATCCAGACCATGGATCCT
AtCat3-M-R	AGGATCCATGGTCTGGATGA
AtCat1-KpnI-L	ATTGGTACCATGGATCCATACAGGGTTCG
AtCat1-KpnI-R	ATTGGTACCGAAGTTTGGCCTCACGT
AtCat1-SalI-L	ATTGTCGACATGGATCCATACAGGGTTCG
AtCat2-KpnI-R	ATTGGTACCGATGCTTGGTCTCACGTTG
AtCat2-XhoI-L	ATTCTCGAGATGGATCCTTACAAGTATCGTC
AtCAt2-SalI-L	ATTGTTCGACATGGATCCTTACAAGTATCGTC
AtCat3-KpnI-R	ATTGGTACCGATGCTTGGCCTTCACGTTCA
AtCat3-SalI-L	ATTGTCGACATGGATCCTTACAAGTATCG