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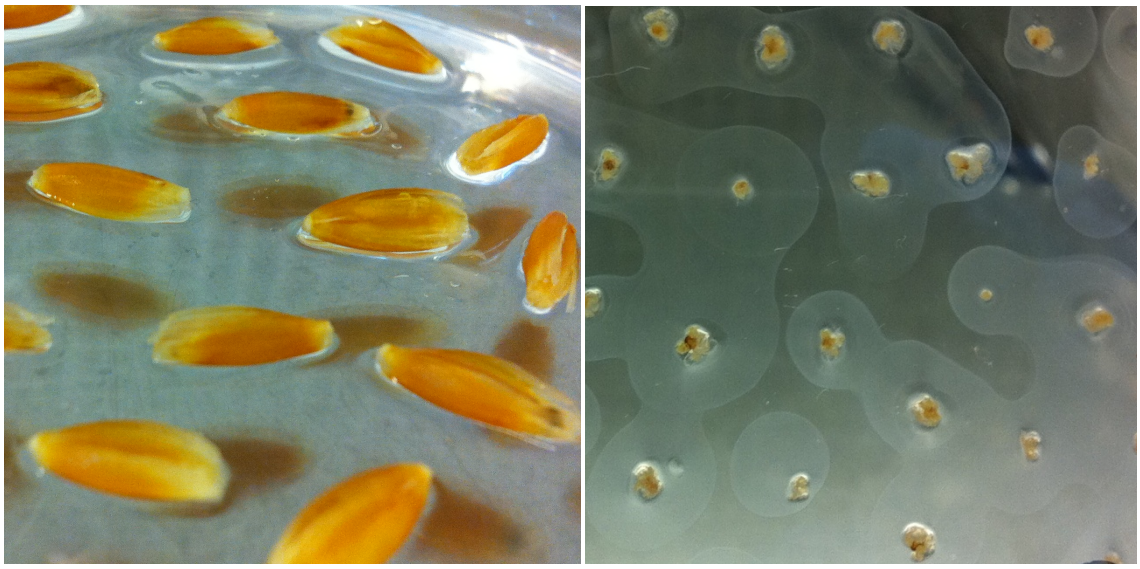
Factors affecting *Agrobacterium* transformation in oat



by

Emelie Ivarson

Swedish University of Agricultural Sciences

Faculty of Landscape Planning, Horticulture and Agricultural Sciences

Department of Plant Breeding and Biotechnology

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SLU, Swedish University of Agricultural Sciences
Faculty of Landscape Planning, Horticulture and Agricultural Sciences
Department of Plant Breeding and Biotechnology

Author Emelie Ivarson

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Supervisor Li-Hua Zhu
Department of Plant Breeding and Biotechnology, SLU

Examiner Anders Carlsson
Department of Plant Breeding and Biotechnology, SLU

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Cover illustration: Sterilized oat seeds and explants of oat infected with a suspension of *Agrobacterium*.
Photo: Emelie Ivarson

Preface and Acknowledgements

Associate Professor Li-Hua Zhu and Associate Professor Anders Carlsson at the Department of Plant Breeding and Biotechnology at SLU, Alnarp, initiated this master project. The project is based on earlier studies in the field.

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Abstract

Oat (*Avena sativa* L.) is one of the crops that have been cultivated by mankind for the longest time (Lásztity, 1998) and today it is an important and traditional agricultural crop in Northern Europe (Bräutigam et al., 2005). In Sweden, most of the oat is used as feed (Bräutigam et al., 2005), but with a superior amino acid composition of the oat proteins (Lásztity, 1998), a high content of desirable soluble fibers (β-glucans), essential vitamins and minerals (Sadiq Butt et al., 2008) and antioxidants (Ryan et al. 2007) the interest in using oats for human consumption has increased (Carlsson, Personal conversation). In comparison to other cereals, oat has a much higher content of lipids. The oil is interesting because its energy content is high, while its content of saturated fatty acids is relatively low. A disadvantage, which may prevent an increase in using oat as food, is the imbalance of Omega-6/Omega-3 fatty acids. The amount of Omega-6 fatty acids is much higher than the amount of Omega-3 fatty acids (Welch & Legett, 1997). The imbalance in the fatty acid composition has shown to be a possible factor behind the increasing number of cases of cardiovascular disease, cancer, diabetes, asthma, depression, obesity, autoimmune diseases and rheumatism in the western countries (Simopoulos, 2004).

With the help of transformation, it is possible to increase the amount of Omega-3 fatty acids, and thus get a better Omega-6/Omega-3-balance in oat. Omega-3 fatty acids have also shown to be potential therapeutic agents for autoimmune and inflammatory diseases (Simopoulos, 2002), the main reason behind the increased interest in using oat as food.

Transformation allows an increase in Omega-3 fatty acids in oats, but previous studies concerning transformation of *Avena sativa* L. has resulted in low transformation frequencies. Oat is a monocotyledonous crop, not a normal host for *Agrobacterium;* it is thus difficult to be infected. The conditions have to be optimal for a successful transformation to occur.

The aim of this work has been to examine various factors affecting oat transformation, and to develop a functional transformation protocol.

Explants from the hypocotyl of the oat cultivar Matilda were infected by *Agrobacterium*-mediated transformation. In total, 15 batches of transformation were carried out, in which different combinations of bacterial strains, vectors and media were tested. GUS- and GFP assays were conducted to confirm Agrobacterium infection of the explants.

No GUS expression was achieved in the GUS assays, but no certain conclusion can be drawn from the result. Endogenous GUS-like activity is triggered by low pH-values, but a

raised pH-value may not only suppress the expression of endogenous GUS, but also the expression of true GUS.

The explants analyzed for GFP expression exhibited whitish-colored spots, but further cultivation and repeated assays of the explants is necessary to confirm GFP expression.

Further trials are needed in order to obtain a well-functioning oat transformation protocol.

Sammanfattning

Havre är en av de grödor som människan odlat under längst tid (Lásztity, 1998) och är idag en viktig och traditionell jordbruksgröda i Norra Europa (Bräutigam et al., 2005). I Sverige används den största delen av havreskörden som foder (Bräutigam et al., 2005), men havreproteinernas goda aminosyresammansättning (Lásztity, 1998), ett högt innehåll av önskvärda lösliga fibrer (β-glucans), viktiga vitaminer och mineraler (Sadiq Butt et al., 2008) samt antioxidanter (Ryan et al. 2007) gör att intresset för att använda havre som människoföda har ökat (Carlsson, Personal conversation).

I jämförelse med andra cerealier har havre ett mycket högre lipidinnehåll. Oljan är intressant eftersom dess energiinnehåll är högt samtidigt som dess innehåll av mättade fettsyror är relativt lågt. En nackdel, som eventuellt kan hindra ett ökat användande av havre som föda, är dess obalans i Omega-6/Omega-3-kvoten. Mängden Omega-6 fettsyror är mycket högre än mängden Omega-3 fettsyror (Welch & Legett, 1997). En obalans i fettsyresammansättningen har visat sig vara en möjlig faktor bakom det ökande antalet fall av hjärt-kärlsjukdomar, cancer, diabetes, astma, depression, övervikt, autoimmuna sjukdomar och reumatism i västländerna (Simopoulos, 2004).

Med hjälp av transformering finns det en möjlighet att höja mängden Omega-3-fettsyror, och därmed få en bättre Omega-6/Omega-3-balans i havre. Eftersom Omega-3-fettsyror har visat sig vara potentiella terapeutiska agenter mot autoimmuna- och inflammatoriska sjukdomar (Simopoulos, 2002), bör ett ökat Omega-3-fettsyreinnehåll leda till ett större intresse av att använda havre som föda.

Transformering möjliggör en ökning av Omega-3-fettsyror i havre, men tidigare studier gällande transformering av *Avena sativa* L. har resulterat i låga transformeringsfrekvenser. Monokotyledoner är inte naturliga värdar för *Agrobacterium*, varför dessa är svårare än dikotyledoner att infektera. Förhållandena måste vara optimala för att en transformering ska ge ett lyckat resultat.

Målet med detta arbete har varit att ta reda på vilka faktorer som påverkar havretransformering, samt att arbeta fram ett transformeringsprotokoll som ger höga transformeringsfrekvenser.

Hypokotylexplantat från havresorten Matilda transformerades med hjälp av *Agrobacterium*. I försöket utfördes 15 transformeringsomgångar, där olika kombinationer av

bakteriestammar, vektorer och medier testades. GUS- och GFP-analyser genomfördes för att bekräfta att transformeringarna lyckats.

Vid analyserna av GUS-uttryck uppvisade inget av explantaten något GUS-uttryck, men det går inte att dra någon säker slutsats av resultatet. Låga pH-värden triggar uttryck av endogen GUS-liknande aktivitet, men ett ökat pH-värde kanske inte bara undantrycker uttryck av endogent GUS utan även uttryck av riktigt GUS.

Explantaten som analyserades för uttryck av GFP uppvisade vitaktiga fläckar, men ytterligare odling och analyser av explantaten krävs för att kunna bekräfta uttryck av GFP.

Fler försök krävs för att hitta ett välfungerande havretransformeringsprotokoll.

Introduction

Oat

Oat is a member of the Poaceae family (Kellogg, 1998). The cultivars used in cultivation are hexaploids (Bennet & Smith, 1991). Oat has a long history in cultivation, being one of the crops cultivated by mankind for longest time (Lásztity, 1998). In 2009 the world production of oat was estimated to 23 millions of tonnes, with Russia being the greatest producer (FAOSTAT, 2011).

In Northern Europe oat is an important and traditional agricultural crop (Bräutigam et al., 2005). In Sweden most of the oat is used as animal feed (Bräutigam et al., 2005), but the recent high oil prices and the low oat prices have led to an increase in using oat in combustion. A minor part of the oat production (approximately 5 %) is used as food. This part is important though, since several parts of the food chain are affected. The greatest part of the production is based on contracts, where both traders and producers are involved (Carlsson, Personal conversation).

The nutritional advantage of oat is being more and more emphasized, which has led to an increase rather than a decrease in using oat as food (Carlsson, Personal conversation). In comparison to proteins of other cereals, the amino acid composition of the oat proteins is nutritionally superior (Lásztity, 1998). Oat also has a high content of desirable soluble fibers (β-glucans), important vitamins and minerals (Sadiq Butt et al., 2008) and antioxidants (Ryan et al., 2007).

Compared to other cereals, oat has a much higher content of lipids; reaching from 2-15.5 %, depending on environmental and genetic factors as well as the method chosen for determination (Zhou et al., 1999). The oil is interesting since it has high energy content and a relatively low portion of saturated fatty acids. A drawback, which may hinder a future increase in using oat as feed and food, is its imbalance in Omega-6/Omega-3 fatty acids. The level of the unsaturated fatty acid 18:2 (ω-6) is much higher (36-47 %:1-2 %) than the level of the unsaturated fatty acid 18:3 (ω-3) (Welch & Legett, 1997). Studies have shown that an imbalance in the fatty acid composition is a possible factor behind the increasingly frequent cases of cardiovascular disease, cancer, diabetes, asthma, depression, obesity, autoimmune diseases and rheumatism in the Western countries (Simopoulos, 2004).

Omega-3- & Omega-6 Fatty Acids

Polyunsaturated fatty acids (PUFAs) are an important part of our diet. On basis of the location of their first double bond, they are divided into two subcategories: omega-3 (n-3) and omega-6 (n-6) fatty acids. The first double bond is found on the third carbon molecule on the omega-3 fatty acids, while it is situated on the sixth carbon molecule on the omega-6 fatty acids (Lee & Lip, 2003).

Studies have shown that omega-3 fatty acids are capable of modifying inflammatory and immune reactions, which makes them potential therapeutic agents for autoimmune and inflammatory diseases (Simopoulos, 2002).

PUFAs are classified as essential nutrients for human health since mammals lack the compounds involved in the synthesis of PUFAs. The western diet contains a sufficient amount of omega-6 fatty acids, but the level of omega-3 fatty acids is generally much lower. Today, plant oils constitute the main source of omega-6 fatty acids while fish- and algal oils are the sources richest in omega-3 fatty acids. Since fish- and algal oils are not always suitable or economical for human use, a more economically dietary source of omega-3 fatty acids is of commercial interest (Pereira et al., 2004).

Genetic improvement

Oat improvement by conventional breeding

Traditional breeding by crossing is a common way for breeding new cultivars, in which one donor plant with the trait of interest is crossed with a recipient plant with only one or few drawbacks. The problem with crossing is that the whole genomes of both plants are mixed and recombinant, leading to an incorporation of both wanted and unwanted genes. In order to achieve an improved variety, several backcrosses are necessary to get rid of the unwanted genes/traits (Roberts, 1984).

Genetic transformation

Genetic modification is an efficient and straightforward method for directly introducing novel genes, conferring desirable traits, into the target plant genome. In combination with conventional breeding programs, transformation enables insertion of transgenes encoding useful traits into crops within a workable time frame. By genetic manipulation, the productivity of crops can be greatly improved through increased resistance against diseases,

pests and environmental stress factors together with a qualitative change of the seed composition. By designing plants that produce high volumes of pharmaceuticals, nutraceuticals and other beneficial substances the nutritional value of crops can be improved. In addition to the possibility of improving crops, transformation also enables study of gene function and the regulation of physiological and developmental processes (Hansen & Wright, 1999). Genetic transformation also enables analysis and understanding of the underlying mechanisms behind expression of transgenes or endogenous genes (Gasparis et al., 2008).

Plant genetic transformation consists mainly of two methods: biolistic and *Agrobacterium* transformation. In most applications, the *Agrobacterium*-mediated method is the most reasonable one (Gasparis et al., 2008).

Agrobacterium-mediated transformation

Binns & Thomashow (1988) discovered that the tumor-inducing (Ti) plasmid of *Agrobacterium* is capable of transferring a DNA segment (T-DNA) into the nucleus of the host plant cell. The T-DNA transfer is controlled by border sequences on the T-DNA. The finding made plant genetic transformation via *A. tumefaciens* possible.

A transfer of the T-DNA is not possible without expression of the *vir* genes located on the Ti-plasmid in the bacterium. Only a few *vir* genes are expressed under normal bacterial growth conditions, while most of them are induced by plant cell exudates. In uninjured plants the plant cell exudates (phenolic compounds such as acetosyringone) that trigger the expression of *vir* genes are present only at very low levels. In order to increase the level of such compounds, the tissue has to be wounded. The increased level of cell exudates triggers *A. tumefaciens* to initiate vir gene expression and related responses that are necessary for a successful plant cell transformation (As reviewed by Binns & Thomashow, 1988).

The capacity that *A. tumefaciens* is capable of sensing the phenolics probably results in a recruitment of the bacterium to the regions of wounded tissues (As reviewed by Binns & Thomashow, 1988).

The T-DNA contains two types of genes; the oncogenes and the opine biosynthetic genes (Binns & Thomashow, 1988). The oncogenes are encoding enzymes that are involved in the production of auxins and cytokinins, resulting in the tumor structures (Opabode, 2006). The opine biosynthetic genes catalyze the production of unusual amino acids and sugar derivatives (opines), which are used by the bacteria either as a carbon and

nitrogen source or as an inducer of plasmid transfer between bacteria (Binns & Thomashow, 1988).

Biolistic transformation – Particle bombardment

Particle bombardment is a technique enabling a direct transfer of genetic material into plant tissues. The principal of this method is that DNA or RNA is coated to particles of gold or tungsten and shooted into the target tissue with help of streams of pressurized helium, (Ziolkowski, 2007).

Particle bombardment is one of the techniques that has made it possible to introduce traits that are of agricultural value to crops, such as insect resistance, leading to an increase in both productivity and efficiency of the crop (Ziolkowski, 2007). However, this method often results in low transformation efficiency and multiple copies of transgene integration.

Factors affecting *Agrobacterium* transformation of monocots

Monocotyledons are not natural hosts of *Agrobacterium* (De Cleene & De Ley, 1976), which is why *Agrobacterium*-mediated transformation of monocots has been very difficult and unreliable (Sood et al., 2011). A great number of factors affect *Agrobacterium* transformation, which often makes the development of a new transformation protocol for a given species a difficult and time-consuming process, especially for monocotyledonous species.

Genotype, age & physiological state of explants

Genotype variation in regeneration has been reported in many plant species and this has also been found to be true for oat (Gasparis et al., 2008). The age and the physiological state of the explant also greatly affect the transformation result. The cells that are receiving the transgene need to recover quickly from the shock that the transformation event brings about. Apart from a fast recovery, the cells have to be competent for regeneration and be able to grow into a complete plant. The recovery of the infected cells has shown to be very difficult in monocots, why the focus of earlier trials to a great extent has been on optimization of the factors influencing the plant regeneration capacity (as reviewed by Sood et al., 2011).

Agrobacterium strains & vectors

The choice of bacterium strains and vectors has been show to be of great importance in transformation of monocotyledons. Only a few *Agrobacterium* strains have resulted in successful transformations of monocotyledons. The *Agrobacterium* strain A281 is a so-called supervirulent strain with a wide host-range and an inducement of large tumors (Wei et al. 2000) due to its additional *vir* genes (Jones et al. 2005).

Agrobacterium attachment

A surfactant is a type of wetting agent that has shown to increase the efficiency of the T-DNA delivery in immature embryos of wheat (Cheng et al., 1997). The surfactant facilitates the attachment of *Agrobacterium* to the surface and/or eliminates substances that inhibit attachment of the bacterium (as reviewed in Opabode, 2006). Apart from chemical agents and surfactants such as Tween 20, Silwet L77 and Pluronic acid F68, (as reviewed in Opabode, 2006), an optimal density of *Agrobacterium* can also facilitate the attachment of the bacterium (as reviewed in Sood et al., 2011).

Co-cultivation

The duration, temperature, irradiance, medium composition and pH need to be optimal during co-cultivation, since this step comprises the delivery and the integration of the T-DNA. Optimization of parameters as medium strength, sugars, *vir* inducing chemicals and plant growth regulators have resulted in successful transformations of monocots. A reduce in the salt strength of inoculation- and co-culture media has proven to result in a more successful transformation of wheat (as reviewed in Sood et al., 2011).

Elimination of residual *Agrobacterium*

High levels of *Agrobacterium* can lead to necrosis and bacterial overgrowth of the transformants. To obtain a good recovery of the transformants and a higher efficiency of the transformation, it is important to get rid of residual *Agrobacterium* (as reviewed in Sood et al., 2011).

Current state of oat transformation

Agrobacterium-mediated transformation of oat (Avena sativa L.) cultivars via immature embryo and leaf explants

In a trial, Gasparis et al. (2008) transformed oat using *Agrobacterium* with immature embryos or leaf base segments as explants of three different cultivars and three combinations of strain/vector in combination with different selection genes. Among all different combinations, only one of the strain/vector combinations resulted in transgenic plants. The highest transformation rate generated by one of the three cultivars was 12.3 % for the immature embryo explants and 8.2 % for the leaf base segment explants. For the other two cultivars, the transformation rates were 1.1 and 3.4 % respectively and transgenic plants were only recovered from the immature embryos.

In a second step of the trial, Gasparis et al. (2008) evaluated the suitability of the pGreen binary vector in oat transformation. The vector was combined with four different selection cassettes: nos::nptII, 35S::nptII, nos::bar and 35S::bar. All cassettes except one (35S::bar) generated putative transgenic plants. The highest transformation efficiency achieved was 5.3 %.

Project aim

Earlier trials concerning transformation of oat have resulted in low transformation frequencies or no success at all. In order to enable efficient modification of important traits, such as omega-3 fatty acid in oat, a well-functional transformation protocol must be first developed.

The aim of this project was to evaluate several factors affecting *Agrobacterium*-mediated transformation of oat. Furthermore, the aim was also to obtain a functional transformation protocol.

Material & Method

Plant material

The plant material was seeds of the oat cultivar *Matilda,* which were kindly provided by Svalöv Weibull.

Strains and Vectors

Different combinations of strain/vector were used to find the combination optimal for a successful transformation. Among them, most combinations (EHA101/pSCV1.6,

LBA4404/pSCV1.6, GV3850/pSCV1.6, GV3101/pCW498GFP, EHA105/pBract, AGL1/pW33 (without KCS), LBA4404/pAC12 and LBA4404/pAC10 se1) were ready to use, while others (LBA4404/pCW498GFP andAGL1/pCW498GFP) were prepared in this study prior to trial.

Transformation of pCW498GFP into *Agrobacterium* by electroporation

The strain/vector combination: LBA4404/pCW498GFP was prepared by use of electroporation.

The prepared competent cells of *Agrobacterium* were added to microfuge tubes and incubated on ice for 60 seconds. The cell mixture was then pipetted into cold electroporation cuvettes, which then were placed in the electroporation device. A pulse of 25 μF capacitance, 2.5 kV, and 200 Ohm resistance was delivered by the apparatus. After the pulse, the cuvette was removed and, at room temperature, 1 ml of SOC medium was quickly added. The cells were transferred to tubes and the cultures were incubated for one hour at 28°C with gentle rotation.

After incubation, the cells were placed onto Petri dishes containing LB medium supplemented with antibiotics. When the liquid was totally absorbed by the medium, the plates were inverted and placed in an incubator (28°C) until colonies appeared.

Transformation of plasmids into *Agrobacterium* by freeze and thaw method

The strain/vector combinations: AGL1/pCW498GFP and LBA4404/pCW498GFP were also prepared by freeze and thaw transformation method.

The prepared competent cells of *Agrobacterium* were thawed on ice for 2-5 minutes and then transferred to sterile tubes. Plasmid DNA (0.5 μg) was added to the tubes and mixed gently. The tubes were incubated on ice for 40 minutes, and then the *Agrobacterium*-cells were heat-shocked at 37°C for 5 minutes. The tubes were moved to ice and allowed to incubate for 20 minutes. After incubation, 0,5 ml liquid LB was added to the tubes before the cells were incubated at 28°C for three hours (Sambrook & Russell, 2001).

After incubation, the culture solution was pipetted into Petri dishes containing LB medium supplemented with antibiotics. The plates were allowed to absorb the solution totally before they were incubated at 28°C until single colonies appeared (Sambrook & Russell, 2001).

Small scale preparation of plasmid DNA

Single colonies were cultivated in LB medium supplemented with antibiotics over night. Half of the amount bacterial solution (1.5 ml) was pipetted into Eppendorf tubes and centrifuged at 12 000 rpm and 4°C for 30 seconds. The supernatant was discarded and the rest of the bacterial solution (1.5 ml) was added to the tubes, and then centrifuged as above. The supernatant was poured off and the tube with tissues was allowed to dry. The pellet was then suspended in 100 µl of ice-cold solution I (50 mM Glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)) and vortexed vigorously until the pellet was totally dispersed. Solution II (0.2 N NaOH and 1 % SDS) was prepared and 200 µl of the solution was added to the tube. The content was mixed gently by five rapid inversions of the tube. The tube was stored on ice for five minutes before 150 µl of ice-cold solution III (5 M potassium acetate, glacial acetic acid and H_2O) was added. The content was mixed by 10 seconds of gentle inversion of the tube. The tube was stored on ice for five minutes, and then centrifuged at 12 000 rpm and 4°C for five minutes. The supernatant was transferred to a fresh tube and an equal volume of phenol:chloroform (1:1) solution was added, and the content was mixed vigorously by hand. The tube was centrifuged again as above, and the supernatant was transferred to a new tube. An equal volume of chloroform solution was added, the tube was centrifuged as earlier and then the supernatant was transferred to a fresh tube. The double stranded DNA was precipitated in two volumes of 100 % ethanol at room temperature (RT). The tube was vortexed and then allowed to stand for two minutes at RT before it was centrifuged at 12 000 rpm at 4°C for five minutes. The supernatant was discarded and the tube was placed in an inverted position on a paper towel to enable drainage of all the fluid. The DNA pellet was rinsed with 1 ml of 70 % ethanol at 4°C. The pellet was allowed to dry in a laminar flow cabinet for approximately 15 minutes and then redissolved in 50 µl of TE (pH 8.0) containing RNase (100 µg/ml). After a brief vortexing, the tube was left at 37°C for one hour. Prior to PCR analysis the DNA concentration was measured by nano-drop and, in cases necessary, diluted in TE-buffer.

PCR and Gel Electrophoresis

A PCR was run to amplify the DNA, which then was analyzed on a gel to confirm a successful transformation of *Agrobacterium*.

Factors affectiong *Agrobacterium*-mediated transformation tested

Strains and vectors

Nine different combinations of strain/vector were utilized to find out if there is any difference in regeneration and transformation efficiency among different combinations.

Batch 1 was transformed with EHA101/pSCV1.6, half of batch 2 was transformed with LBA4404/pSCV1.6 and the other half with GV3850/pSCV1.6, batch 3-5 and 7 were transformed with LBA4404/pSCV1.6, batch 6 with LBA4404/pCW498GFP, batch 8-10 with GV3101/pCW498GFP, batch 11-13 with GV3850/pSCV1.6, in batch 14 one third was transformed with GV3850/pSCV1.6, one third with LBA4404/pSCV1.6 and one third with AGL1/pSCV1.6. The last batch was divided into four, with one fourth transformed with EHA105/pBract, one fourth with AGL1/pW33 (without KCS), one fourth with LBA4404/pAC12 and one fourth with LBA4404/pAC10 se 1.

Medium composition, selection agent and light conditions

To see the effect of light conditions on regeneration, some of the batches were placed in dark, while others in light.

An addition of copper in media has shown to have a positive effect on both callus induction and plantlet regeneration (Nirwan & Kothari, 2003). Copper sulphate was added in some of the selection media in this study to evaluate if it also has a possible effect on oat regeneration and transformation.

Some plant species are sensitive to the selection agent kanamycin, leading to inhibition of growth and development (as reviewed by Mihaljević et al., 2001). Paromomycin is an aminoglycoside analog to kanamycin (as reviewed by Mihaljević et al., 2001), and was used in selection of some of the batches to compare the regeneration capacity between the two selection agents.

The first six batches of transformation were placed on a callus induction medium with selection (Full MS (Murashige & Skoog, 1962) with Gamborg's (Gamborg et al., 1968) vitamins, 30 g l^{-1} sucrose, 2 g l^{-1} gelrite, 3 mg l^{-1} 2,4D, 150 mg l^{-1} timentin, 50 mg l^{-1} kanamycin, pH 5.8) (Gasparis et al., 2008, modified by Leonova, personal communication), and placed in dark. The following six batches were instead placed directly either on a shoot

induction medium with selection (Full MS (Murashige & Skoog, 1962), Gamborg's vitamins (Gamborg et al., 1968), 30 g l^{-1} sucrose, 500 mg l^{-1} MES, 2 g l^{-1} gelrite, 0,2 mg l^{-1} IAA, 1 mg l^{-1} BAP, 50 mg l^{-1} kanamycin, 150 mg l^{-1} timentin, pH 5.8) (Gasparis et al., 2008, modified by Leonova) or that medium supplemented with 5 µM $CuSO_4$. Half of the explants were placed in dark and half of them were placed in light. The last three batches were placed either on shoot induction medium with selection (same as earlier, but this time with 50 mg l^{-1} paromomycin instead of kanamycin) or on callus induction medium with selection (same as earlier, but with 50 mg l^{-1} paromomycin instead of kanamycin). All of the explants were placed in dark.

Table 1. Different strain/vector combinations used in different transformation batches.

Strain/vector	**Batch nr.**
EHA101/pSCV1.6	1
LBA4404/pSCV1.6	1/2 2, 3-5 & 7
GV3850/pSCV1.6	1/2 2, 11-14
LBA4404/pCW498GFP	6
GV3101/pCW498GFP	8 & 9
EHA105/pBract	1/4 15
AGL1/FW33	1/4 15
LBA4404/pAC12	1/4 15
LBA4404/pAC10 se 1	1/4 15

Table 2. Schematic view over the different treatments with different light conditions and medium compositions.

Selection week 1-3	**Light/dark**	**Selection week 4-6**	**Light/dark**	**Selection week 7-9**	**Light/dark**	**Selection week 10-12**	**Light/dark**	**Batch nr.**
CITK*	Dark	CITK	Dark	SRTK* + $CuSO_4$	Light	SRTK + $CuSO_4$	Light	1
CITK	Dark	½ SRTK + $CuSO_4$ ½ SRTK*	½ dark ½ light	½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	2
½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	½ SRTK + $CuSO_4$ ½ SRTK	½ dark ½ light	3, 7 & 8
SRTK + $CuSO_4$	½ dark ½ light	SRTK + $CuSO_4$	½ dark ½ light	SRTK + $CuSO_4$	½ dark ½ light	SRTK + $CuSO_4$	½ dark ½ light	4-6 & 9-12
½ SRTP ½ CITP	Dark	½ SRTP* ½ CITP*	Dark	½ SRTP ½ CITP	Dark	½ SRTP ½ CITP	Dark	13
CITP	Dark	CITP	Dark	CITP	Dark	CITP	Dark	14 & 15

*CITK = Callus induction medium with timentin (150 mg l^{-1}) and kanamycin (50 mg l^{-1}) *SRTK + $CuSO_4$ = Shoot induction medium with timentin (150 mg l^{-1}), kanamycin (50 mg l^{-1}) and $CuSO_4$ (5 µM) *SRTK = Shoot induction medium with timentin (150 mg l^{-1}) and kanamycin (50 mg l^{-1}) *SRTP = Shoot induction medium with timentin (150 mg l^{-1}) and paramomycin (50 mg l^{-1}) *CITP = Callus induction medium with timentin (150 mg l^{-1}) and paramomycin (50 mg l^{-1})

Transformation method

The seeds were surface sterilized in sodium hypochlorite and Tween for 35 minutes, and then rinsed five times with sterilized water. The sterilized seeds were placed on germination medium (half MS (Murashige & Skoog, 1962) with Gamborg's (Gamborg et al., 1968) vitamins, 20 g l^{-1} sucrose, 2 g l^{-1} gelrite, pH 5.8) and allowed to germinate in dark for three days (Gasparis et al., 2008, modified by Leonova).

After germination, hypocotol explants were excised and placed on callus induction medium (Full MS (Murashige & Skoog, 1962) with Gamborg's (Gamborg et al., 1968) vitamins, 30 g l^{-1} Sucrose, 2 g l^{-1} Gelrite, 3 mg l^{-1} 2,4-D, pH 5.8) and cultivated in dark for four days before they were inoculated (Gasparis et al., 2008, modified by Leonova).

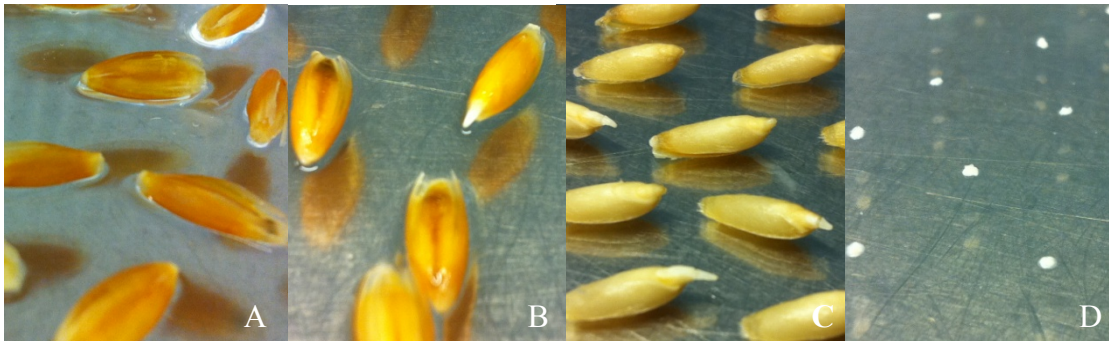

Figure 1. Sterilized oat seeds (A), oat seeds germinated for three days (B), oat seeds without seed coat (C) & excised hypocotyl explants (D).

Prior to transformation, *Agrobacterium* was cultivated in 20 ml LB-medium containing antibiotics and acetosyringone (400 µM) over night (200 rpm in 28°C). The bacterium was then centrifuged (3500 rpm for 15 min) and suspended in MS (full MS (Murashige & Skoog, 1962) with Gamborg's (Gamborg et al., 1968) vitamins, 20 g l^{-1} sucrose, pH 5.8) with an OD-value around 1.5-2. To make the bacterium more efficient in infecting the tissue, 400 µM acetosyringone was added to the bacterial solution. The explants were inoculated by pipetting 10 µl of bacterial solution onto each explants (Gasparis et al., 2008, modified by Leonova).

After inoculation, the explants were co-cultured in dark for three days. After co-cultivation, the explants were washed in water (three times) and then in water with an addition of timentin (150 mg/L) for two minutes. After washing, the explants were transferred to selection medium (Gasparis et al., 2008, modified by Leonova).

The explants were transferred to new selection medium every third week (Gasparis et al., 2008, modified by Leonova).

GUS staining

Explants transformed with constructs harboring the *gus* gene were assayed by histological GUS staining, to ensure GUS expression and thereby a successful transformation. The batches were tested three, five and seven days after inoculation. Explants (three-four) were placed in Eppendorf tubes containing 150 µl X-Gluc solution (0.2 M NaH_2PO_4 buffer pH 7.0, H_2O, 0.1 M $K_3(Fe(CN)_6)$, 0.1 M $K_4(Fe(CN)_6*6H_20$, 0.5 M Na_2EDTA and X-Gluc) and allowed to

incubate at 37°C over night. The following day, in cases necessary, the explants were rinsed with ethanol (absolute) to get rid of chlorophyll (Jeffersson, 1987).

Since a low pH value can trigger the expression of endogenous GUS-like activity (Solís-Ramos et. al., 2010), tests with different pH were conducted using leaf segments from transgenic *Lepidium campestre* in comparison with non-transgenic ones. After having obtained reliable results on *Lepidium*, similar tests were carried out in oat.

GFP analysis

To analyze the presence of green fluorescent protein (GFP), the explants infected with *Agrobacterium* carrying the vector pCW498GFP were analyzed in Bio-Rad's Versa Doc Imaging System.

Results & Discussion

Transformation of *Agrobacterium* by electroporation

No colonies appeared after incubation of LBA4404/pUC52AtWRI1 and LBA4404/pCW498GFP, which must be due to failure in the electroporation event.

Transformation of plasmids into *Agrobacterium*

After incubation, colonies appeared on the plate containing LBA4404/pCW498GFP, while no colonies appeared on the plates containing AGL1/pUC52AtWRI1 and AGL1/pCW498GFP. The bacteria's inability to grow indicates sensitivity to antibitotics, which means that the plasmids were not successfully transformed into the competent cells of *Agrobacterium*. A possible explanation to the transformation failure is that the competent cells might not have been competent, and thus not able to be transformed.

Small scale preparation of plasmid DNA

The DNA-concentration of the *Agrobacterium* sample was low (260/280 = 2,01 and 260/230 = 1,36) so the sample was divided into two, with one part of the sample undiluted and one part of the sample diluted by 10. The reason behind dilution of DNA is that a too high DNA concentration can hinder amplification of the DNA in the PCR analysis.

PCR & Gel Electrophoresis

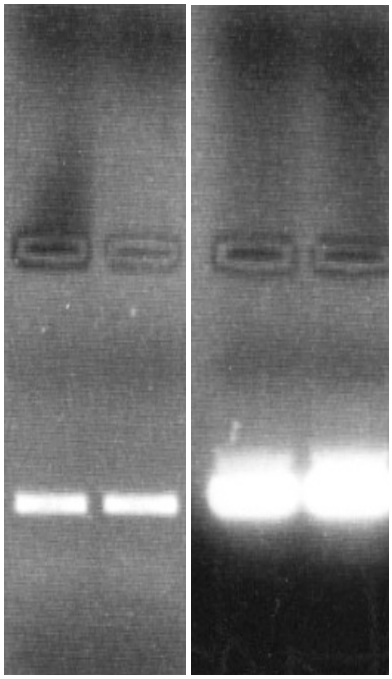

Figure 2. Results from the gel electrophoresis. Upper two bands representing *gus* and lower bands representing *nptII*.

When running the *Agrobacterium* samples in a PCR and a gel electrophoresis, indications of contaminations and too high amounts of DNA-template was found. If time had allowed it, another PCR analysis had been run to confirm the results.

GUS staining

When testing the first batch for GUS expression, no staining of the callus was visible. A too old X-Gluc solution was thought to be the reason. A new solution was thus prepared. When testing the first batch with the fresh X-Gluc solution, staining was found not only in the infected explants, but also in the non-infected ones, indicating probably some endogenous GUS activity.

It has been reported that a low pH value can trigger the expression of endogenous GUS-like activity (Solís-Ramos et. al., 2010), which is apparently the reason to the staining of the non-transformed control in this study. To confirm that the pH-value of the X-Gluc solution affected the GUS expression, different pH-values were evaluated. When testing control explants in the original pH-value (pH 6), pH-value 7,5, pH-value 8 and pH-value 9, GUS expression was only achieved in pH 6. X-Gluc solutions with pH-values from 7,5 and upwards suppressed the expression of endogenous GUS-like activity.

The pH-value was kept at 7.5 in analysis of all of the following oat batches. No staining was achieved in any explants of the batches of transformation. A lack of GUS expression indicates a failure in transformation, but that conclusion cannot be drawn with certainty in this trial. Maybe the histological GUS staining method is not a trustworthy or suitable method to confirm a successful transformation of oat. The increased pH-value might not only suppress the expression of endogenous GUS-like activity, but also hinder a working enzyme activity of true GUS. To confirm transformation of oat, the finding of methods other than histological GUS staining is of interest.

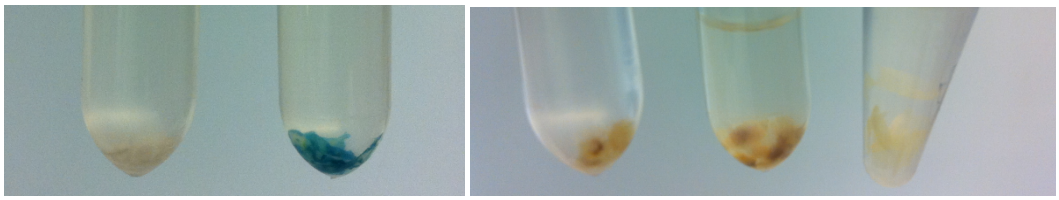

Figure 3. Results from GUS-assays; left picture showing the absence of GUS-expression in the leaf fragments of the non-transgenic *Leipium* (left tube) and GUS-expression in the leaf fragments of the transgenic *Lepidium* (right tube). Right picture shows no GUS-expression in any of the oat calli (first and second tube) or in the leaf fragments (third tube).

GFP analysis

Figure 4. Result from a GFP analysis. Whitish-colored spots indicate a possible GFP expression.

No absolute conclusion can be drawn from the analysis of explants transformed with constructs harboring the green fluorescent protein (GFP) in this trial. The non-transformed explants exhibited an even dark color, but the transformed explants exhibited small spots that were whitish-colored. The spots might have been GFP expression, but the time limitations in this trial did not allow further analysis to confirm that the spots actually expressed GFP.

In future trials, the GFP analysis will be repeated in the explants transformed in this trial. The additional cultivation of the explants may increase the possible GFP expression.

Regeneration

Both the transformed explants and the non-transformed controls exhibited a poor regeneration. Regeneration of transformed monocots has showed to be difficult, but good regeneration has been achieved in both transformants and controls in trials by Leonova (Leonova, Personal conversation). The poor regeneration generated in this trial indicates that the seeds are too old.

Perspective

The aim to achieve a well-functional transformation protocol will not end with this master project, but will continue for a further trial. The first parameter that will be altered is the plant material. New and fresh oat seeds will be used, which hopefully will result in a better regeneration. In some batches of transformation, acetosyringone will be added not only during co-cultivation, but also in the selection medium to see if a better infection is achieved. A surfactant will be added to the inoculation medium in some of the batches to see if a better attachment (and thereby a more effective infection) is generated. An addition of silver nitrate in the co-culture medium can suppress *Agrobacterium* growth and lead to a more stable transformation (as reviewed in Sood et al., 2011), and thus will be utilized in some of the future transformation batches. Hopefully these alterations in the transformation method will result in a well-functional oat transformation protocol.

References

Bennet M. D. & Smith J.B. (1991). Nuclear DNA amounts in angiosperms. *Philosophical Transactions of the Royal Society: B* 334, 309-345.

Binns A. N. & Thomashow M. F. (1988). Cell Biology of *Agrobacterium* Infection and Transformation of Plants. *Annual Review of Microbiology* 42, 575-606.

Bräutigam M., Lindlöf A., Zakhrabekova S., Gharti-Chhetri G., Olsson B. and Olsson O. (2005). Generation and analysis of 9792 EST sequences from cold acclimated oat, *Avena sativa. BMC Plant Biology* 5:18.

Cheng M., Fry J. E., Pang S., Zhou H., Hironaka C. M., Duncan D. R., Conner T. W. & Wan Y. (1997). Genetic Transformation of Wheat Mediated by *Agrobacterium tumefaciens*. *Plant Physiology* 115, 971-980.

De Cleene M. & De Ley J. (1976). The Host Range of Crown Gall. *The Botanical Review* 42, 389-466.

FAOSTAT, Food and Agriculture Organization of the United Nations. Homepage [online] (2011-04-06) Available: http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor [2011-04-06]

Gamborg O. L., Miller R. A., Ojima K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50:1, 151-158.

Gasparis S., Bregier C., Orczyk W., Nadolska-Orczyk A. (2008). Agrobacterium-mediated transformation of oat (Avena sativa L.) cultivars via immature embryo and leaf explants. *Plant Cell Reports* 27, 1721-1729.

Hansen G. & Wright M. S. (1999). Recent advances in the transformation of plants. *Trends in Plant Science* 4:6, 226-231.

He Y., Jones H. D., Chen S., Chen X. M., Wang D. W., Li K. X., Wang D. S. & Xia L. Q. (2010). Agrobacterium-mediated transformation of durum wheat (Triticum turgidum L. var. durum cv Stewart) with improved efficiency. *Journal of Experimental Botany* 61:6, 1567-1581.

Hiei Y., Komari T. & Kubo T. (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Molecular Biology* 35, 205–218.

Jefferson R. A. (1987) Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* 5, 387-405.

Jones H. D., Doherty A. & Wu H. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. *Plant Methods*, 1.

Kellogg E. A. (1998). Relationships of cereal crops and other grasses. *Proceedings of the National Academy of Sciences* 95, 2005-2010.

Lai E-M., Shih H-W., Wen S-R., Cheng M-W., Hwang H-H. &
Chiu S-H. (2006). Proteomic analysis of *Agrobacterium tumefaciens* response to the *vir* gene inducer acetosyringone. *Proteomics* 6, 4130–4136.

Lásztity R. (1998). Oat grain – a wonderful reservoir of natural nutrients and biologically active substances. *Food Reviews International* 14:1, 99-119.

Lee K. W. & Lip G. Y. H. (2003). The role of -3 fatty acids in the secondary prevention of cardiovascular disease. *Quarterly journal of medicine* 96, 465-480.

Leonova S. Postgraduate student, Department of Plant Breeding and Biotechnology, SLU, Alnarp. Modification of protocol by Gasparis et al. (2008).

Mihaljević S., Perić M & Jelaska S. (2001). The sensitivity of embryogenic tissue of *Picea omorika* (Panc˘.) Purk. to antibiotics. *Plant Cell, Tissue and Organ Culture* 67, 287–293.

Murashige T. & Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15, 473-497.

Nirwan R. S. & Kothari S. L. (2003). High copper levels improve callus induction and plant regeneration in *Sorghum Bicolor* (L.) Moench. *In Vitro Cellular & Developmental Biology – Plant* 39, 161-164.

Opabode J. T. (2006). Agrobacterium-mediated transformation of plants: emerging factors that influence efficiency. *Biotechnology and Molecular Biology Review* 1:1, 12-20.

Pereira S. L., Huang Y-S., Bobik E. G., Kinney A. J., Stecca K. L., Packer J. C. L. & Mukerji P. (2004). A novel ω3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. *Biochemical Journal* 378, 665–671.

Roberts L. (1984) *Genetic Engineering of Plants: Agricultural Research Opportunities and Policy Concerns*. Washington, D.C. : National Academy Press.

Ryan D., Kendall M., & Robards K. (2007). Bioactivity of oats as it relates to cardiovascular disease. *Nutrition Research Reviews* 20, 147-162.

Sadiq Butt M., Tahir-Nadeem M., Khan M. K. I., Shabir R., Butt M. S. (2008). Oat: unique among the cereals. *European Journal of Nutrition* 47, 68-79.

Sambrook J. & Russell W. (2001). *Molecular cloning*. 3rd edition. Cold Spring Laboratory Press.

Simopoulos A. P. (2002) Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases *Journal of the American College of Nutrition* 21:6, 495–505.

Simopoulos A. P. (2004). Omega-6/Omega-3 Essential Fatty Acid Ratio and Chronic Diseases. *Food Reviews International* 20:1, 77-90.

Solís-Ramos L. Y., González-Estrada T., Andrade-Torres A., Godoy-Hernández G., Castaño de la Serna E. (2010). Endogenous GUS-like activity in *Capsicum chinense* Jacq. *Electronic Journal of Biotechnology* 13:4.

Sood P., Bhattacharya A. & Sood A., (2011). Problems and possibilities of monocot transformation. *Biologia Plantarum* 55, 1-15.

Wei L., Guangqin G. & Guochang Z. (2000). *Agrobacterium*-mediated transformation: state of the art and future prospect. *Chinese Science Bulletin* 45, 1537-1546.

Welch R. W. & Leggett J. M. (1997). Nitrogen Content, Oil Content and Oil Composition of Oat Cultivars (A. sativa) and Wild Avena Species in Relation to Nitrogen Fertility, Yield and Partitioning of Assimilates. *Journal of Cereal Science* 26, 105-120.

Zhou M., Robards K., Glennie-Holmes M. & Helliwell S. (1999). Oat lipids. *Journal of the American Oil Chemists' Society* 76:2, 159-169.

Ziolkowski M. J. (2007). Advancements in Biolistics and Applications for Agriculturally Significant Crops. *Basic Biotechnology eJournal* 3, 34-39.