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Evaluation of transgenic *Campanula carpatica* plants

by

Stefan Bengtsson

Biology
Supervisors
Sridevy Sriskandarajah
Margrethe Serek
Department of Agricultural Sciences
The Royal Veterinary and Agricultural University
Thorvaldensvej 40
DK-1871 Fredriksberg C
Denmark

Foreword

This Master's thesis forms part of my studies as a horticulturist at the University of Agricultural Sciences, Sweden. The work was carried out at the Royal Veterinary and Agricultural University, Denmark during the period April - November 2005. I am very happy to have had this opportunity to do my Master's project in Denmark.

I thank all the staff at the Department for support and help, especially since we had difficult circumstances to work under when the labs had to move and so on.

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Stefan Bengtsson

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Text to the figure on cover page:

Fully flowering Blue Uniform control plant of *Campanula carpatica* taken from above.

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Abbreviations

A	Adenine
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
Aglo	The strain harbouring the plasmid pBEO210
AOA	Aminooxyacetic acid
AP	Apetala promoter
AVG	Amino-ethoxyvinylglycine
BA	6-benzylamino purine
BSA	Bovine serum albumin
BU	Blue Uniform
C	Cytosine
Ca^{2+}	Calcium ion
CaMV	Cauliflower mosaic virus
CMB	Carnation MADS box gene
CO_2	Carbon dioxide
CTR	Copper transporter
DACP	Diazocyclopentadiene
dATP	Deoxyadenosine-5'triphosphate
dCTP	Deoxycytidine-5'triphosphate
dGTP	Deoxyguanosine-5'triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxyribonucleotide tri-phosphate
dTTP	Deoxythymidine-5'triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetate
EIN	Ethylene-insensitive
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERF	Ethylene responsive factor
ES	Embryo sac

ETR	Ethylene receptor gene
etr	Ethylene resistant mutant
FCR	Fluorochromatic reaction
FBP	Fructose-1,6-bisphosphatase
fbp	Floral binding protein
G	Guanine
GA_3	Gibberellic acid
GACC	1-(gg-L-glutamylamino) cyclopropane-1-carboxyl acid
GH	Greenhouse
GUS	β-glucuronidase
HCl	Hydrochloride
HCN	Hydrogen cyanide
MADS box	MCM1, Agamous, Deficiens and SRH (serum response factor) box
mas	Mannopine synthase
MAPK(KKK)	Mammalian Raf protein kinase family
MTT	2,5-diphenyl monotetrazolium bromide
nos	Nopaline synthase
*npt*II	Neomycin phospotransferase II
Nr	Never-ripe mutant gene
ocs	Octopine synthase
OD	Optical density
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
RNA	Ribose nucleic acid
rolC	Root locus
ppb	Parts per billion
RT-PCR	Reverse transcriptase polymerase chain reaction
SAM	S-adenosyl-methionine
SF	'Stefan Frello'
STS	Silver thiosulphate
T	Thymine
TAE buffer	Tris-acetate-EDTA buffer

Taq	*Thermus aquaticus*
TTC	2,3,5-triphenyl tetrazolium chloride
UV	Ultraviolet
WU	White Uniform
X-gal	5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside
Å	Angstrom
1-MCP	1-methylcyclopropene

Sammanfattning

Det generella problemet hos *Campanula carpatica,* är att blommorna är mycket känsliga mot etylen. Blommor som exponeras med etylen vissnar inom två till tre dagar. Behandling av plantorna med kemikalier kan förhindra problemen med etylen. Men dessa kemikalier är väldigt kostsamma och farliga för miljön. Framstegen inom den genetiska ingenjörskonsten och genförändringen med den muterade *etr1-1* genen i t.ex. blåklocka och petunia har lett till plantor av *Campanula carpatica* sorten 'Blue Uniform' blivit genförändrade med genen *etr1-1* så att sorten har motståndskraft mot effekterna av etylen. Genförändringen av plantorna resulterade i genförändrade skott som odlades i vävnadskultur och planterades senare ut i växthus. Dessa olika genförändrade skott kallades för linjer och fick olika nummer. De genförändrade plantorna testades för känslighet för etylen. Totalt 20 olika transgena linjer av *Campanula carpatica* utvärderades. För att kunna fastställa om det fanns någon skillnad hos morfologin efter att plantorna har blivit genförändrade med *ert1-1* genen gjordes morfologiska studier. För att de transformerade plantorna skulle kunna vara lämpliga för vidare växtförädling gjordes studier av blommorna och särskilt pollenet utreddes. Pollen testades för deras livsduglighet, tillväxt av pollenslangen och slutligen korsningar gjordes för att bevisa livsdugligheten. Linjerna delades upp i tre grupper beroende på den procentuella livsdugligheten eller procentuella tillväxten av pollenslangen. Grupp A inkluderar linjerna Aglo 2, Aglo 3, Aglo 4 and SF 28-1. Grupp B inkluderar linjerna SF 15-1 och 'Blue Uniform' kontroll. Grupp C inkluderar linje SF 13-1. För att bekräfta att genen *etr1-1* var insatt hos plantorna, testades plantorna också med PCR. ELISA analys utfördes för att utreda den procentuella mängden av *npt*II hos de genförändrade linjerna. Det slutliga resultatet visade att linjerna SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 och GH 4 hade väldigt låg känslighet mot etylen. Dessa plantor hade inga morfologiska förändringar. Men linjerna Aglo 1, Aglo 2, Aglo 3, Aglo 4 och Aglo 8.3 saknade hår under blad och på stam delar. Linje SF 11-2 visade ett annat mönster hos planttillväxten då den hade ett spetsigare tillväxt beteende av knoppar och blad. Linjerna SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 och GH 4 (speciellt SF 15-1) hade fertilitets värden väldigt nära kontroll plantornas. I PCR bekräftades att linjerna SF 15-1, SF 15-1c, SF 15-1de, SF 15-4, SF 13-1, SF 13-1a och GH 4 amplifierade genen *ert1-1*. Hos linjerna SF 13-1 och SF 13-1a genen *ert1-1* var amplifierad men dessa plantor var känsliga till etylen. Jag tror att genen *etr1-1* inte var aktiv i blommorna eller i hela växten.

Summary

In *Campanula carpatica,* the general problem is that the flowers are very sensitive to exogenous ethylene. Flowers that are exposed to ethylene wilt in two to three days. Treating the plants with anti-ethylene compounds can prevent the problems with ethylene. However, these compounds are very expensive and environmentally dangerous. The advances in genetic engineering and transformations with the mutant *etr1-1* gene in, *e.g.* carnations and petunia have led to plants of the *Campanula carpatica* cultivars 'Blue Uniform' being transformed with the gene *etr1-1* in order to resist the effects of ethylene. The transformation of the plants resulted in transformed shoots that were grown in tissue culture and later planted in greenhouses. These different shoots were called lines and were given different numbers. The transformed plants were tested for their sensitivity to ethylene. A total of 20 different transgenic lines of the *Campanula carpatica* were evaluated. To determine whether there were any differences in plant morphology after they had been transformed with the *etr1-1* gene, morphological studies were performed. For the transformed plant to be suitable for use in further plant breeding, the flowers, and especially the pollens, had to be investigated. Pollen was tested for viability, growth of the pollen tube, and finally, crossing was done to prove the viability. The lines were divided into three groups depending on percentage viability or percentage of grown pollen tubes. Group A included lines Aglo 2, Aglo 3, Aglo 4 and SF 28-1. Group B included lines SF 15-1 and 'Blue Uniform' control. Group C included line SF 13-1. To confirm that the gene *etr1-1* was inserted in the plants, they were also tested using polymerase chain reaction (PCR). ELISA analyses were performed to investigate the presence of *npt*II in the transformed lines. The final results revealed that the lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 and GH 4 had a low sensitivity to ethylene. These plants had no morphological differences. However, the lines Aglo 1, Aglo 2, Aglo 3, Aglo 4 and Aglo 8.3 lacked hairs under the leaves and on the stem parts. Line SF 11-2 displayed a different pattern of plant growth with a pointier growth behaviour of the buds and leaves, while the lines SF 13-1 and SF 13-1a grew much taller than the other lines. The lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 and GH 4 (especially SF 15-1) had fertility values that were close to the control plants. In the PCR, lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4, SF 13-1, SF 13-1a and GH 4 amplified the gene *etr1-1*. In lines SF 13-1 and SF 13-1a the gene *etr1-1* was amplified, but these plants were sensitive to ethylene. I think the gene *etr1-1* was silenced in the flowers or in the plant itself.

General introduction

In 2005, the production of Campanula amounted to 11.7 million pots in Denmark (S. I. Pedersen per. comm.). The turnover for *Campanula carpatica* was 12 million DKR in Denmark in 2005 (Flora-Dania Marketing A/S, 2006). The biggest growers of Campanula are found in the area around Odense, Denmark. The increasing trade over the world has led to demand for increasing quality and longevity of the Campanula plants. This has prompted the development of new methods to achieve the goals by conventional breeding. The developments in plant genetic engineering can be the solution to meet the plant quality goals.

Problem statement

The problems with the wilting effects of ethylene on *Campanula carpatica* plants have led to much research to find methods to prevent such effects. Ethylene affects the Campanula plants in that the buds and flowers wilt. To prevent this effect, anti-ethylene compounds, which can be found in the market today, are used. The anti-ethylene compounds, ***e.g.*** Silver thiosulphate that are used today will be banned by the year 2007 in the European Union. Silver thiosulphate (STS) works by blocking the receptor for ethylene. However, these compounds are expensive, environmentally dangerous and not sufficiently effective for full treatment. The finding of ethylene-mutated genes such as *etr1-1*, *rolC* and *Nr* led to the suggestion that these genes could be used to develop plants that are not sensitive to ethylene. During the past 20 years, transformation with these ethylene-mutated genes of important crops, *e.g*. in carnation, petunia and tomato, has been developed. Insertion of the gene *etr1-1* into the important ornamental crop Campanula has been done recently. Even though the gene was successfully inserted into *Campanula carpatica*, to be able to study the effects of the transgene and release the transgenic crop into the market a full evaluation had to be performed. In the evaluation various important parameters had to be investigated, ***e.g.*** tolerance on the transgenic plants to exogenous ethylene, morphological studies to determine differences after transformation and investigation on fertility. Moreover, the fact that the gene has actually been inserted had to be proven by various molecular methods such as ELISA-test and PCR.

1 Literature review

1.1 Campanula species

1.1.1 Taxonomy of *Campánula carpatica* Jacq.

The taxonomy of *Campánula carpatica* Jacq. is in the following order: Plantae (Kingdom), Vascular Plants (Group), Magnoliopsida (Class), Magnoliidae (Dicot), Asteriflorae (Sub Order), Campanulaceae (Family) Bellflower family, *Campánula carpatica* Jacq. (Teknica Ltd., 2005).

1.1.2 Description of Campanulaceae family

The family includes species that are annual or perennial herbs and they occasionally contain white latex in the plant tissue. The plants have a simple leaf with entire or dentate leaf margins that do not have stipules. The flowers are hermaphroditic and the sepals are free. Petals are fused together and sometimes can be labiate (*Lobelia),* with 1 style and 5 stamens. Anthers are fused together (*Lobelia*), only partly fused (*Jasione*) or free. The ovary is inferior and the fruit is a capsule. The Campanulaceae comprise 87 families and nearly 2000 species that are spread over the world. The biggest species in the family is *Campanula*, with 300 subspecies (Anderberg, 2005).

1.1.3 Description of *Campanula* L. species

The species contains annual to perennial herbs, which are bare to stiff and hairy on the leaves and stems, and contain white latex in the plant. Leaves are alternate, simple, and oval to cordate. Stipules are not present. The flowers are actinomorphic and sepals triangle-like or awl-like. The flower is campanulate shallow or deep edged, blue, violet or very seldom white. The 5 stamens are free and the 1 style has a 3- or 5-edged stigma. The fruit is a poricidal capsule with 3 or 5 openings. On suspended capsules, the openings are at the capsule base near the pedicle, while on upright capsules the openings are at the top. The seeds are oval, flat and sometimes with a thin, winged edge (Jacobsen & Jensen, 1999).

1.1.4 Description of *Campanula carpatica* Jacq.This is a clump-forming perennial with rounded to ovate (Brickel & Zuk, 1997) or cordate (Gadella, 1964), toothed, basal leaves 2.5–5.0 cm long. Many long, branched stems bear solitary, large, upturned, open, bell-shaped, blue, violet-blue or white flowers. The flowers are 3 cm or more across and they flower over several months during the summer (Brickel & Zuk, 1997). The calyx appendages are absent. The plant has a glabrous style and the capsule has 3 loci. The capsule is apical dehiscence and has an erect position (Gadella, 1964). The plant is 30 cm high and can be 30-60 cm or more wide (Brickel & Zuk, 1997). The species has a chromosome number 2n = 34 (Gadella, 1964).

1.2 Postharvest quality of ornamentals

The ornamentals include cut flowers and foliage, flowering and foliage pot plants, bedding plants and container shrubs and trees. Ornamentals are a very important sector of the horticultural industry. The cut flower industry represents one third of the total value of the global horticultural industry. The ornamentals have a substantially higher added value when it comes to secondary industries like processing, retailing, plant hire and interior plantscaping compared to fruit and vegetables. Because there are many industries involved in ornamentals that are dependent on fresh ornamentals, postharvest is a very important area (Wills *et al.*, 1998).

The ornamentals are harvested when the visual quality is at the best stage. However, because they are living organisms, they start to deteriorate after they have been harvested. The rate of deterioration varies between individuals because of their overall rate of metabolism. When plants are transported to markets that are situated very close to the farmer, the postharvest deterioration is limited, but the increased changes in production areas in both developing and developed countries, and the growth of marketing systems and international trade imply that the time from farmer to market can be long. In addition, modern marketing chains have increased their requirements regarding the quality of the products. This has led to a need for postharvest techniques that can maintain plant quality over an increasingly longer period. Postharvest losses can be divided into two main groups: physical and chemical. Physical losses can arise from structural damage or microbial spoilage, which leads to tissue degradation, to a stage where the products cannot be displayed. The evaporation of inter- and intracellular water also leads to loss in plant weight. Chemical losses involve a loss of quality

by postharvest due to physiological and compositional changes that affect the appearance and render the product aesthetically unattractive for the end consumer. This loss of quality originates from changes in the normal metabolism or abnormal events from the postharvest environment. All of these different postharvest losses lead to economic losses when the products have to be marketed at reduced prices or thrown away (Wills *et al*., 1998).

1.2.1 Ethylene and postharvest life

Ornamental flowers have a short vase life after harvest. The most important parameter responsible for this is their sensitivity to ethylene. Most ornamentals are non-climacteric, but the climacteric plants produce an ethylene and respiratory peak. There is a difference in the response to ethylene. Some flowers such as non-climacteric delphinium are very sensitive to ethylene, while climacteric carnation is relatively tolerant. Ethylene induces the start of abscission, but the short postharvest life of cut flowers is limited by carbohydrate reserves and a rapid rate of metabolism (Wills *et al.*, 1998).

Potted flowering plants can range from ethylene insensitive to ethylene sensitive. The quality and longevity of the flowers depend on their sensitivity and ethylene exposure. However, if a flowering plant has yellow leaves, the plant has little value even if the flowers are maintaining good quality. The same can be said if the flowers die fast but the leaves have good quality. Problems during transport may not be visible on the plant until after several days. Buds and flowers can drop several days after the plant has been un-boxed and leaves can turn yellow up to 2 weeks after transport. The selection of cultivars and production system has an effect on the response of flowering plants (Nell, 2005). Campanula cultivars are sensitive to exogenous ethylene. The campanula buds and flowers wilt when exposed to ethylene. Yellowing of leaves can also occur. Chrysanthemum and poinsettia cultivars can vary over a wide range in their ability to withstand transport. The hibiscus cultivars drop buds and flowers when the shipping conditions are unfavourable. The major negative effect of transport is exposure of the plants to ethylene. This results in a rapid loss of quality during transport, while the longevity and quality can also be reduced during secondary transport. The control of the temperature during transport is a good method to control the effects of ethylene. Lower temperature leads to decreased respiration and the conservation of carbohydrate reserves. This decreases the problems with ethylene. There are different optimum temperatures for different species, but each should be transported under the lowest possible temperature. The plants produce ethylene themselves and additional ethylene can originate from external sources such

as engines, cigarette smoke and dead organic matter. Ethylene can affect plants at concentrations as low as 25-100 ppb. The possible symptoms of ethylene exposure include dropping of leaves and buds, ageing and leaf yellowing. The symptoms that actually develop depend on the concentration, as well as time duration and cultivars. Ethylene is more damaging as the temperature increases. Carnations, for example, are 1000-fold more sensitive to ethylene when the temperature is increased from 2 to 21°C (Nell, 2005).

Table 1.1

Response of flowering potted plants to ethylene and their symptoms[1,2] (Nell, 2005)

Crop	Symptoms
Achimenes	Flower/bud drop
African violet	Flower wilting
Azalea	Leaf drop
Begonia-elatior	Flower drop
Bougainvillea	Flower/bract drop
Browallia	Flower/bud drop
Carnation	Failure of flower to open
Calceolaria	Flower/bud drop
Clereodendron	Flower/bract drop; Leaf drop
Crossandra	Flower drop
Cyclamen	Flower drop; Flower wilting
Cymbidium	Wilting of the sepal
Exacum	Flower wilting
Geranium	Floret drop
Gardenia	Flower/bud drop
Gloxinia	Flower drop
Hibiscus	Flower/bud drop
Kalanchoe	Failure of flowers to open; Petal drying
Pachystachus	Petal wilting; Bud blasting; Leaf yellowing
Poinsettia	Petiole droop[3]
Streptocarpus	Flower drop

[1] The degree of sensitivity to ethylene varies with plant species, variety, ethylene concentration, and temperature during exposure and duration of exposure.
[2] Adapted from E. J. Woltering, 1987, Scientia Horticulture 31:283-294.
[3] Petiole droop (epinasty) of poinsettia is caused by upward bending of leaf and bract petioles during sleeving.

'Adapted from (Woltering, 1987)'

1.2.2 Biosynthesis of ethylene

Ethylene is synthesised from methionine via a pathway that includes the intermediates S-adenosyl-methionine (SAM) (Wills *et al.*, 1998) and the cyclic amino acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Müller *et al.*, 2003). Conversion of SAM to ACC and S-adenosyl methanethiol by the enzyme ACC synthase (Wills *et al.*, 1998; Müller *et al.*, 2003) is one of the rate-limiting steps in the biosynthesis of ethylene. Methanethiol is reincorporated to methionine into the Yang cycle. Oxidation of ACC by ACC oxidase leads to

production of ethylene, HCN and CO_2. The genes that code for ACC synthase and ACC oxidase constitute multigene families. The reactions catalysed by ACC synthase and ACC oxidase are rate-limiting for ethylene production. Under certain conditions, ACC can be converted to N-malonyl ACC by ACC N-malonyltransferase or to 1-(γ-L-glutamylamino) cyclopropane-1-carboxyl acid (GACC) (Müller *et al.*, 2003). In higher plants forming malonyl ACC or glutamyl ACC, these can be hydrolysed to give ACC. The ACC in preclimacteric fruit results in a very small increase in ethylene development, showing that ethylene-forming enzyme, ACC oxidase, has very low activity at this stage ACC is very sensitive to oxygen and is a pyridoxal enzyme, meaning that it requires pyridoxal phosphate to have the highest activity and is very strongly inhibited by aminooxyacetic acid (AOA), rhizobitoxine and L-2-amino-4-(2-aminoethoxy)-trans-3-butenoic acid (AVG), which are known inhibitors of pyridoxal phosphate-dependent enzymes. ACC oxidase is inhibited by anaerobiosis, temperatures over 35°C and cobalt ions. Ethylene is also developed in plant tissues from oxidation of lipids involving a free-radical mechanism (Wills *et al.,* 1998).

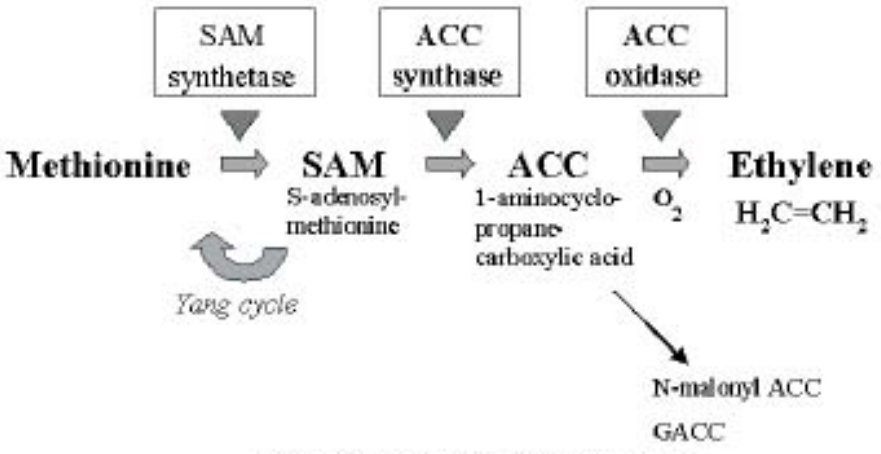


Figure 1.1

Overview of the biosynthesis of ethylene, signal transduction and effects of ethylene. First, ethylene is synthesised from methionine via the intermediates S-adenosyl methionine (SAM) and the cyclic amino acid 1-aminocyclopropane-1-carboxyl acid (ACC). The enzyme SAM synthase converts methionine to SAM and ACC synthase converts SAM to ACC. The ACC oxidises to ethylene, HCN and CO_2 under the ACC oxidase (Müller *et al.*, 2003).

1.2.3 Ethylene effects on ornamentals

Ethylene, in cooperation with other plant hormones such as auxins, gibberellins, kinins and abscisic acid, controls the ripening process. There are two systems that regulate ethylene biosynthesis. In system 1, an unknown factor is inhibited or it controls the regulation of senescence. System 1 triggers system 2, which initiates production of the very large quantities of ethylene necessary for ripening. In system 2, an autocatalytic process triggers further production of ethylene. In non-climacteric plants, system 2 is not active and treatment of their fruits with ethylene will avoid system 1 (Wills *et al.*, 1998).

In the general transduction model for ethylene signals in plants, the receptor *Arabidopsis* ETR1 is often used for description of the transduction model. This receptor is a subfamily 1 receptor, but other receptors work in the same way. General receptors of the subfamily 2 have 4 instead of 3 membrane-spanning domains. Some receptors lack the R (receiver) domain. The binding of ethylene occurs in a hydrophobic pocket in the dimer and requires a Cu ion. The two monomers in the receptor are connected with two disulphide bonds. When ethylene is not present, ETR1 keeps CTR1 active, which is very similar to MAPKKK. When CTR1 is active it keeps EIN2 inactive, possibly via MAPKK and MAPK (hypothesized) intermediates. EIN2 consists of two domains. The N-terminal domain, which is very similar to a mammalian family of divalent metal ion transporters, is one of these two domains. EIN2 is thus considered to be an ion transporter and is placed in the plasma or in the ER membrane. When it is open metal ions (possibly Ca^{2+}, but this is not yet known), which are abundant in the ER and outside the cell, can influx to the cytosol. Ethylene binding to ETR1 leads to inactivation of CTR1 so that the effects of active CTR1 on EIN2 stop. Active (open) EIN2 initiates the binding of the transcription factor EIN3 to the promoter of the ERF1 gene. ERF1 is produced and binds to many different promoters. These result in changes in gene expression, leading to the ethylene response (Müller *et al.*, 2003).

The sensitivity of the receptors increases when oxygen is present and decreases in the presence of carbon dioxide. Treatment with silver ions inhibits the effects of ethylene, substitutes the Cu ions in the receptors, and ethylene cannot bind to Ag-containing receptors. The gaseous cyclic olefins 2,5-norbornadiene and 1-methylcyclopropene (1-MCP) are very effective in preventing the effects of ethylene. 1-MCP binds irreversibly to ethylene receptors in the plant tissue and when treated for a few hours with a low concentration, the effects can last for several days (Wills *et al.*, 1998). It works at very low concentrations (0.5 $nl.l^{-1}$)

(Müller, 1996). Another anti-ethylene compound is DACP (diazocyclopentadiene) that is a gaseous compound that blocks the ethylene binding permanently and is very effective against endogenous and exogenous ethylene. Aminooxyacetic acid (AOA) and Rhizobitoxine (AVG) work in that they block the synthesis of ethylene. The compounds affect the activity of enzymes necessary in ethylene biosynthesis. Plant hormones of the cytokinin group can stop senescence, *e.g*. the cytokinin BA and (GA_3) gibberellins can stop the senescence of leaves and shoots (Müller, 1996).

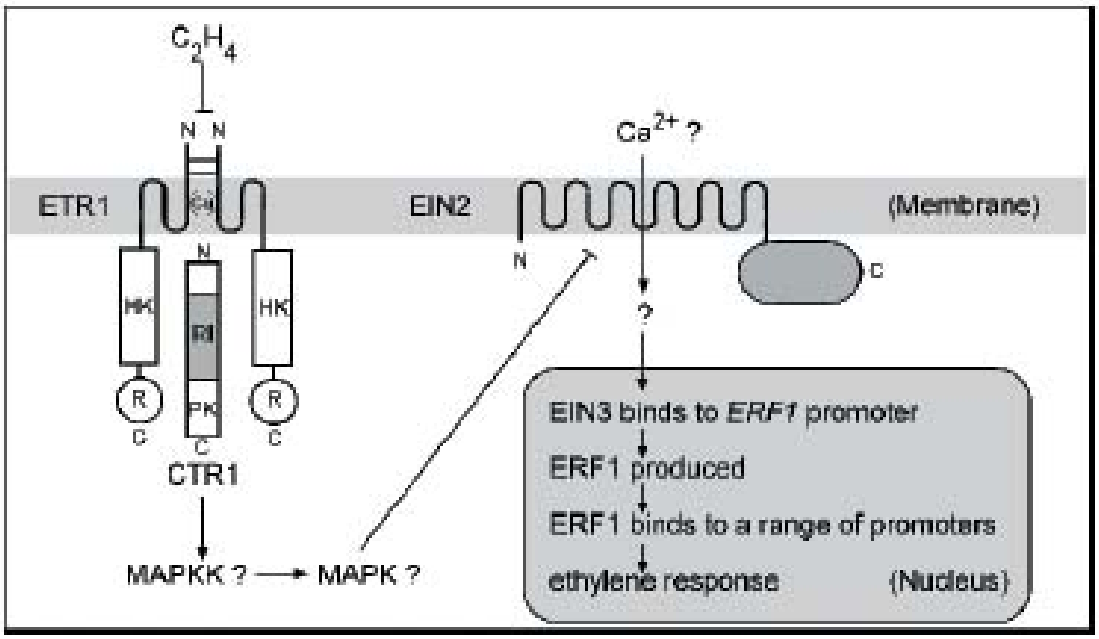


Figure 1.2

Standard model of ethylene signal transduction in plants. N- and C-terminal ends of ETR1, CTR1 and EIN2 are indicated. The question mark (?) indicates unknown compounds. HK stands for histidine kinase domain, R stands for receiver domain, RI stands for receptor interaction domain and PK stands for protein kinase domain (Müller *et al.*, 2003).

1.3 Reproduction

1.3.1 Development of the flower in *Campanula*

The most common flower type in the *Campanula* genus is protandrous, meaning that they present the pollen before the stigma is receptive. There are differences between species regarding when the phase of separation of the male and female take place. When the flower is in the bud stage, the introrse anthers form a closed cylinder around the immature style. The anthers open and release the pollen when the bud is closed or just when it starts to open. The

pollen is collected on a dense brush of regularly arranged hairs on the upper half of the style. When the flower opens the anthers wither. The style is completely covered with pollen and is exposed. An elongation of the lower part of the style leads to collection of pollen on the hairy part of the style. Such elongation takes part below the hairy zone. After the male phase, the flower transforms to the female phase and the stigma splits into 3 (4) stigmatic branches, which start to bend backwards. When the pollen-collecting hairs invaginate into the style, the pollen grains fall off the style, preventing self-pollination (Nyman, 1992).

1.3.2 Pollen viability

Pollen has a very important role in the flow of genes in plants, especially in plants that are out-crossing. It is especially important to investigate the pollen when transgenic plants are formed and also to compare it with that of non-transformed plants. A risk assessment is important before allowing transgenic cultivars to be released. To get a good risk assessment of the plant, different methods can be used, *e.g.* pollen viability. This is to get an indication of the ability of the pollen grain to perform the function of delivering the sperm cell to the embryo sac during pollination. There are several methods that can be used for evaluation of pollen viability, *e.g.* different staining techniques (aniline blue for detection of the callose in the pollen walls and pollen tubes, iodine to determine starch content, etc.), by in vitro and in vivo germination tests or analysing seed set *e.g.* from crossings. The methods used depend on the crop being investigated. There is currently very little information on which method is best for different crops. Using information from different tests that have been carried out on other crops can help refine methods. For example, the development of a viability test for tall fescue (*Festuca arundinacea*) by Wang *et al*. (2004) is a good method that can be used. The work on tall fescue has provided the background for the development of a viability test for the species *Campanula carpatica*.

1.3.2.1 Staining for pollen viability

The first method for testing pollen involves determining viability by staining. Fresh pollen and dead pollen from plants grown from seeds is tested using:

(a) The X-gal test to determine the content of β-galactosidase (an enzyme involved in the lactose degradation (Atiaksheva *et al*., 2000)). The X-gal test consists of a solution of 1 mg X-gal (5-bromo-4-chloro-3-indoyle-β-galactoside) that is dissolved in 50 μL N,N-dimethyl formamide and 1 mL acetate buffer (50 mmol with pH 4.8). Viable pollen turns blue.

(b) A test for the content of dehydrogenase (an enzyme) using a 1% solution of substrate 2,3,5-triphenyl tetrazolium chloride (TTC) or by 2,5-diphenyl monotetrazolium bromide (MTT) in a 5% sucrose solution. Viable pollens turn deep pink.
(c) A test with aniline blue for detection of callose in pollen walls and tubes. The aniline blue-lactophenol solution contains 5 mL 1% aqueous aniline blue, 20 mL phenol, 20 mL lactic acid, 40 mL glycerine and 20 mL water.
(d) Lugol solution to detect starch content in the pollen. The Lugol solution consists of iodine and potassium iodide. Viable pollen turns black.
(e) The fluorochromatic reaction (FCR) test to determine the esterase activity and the intactness of the cell membrane. The fluorochromatic reaction test consists of fluorescein diacetate dissolved in acetone (2 mg mL^{-1}) and is used on 10^{-6} mol L^{-1} in 0.8 mol L^{-1} sucrose. The pollens are then investigated under a fluorescence microscope (Wang *et al*., 2004).

1.3.2.2 Pollen tube germination

In the second method for testing the viability, germination tests can be carried out to measure pollen viability. There are two major tests, which can be divided in two different parts. In *in vitro* germination, pollen is grown on a specific media. In *in vivo* germination pollen is grown on the stigma of the plant.

1.3.2.2.1 *In vitro* germination for pollen viability

Fresh harvested pollen is grown on a medium containing sucrose, boric acid and calcium nitrate. These compounds have been shown to be very important for pollen germination in different species. The pollen is grown in a humid environment and at room temperature (~20°C). The pollen is considered mature when the pollen tube length is longer than the diameter of the pollen grain (Wang *et al*., 2004).

1.3.2.2.2 *In vivo* germination for pollen viability

In this test, pollen is taken from the flower and grown on the surface of the stigma. The stigma surface is placed on a microscope slide after it has been pollinated and then incubated at room temperature with high humidity for a short period (40+ mins), with different time periods for different species. The style is then stained with 0.05% aniline blue in a potassium phosphate (K_2PO_4) buffer for 2-3 minutes. The stained style is then examined under a UV excitation filter (330-380 nm) and a barrier filter transmitting above 420 nm (Kedar & Clyton, 1998).

1.3.2.3 Crossing

Crossing between different individuals is called out-breeding. Higher plants are hermaphrodites, meaning that the angiosperm flower has an area with pollen-bearing stamens that are surrounded with a gynaecium that contains one or more ovules. To avoid self-pollination these plants have developed self-sterility and in this process self-incompatibility plays an important role. This means that a fertile hermaphroditic plant cannot produce zygotes by self-pollination. The mechanisms that prevent self-pollination are:

1. The plan is heterozygous for the gene *S*. The style and stigma contain nuclei that have a diploid chromosome number. So the style and stigma contain S_1S_2 in a plant that has the genotype S_1S_2.
2. In meiosis, pollen formation and segregation take place. Pollen that contains nuclei with a haploid chromosome number receives one of the two *S* alleles. Then the pollen is positioned depending on the *S* allele genotype it has.
3. The pollen reaches the stigma by the action of wind, insects or other methods.
4. The pollen grain starts to hydrate and germinates. The pollen tube grows intracellularly through an area of transmitting tissue.
5. If the *S* allele in the pollen is also present in the style, the growth of the pollen tube slows down and the tip of the incompatible tube growth becomes occluded with callose. If the pollen and style have different alleles, growth continues and fertilisation will occur (Briggs & Walters, 2000).

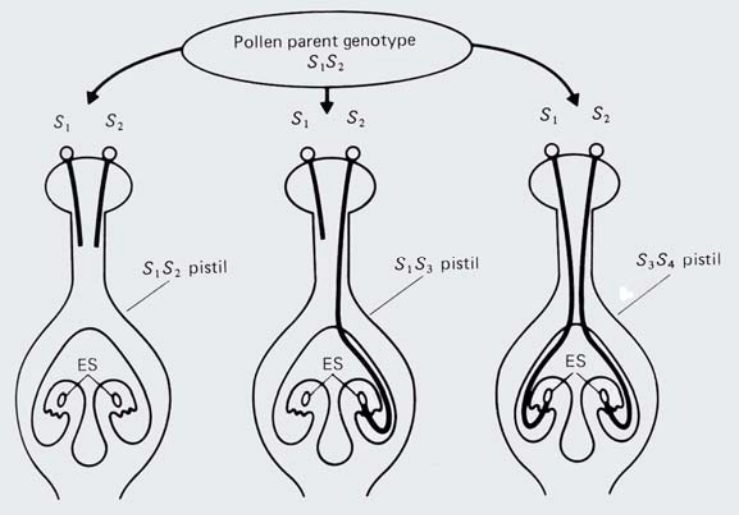


Figure 1.3

Gametophytic self-incompatibility.

The pollen parent S_1S_2 is infertile, semi-fertile or fertile depending on the genotype of the female plant. In most species that have this system, the incompatible pollen tubes are inhibited in the style. ES = embryo sac (Briggs & Walters, 2000).

1.4 Genetic engineering of ornamentals for longer postharvest life

In recent years, the development of genetic engineering has made it possible to develop and improve ornamental flowers. The first transgenic commercial flower was the carnation 'Moon', which is marketed in North America, Australia and Japan (Tanaka *et al*., 2005).

As discussed earlier, one of the biggest problems in ornamentals is the post-harvest life. One way to avoid this problem can be to treat the flowers with silver thiosulphate (STS), but the treatment has a wide variation depending on the time of application, the concentration and flower type. The silver interferes with the reception of ethylene by binding to the membranes that are associated with the ethylene receptors. This renders the flowers insensitive to endogenous and exogenous ethylene. When the silver becomes depleted, it is less effective in the resistance to ethylene. When treating using STS, there is a risk that parts of the flower will not get exposed, thereby leading to lower ethylene protection. STS is also classed as a toxic chemical, thereby increasing the pressure on the horticultural industry to reduce the use of chemicals. Understanding of the processes that are involved in the senescence has lead to opportunities for genetic engineering to achieve longer post-harvest life of ornamentals (Tanaka *et al*., 2005).

There are a number of different genetic methods used to achieve longer postharvest life. The first method, developed in carnation flowers, is the down regulation of ethylene production in flowers via post-transcriptional floral-specific gene silencing of a gene encoding ACC Oxidase (ACO) or ACC Synthase (ACS). These enzymes catalyse the two penultimate teps in ethylene biosynthesis. This leads to flower resistance to exogenous ethylene comparable to that achieved by treatment with STS. However, exogenous ethylene is not a problem in the transport chain of carnation (Tanaka *et al*., 2005).

In the plant *Arabidopsis,* the gene that encodes ethylene receptors (ETR1) has been isolated (Tanaka *et al.*, 2005). This has led to the development and chemical-free production of carnation flowers with longer post-harvest life (Tanaka *et al.*, 2005). The transgenic plants of carnation harbour the *etr1-1* gene under the control of the promoter CaMV 35S or an *fbp1* (floral binding protein). The outcome is that the senescence is delayed up to 16 days and there is a three-fold increase in the vase life. Similar results were obtained using *etr1-1* with the *CMB2* promoter (Tanaka *et al.*, 2005). The second genus that the *etr1-1* gene was introduced

into was petunia, under the control of CaMV 35S promoter (Tanaka *et al.*, 2005). The transgenic petunia flowers have 2-4 times longer flower life and abscission is delayed compared with plants that were not transgenic. The *etr1-1* gene was also introduced into petunia under the control of a floral binding protein (*fbp1)* or an apetala (*AP3)* promoter. This led to 70% and 30%, respectively, of the plants having a twice as long post-harvest life than the non-transgenic plants (Tanaka *et al*., 2005).

1.4.1 Campanula

In the transformation of *Campanula carpatica* an established method with an *Agrobacterium tumefaciens* strain was used. The strain AGL0 was used harbouring the plasmid pBEO210, which consists of gene *etr1-1* under the control of the *fbp1* promoter, which is flower-specific, and gene *npt*II under the control of the CaMV 35S promoter. Seeds were surface-sterilised and germinated on MS medium. Cotyledons and hypocotyls were taken from grown seedlings. Bacterial suspensions were used for the inoculation and the explants were placed in the suspensions. After 20 min, the explants were planted on co-cultivation medium. In the co-cultivation, two mediums were used. The explants were incubated for 72 h in darkness and after incubation the explants were transferred to selection medium. The post co-cultivation medium contained 100 mg/l kanamycin, which stops regeneration and kills non-transformed explants. After four weeks, the explants were transferred to fresh selection medium. The shoot clumps were cut into smaller parts and planted in different mediums. After 5 weeks, the shoots were tested with enzyme-linked immunoabsorbent assay (ELISA) for *npt*II activity. The ELISA test for *npt*II activity indicated that the *npt*II gene was expressed in the transformed shoots. This test proved that the shoots had been transformed with the *etr1-1* gene (Sriskandarajah *et al*., 2004).

In the Campanula specie *Campanula glomerata* ‘Acaulis’ transformation with the *ipt* gene that resulted in increased number of shoots and flower-stems. Transformation was done by the established method with *Agrobacterium tumefaciens* strains. The strain that were used was LBA4404 that contain the pBC34 vector with the gene *ipt* controlled by CaMV 35S promoter and the *npt*II gene under the nos promoter. The leaf explants were placed in the *Agrobacterium* suspension for 15 min and after they were transferred to sterile paper to remove excess bacterial suspensions. The explants were placed abaxial side down to co-cultivation medium and incubated for 2-3 days. After the co-cultivation the explants were transferred to regeneration medium that contained 50 mg/l kanamycin to selection and both

400 mg/l vancomycin and 100 mg/l cefotaxime to elimination of *Agrobaterium*. The explants were cultured on fresh medium until shoots regenerated. DNA was extracted from putative transformants for the PCR. The *ipt* gene was amplified by this method (Joung *et al*., 2001)

1.4.2 Carnation

Ethylene production was down-regulated by insertion of the post-transcriptional floral-specific gene that silences the gene encoding ACC Oxidase or ACC Synthase, two enzymes catalysing the two penultimate steps in ethylene biosynthesis. However, carnations were still sensitive to exogenous ethylene. When the mutated *Arabidopsis* ethylene receptor gene (*etr1-1)* was inserted, carnations insensitive to both endogenous and exogenous ethylene were produced. The gene is harboured in the transgenic carnation with control of its own promoter. This resulted in the carnations having senescence delayed by 6 days to a maximum 16 days. The same results were achieved when the gene *etr1-1* was inserted by a *CMB2* promoter (this promoter is a carnation MADS box gene) (Tanaka *et al*., 2005). Also, the gene ACO1 was introduced into carnations, causing ethylene biosynthesis in the flowers to be blocked by co-suppression of the gene encoding ACC oxidase. Gene expression in only the flowers was achieved under the control of the petunia *fbp1* promoter, thus avoiding the negative effects of ethylene-dependent processes. Three binary plasmids (pBEO210, pBEO220 and pETR1-1) were transformed from *E. coli* to *Agrobacterium tumefaciens* (Figure 1.4). The transgenic carnations were then transformed with these three plasmids. The promoter Petunia *fbp1* was tested in the transgenic plants by expression of the GUS-A reporter gene under the control of the *fbp1* promoter. The results for the transformation with the constructs pBeO210 (Pfbp1-etr1-1), pBEO220 (P35s-etr1-1) and pETR1-1 (Petr1-etr1-1) were 5.8%, 4.2% and 3.8%, respectively. Transformation was lower when used with a promoter that was active in the whole plant. Plants derived from the transformation with the construct pBEO220 died after growth in tissue culture and in a greenhouse. This indicates that inhibition of the ethylene response in the whole plant has negative effects (Bovy *et al.*, 1999).

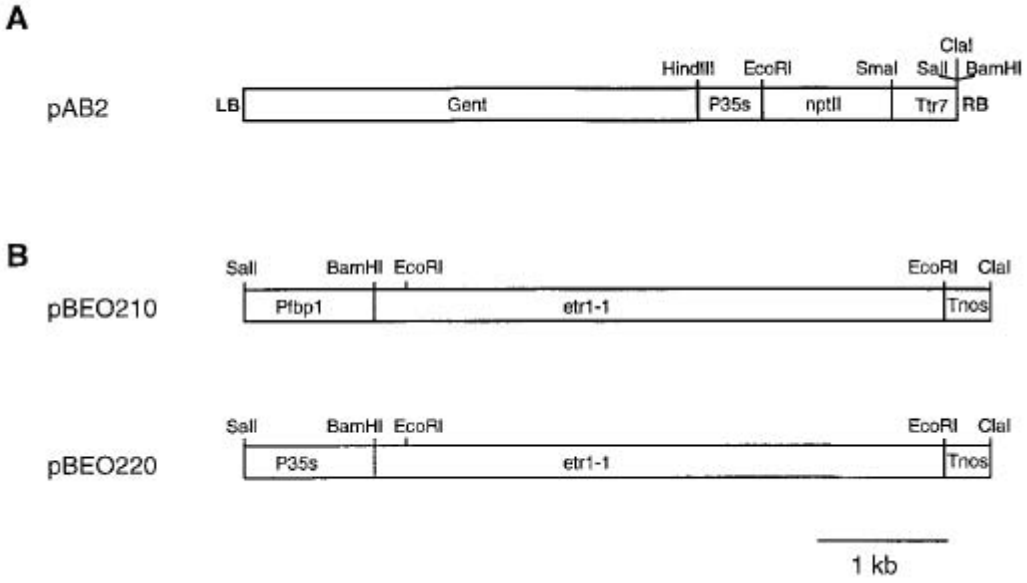


Figure 1.4.

Schematic drawing of three gene constructs. A. The T-DNA region of the plasmid pAB2. B. *Sal*I/*Cla*I inserts of the plasmids pBEO210 and pBEO220. Abbreviations: LB= T-DNA left border, RB= T-DNA right border; Gent, gentamycin resistance gene; P35s= CaMV 35S promoter; *nptII*= neomycin phosphotransferase gene, conferring kanamycin resistance; Ttr7= transcript 7 polyadenylation region from the octopine Ti plasmid pTi15955; Pfbp1= *Petunia hybrida fbp1* promoter; *etr1-1= Arabidopsis thaliana etr1-1* allele; Tnos= polyadenylation region of the *Agrobacterium tumefaciens* nopaline synthase gene (Bovy *et al*, 1999).

1.4.3 Petunia

The gene e*tr1-1* was inserted into petunia with the control of the CaMV 35S promoter, resulting in the flowers having a much longer life. The gene was also inserted with the control of *fbp1* or the *AP3* promoter. This resulted in 70% and 30%, respectively, having a flower life that was twice as long as that of the non-transformed plants (Tanaka et al., 2005). In another transformation of petunia, the petunia 'Mitchell' was transformed with the gene *rolC* from Agrobacterium rhizogenes with the control of the CaMV 35S promoter. The plants that were transformed with the gene *rolC* had changes in growth pattern and in plant morphology. The plant was shorter, more compact and had increasing branching. The stem was 50% shorter than in the plants that were not transformed, while the internode and leaf area decreased. The flower size was also, changed; the petal limb widths were 40-60% shorter than in the control plants. The petal tube length was also shortened by 5-10% in the transgenic lines. The ovaries, pistils and anthers were also smaller and the pollen grains were less abundant. Pollen viability and germination were reduced with 15-25% of the pollen from the transformed lines being viable compared with 60-80% of the pollen from non-transformed lines. Pollen germination

and seed set were reduced in the transformed lines (Winefield *et al*., 1999). This shows that when plants are transformed, different changes in morphology and pollen viability can arise.

1.4.4 Tomato

In the climatic fruit tomato, the ripening process is controlled by the biosynthesis of ethylene. The genes that code for the enzymes of the ethylene biosynthetic pathway regulate the fruit-ripening ethylene biosynthesis. These processes are also affected by ethylene via some unknown signals. In tomato fruits, mutations in the fruit ripening process have been found *e.g.*, *rin*, *nor* and *Nr* involved in the production and/or perception of primary compounds that initiate the ripening process. The gene *Nr* is homologous to the *ERT1* gene in Arabidopsis. The gene *Nr* affects the normal ethylene responses in tomato, seedling triple response, epinasty, pedicel abscission, petal senescence and fruit ripening (Yen *et al.*, 1995).

1.4.5 Polymerase chain reaction (PCR)

To detect a certain DNA sequence, the sequence has to be copied in large amounts using two oligonucleotide primers because the chain elongation always occurs in the 5' → 3' direction. This method is called polymerase chain reaction (PCR). In PCR, one uses a heat-stable DNA polymerase and a pair of short, synthetic oligonucleotide primers, typically 17-30 nucleotides in length (Primrose *et al.*, 2003), which are designed from the sequence at the ends of the DNA sequence that is to be amplified (Hartl & Jones, 2001). The original duplex molecule is mixed with a vast excess of primer molecules, DNA polymerase and all four nucleoside triphosphates. When the temperature is increased, usually to around 95°C, the strands of the duplex denature and separate. The temperature is then lowered so that the primers become annealed to the separated template strands, usually around 50°C-60°C depending on the G+C content of the oligonucleotides. The primers are placed with the 3' ends in the direction of the region that is being amplified. After the primers have annealed, they are elongated at 70°C by DNA polymerase with the original strand as a template. To complete the cycle, the temperature increased up to 95°C for the second cycle of PCR amplification to denature the duplex DNA. This cycle is repeated around 20-30 times so that the new daughter strand dilutes the original parental strands out so and every molecule that is produced in the PCR has the desired length (distance between the primer 5´ends). In PCR, the copies of the template strand increase exponentially: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, etc. This is the strength of the PCR method. The process of PCR with conventional DNA polymerases is not convenient because the high temperature that is needed for the denaturation leads to the

polymerase itself unfolding and becoming inactive. Thus polymerase must be added in each cycle. However, DNA polymerase is found in organisms that are living in hot springs with temperatures over 90°C. The most commonly used DNA polymerase is called *Taq* polymerase and comes from the bacteria *Thermus aquaticus* (Hartl & Jones, 2001).

1.4.6 Promoters

To get high expression of a transgene in plants, an active promoter has to be used. In dicots there are a number of different promoters used. Promoters from the *Agrobacterium* nopaline synthase (*nos*), octopine synthase (*ocs*) and mannopine synthase (*mas*) genes are widely used, but the most commonly used promoter is the 35S promoter from the cauliflower mosaic virus (CaMV 35S). This promoter is very active and can be improved by duplicating the enhancer region. However these promoters have a low activity in monocots and for such plants, other promoters have to be used for transgene expression (Primrose *et al.*, 2003).

1.4.6.1 Cauliflower mosaic virus and its 35S promoter

The cauliflower mosaic virus (CaMV) has an 8 kb DNA genome that has been completely sequenced and has eight tightly-packed genes expressed as two major transcripts: 35S RNA and 19S RNA. The promoter and terminator for both transcripts have been utilized for expression, but the most used sequence is the 35S promoter. Two of the genes in the CaMV genome are not essential for replication (gene II and gene VII) and since CaMV has an icosahedral capsid, the size of the genome cannot increase very much without affecting the efficiency of packaging (Figure 1.6). The highest capacity of the CaMV capsid is 8.3 kb and if the non-essential genes are removed, the insert size is less than 1 kb. This restriction in the capacity for foreign DNA insertion represents the limitation of CaMV as a vector (Primrose *et al.*, 2003). A problem when using 35S promoter in transformation of plants with delayed flower senescence is that this will have negative effects in seed germination and adventitious rooting due to ethylene insensitivity in the entire plant (Jones *et al.*, 2006). Another promoter is the floral-binding protein (*fbp*) from petunia, which is flower-specific. This means that the promoter gene is expressed only in the petals and stamens. By using *fbp1* promoter instead of 35S promoter, the expression of a transgene in the plant can be restricted to the flower parts instead of expression in all tissues of the plant, which will be the case when using 35S promoter.

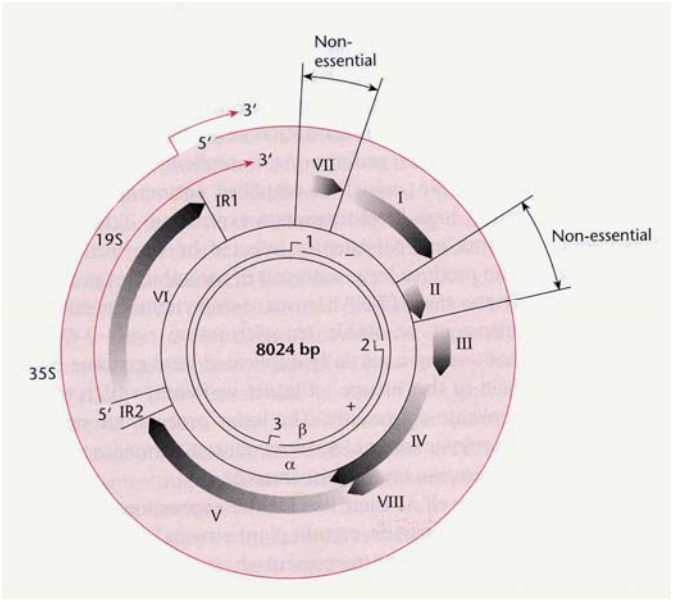


Figure 1.5
Map of the cauliflower mosaic virus genome. The coding regions are represented by thick grey arrows and the reading frames are indicated by radial positions of the boxes. The lines in the centre indicate the DNA strands with the three discontinuities. Major transcripts, 19S and 35S, are shown around the outside (Primrose *et al.*, 2003).

1.4.6.2 Floral-binding protein (*fbp*) as a promoter

The floral-binding protein 1 has been isolated from petunia and is flower-specific. The FBP1 and FBP2 are putative transcription factors that are involved in the MADS box DNA binding. The *fbp1* gene is only expressed in the petals and stamens and not in the sepals, carpels or vegetative parts of the petunia, but the FBP1 protein is only found in the petals (Angenent *et al.*, 1992). Expression of *fbp1* is initiated before the petals and stamens are determined primordially. The *fbp1* is active during flower development and remains restricted to the petals and stamens. The *fbp1* gene also regulates the fate of whorl 2 and 3 organs (Angenent *et al*., 1993)

1.4.7 Gel electrophoresis

DNA fragments that have been produced by an enzyme can be separated according to size by using the fact that DNA is negatively charged and will move when it is placed in an electric field. The DNA molecules move to the positive pole and the speed of movement of the DNA fragments is dependent on the shape and size of the molecules. The most common movement of charged molecules in an electric field is called electrophoresis. In genetics, gel electrophoresis is used. This involves a thin slab of gel: agarose for DNA fragments that are from a few hundred base pairs to around 20 kb, or acrylamide for DNA fragments that are smaller than a few hundred base pairs (Primrose *et al.*, 2003). Small slots (wells) are prepared

where the samples are placed. An electric field is applied and the negatively-charged DNA molecules penetrate into the gel and move towards the anode. The gel is comprised of narrow and small passages, which means that smaller DNA molecules can move easily and the rate of movement increases as the size of the DNA fragments decreases. This results in different discrete regions or bands containing DNA. The bands can be seen under ultraviolet light after the gel has been soaked in the dye ethidium bromide (or the dye can been added to the gel when it is being made). Each band in the gel represents all DNA fragments of a given size that have migrated to the same position in the gel. The smallest visible bands contain about 5 ng of DNA, which for fragments of size 3 kb equals about $1.5 \cdot 10^9$ molecules. This means that a very large amount of copies of the particular DNA fragment has to be present to get a visible band (Hartl & Jones, 2001).

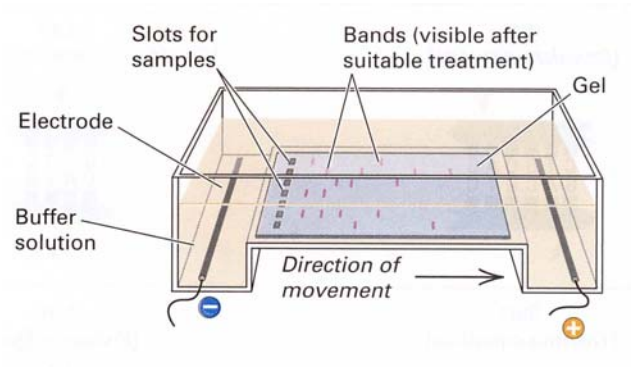


Figure 1.6

Ordinary apparatus used for gel electrophoresis. A gel is made with the desired shape of mould to form the wells used for the samples. After the electrophoresis, the DNA fragments can be visualized on a UV- transilluminator after the gel has been immersed in a solution containing a reagent that binds to the DNA fragments (Hartl & Jones, 2001).

1.4.8 Enzyme-linked immunosorbent assay (ELISA)

The most commonly used solid phase in the ELISA is the 96-well microlitre plate made of polyvinyl chloride or polystyrene. Flat-bottomed wells are best for the ELISA test because they can be used for spectrophotometric reading. The important factor in the solid phase of ELISA is that antigens or antibodies are attached to the surface very easily. The proteins bind to the surface of the plastic because of hydrophobic interactions between the non-polar protein structures and the plastic matrix. When the temperature is high, a higher rate of interaction takes place. The most common incubation schedule is 37°C for 1 to 3 h or overnight at 4°C. The coating buffers that are mostly used are 50 mM carbonate, pH 9.6; 20 mM HCl, pH 8.5 and 10 mM phosphate-buffer saline (PBS), pH 7.2. After the incubation time, the wells are

washed to separate bound and unbound reagents. In the ELISA, it is important to have accurate dispersal of reagents. The volumes that are used in the ELISA range from 50 to 100 µL per well for reagents and 2-10 µL for samples. A reaction between the antigens and antibodies depends on the effectiveness of the concentration, distribution, time and temperature of incubation. There are two incubation conditions that are common: 1) incubation of rotating plates, or 2) incubation of stationary plates. When incubating rotating plates, the purpose is to mix the reactants. This is independent of temperature. When incubating stationary plates, the time and temperature are very important. The temperature that is used is 37°C and the time is 1-3h. Incubation can also take place at 4°C overnight. In the ELISA, there is a risk of non-specific uptake of proteins by the wells from the samples that are added after coating. To prevent this type of binding, there are several methods that can be used. One method is the addition of a highly-concentrated, immunologically-inert substance to the dilution of buffer of the reagent that is added. One of the most important steps in the ELISA is the step when reagents are conjugating to enzymes. The assays are visualised by the development of coloured products after combination of substrates and dye. The antibodies are conjugated to enzymes in this step. Before conjugation, they have to be purified so that unconjugated antigens or antibodies and free enzymes are removed. When the enzymatic reaction is finished, stopping reactions are added to prevent further reaction in ELISA. The product of the substrate catalysis is coloured and can be read by the eye or by a spectrophotometer. Strong positive samples have a strong colour; weak positive results have a partial colour and negative results have no colour (Crowther, 2001).

2 Materials and Methods

2.1 Plant material

Transgenic plants from the *Campanula carpatica* cultivar 'Blue Uniform' were obtained from shoots grown in tissue culture media. The rooted shoots were transferred to small pots with sterilised peat (grade No 1) and covered with plastic to maintain high humidity. The shoots were placed in a basement with 12h light (50 μmol $m^{-2}s^{-1}$) from cool-white fluorescent tubes at room temperature (20°C). After 2-3 weeks, the plants were re-potted into 14 cm diameter pots with peat soil and grown under continuous greenhouse light (100 μmol $m^{-2}s^{-1}$) from SON-T lamps in the basement at room temperature (20°C). After further 3-4 weeks further, the plants were moved to a greenhouse under the conditions: 25°C day and 20°C night temperatures with normal daylight and supplementary irradiance of 100 μmol $m^{-2}s^{-1}$ from SON-T lamps.

2.2 Ethylene sensitivity tests

The test was done eight times with lines in every test. Every test contained four or five plants from the different transgenic lines and 'Blue Uniform' control plants (Table 2.1). The experiments for testing the transgenic lines for their tolerance to ethylene were performed by placing chosen plants in a 160-litre glass chamber under 12h light (50 μmol $m^{-2}s^{-1}$) from cool-white fluorescent tubes at room temperature (20°C). Each plant was given a specific number starting from 1, 2, 3, etc. The target buds on each plant were consequently labelled 1-1, 1-2, 1-3, etc, the first number referring to the number of the plant. If the plants had flowers when the experiment started, the flowers were cut away since the flower could have an influence on the results. The test plants were photographed before they were placed in the aquarium. Petri dishes with silica gel were also placed in the aquarium (Figure 2.1). The silica gel absorbed the moisture that developed in the aquarium during the experiment. To start the experiment, the aquarium was sealed with tape and ethylene gas was introduced through the tape by using a syringe to get the final concentration of 2 ppm. The flowers and buds were observed every day by taking the plants out and recording the condition of each flower and/or bud. After they were recorded, the plants were placed back in the aquarium, which had been ventilated for 1h and the silica gel was replaced. The aquarium was sealed and ethylene was added to achieve the concentration again. After the test, the plants were photographed. The resulting pictures

were used to compare the plants before and after the experiment. After 5 to 7 days, depending on the plants' sensitivity to ethylene, the experiment was terminated. A total of eight tests were performed during the period from 2005-05-24 to 2005-09-25.

Figure 2.1
Ethylene test in a 160-litre aquarium. Blue silica gel was used for absorption of moisture.

Table 2.1

The transgenic lines that were tested for ethylene sensitivity and the number of times they were tested.

Tested lines	SF 15-1	SF 15-1c	SF 15-1de	SF 15-4	Aglo 1	Aglo 2	Aglo 3	Aglo 4	Trans 1	GH 4	Aglo 8.3	SF 11-2	SF 13-1a	SF 13-1	BU control	WU control
Number of tests	3	1	1	4	1	1	3	1	2	**1**	1	2	1	3	11	1

2.3 Morphological studies

Morphological studies were performed on control plants and transgenic lines. Common characters and the differences were documented. The morphological investigation comprised the following characters:

- Plant height
- Plant diameter
- Diameter of the flowers
- Number of petals
- Colour
- Stigma
- Stamens and ovary
- Leaf shape
- Leaf and plant hairiness

2.4 Pollen fertility tests

The purpose of the pollen viability test was to study whether there were any differences between transgenic and control plants regarding pollen physiology, viability and pollen growth. It is important to ensure that the insertion of *etr1-1* of the plants had not influenced the pollen viability. A method for pollen viability was chosen based on work on buckwheat by Kedar & Clyton (1998).

2.4.1 Aniline blue staining test of pollen

Aniline blue was diluted with water with a 1:1 proportion. Pollen grains from newly opened flowers were placed on microscope slides and then one drop from the diluted aniline blue solution was placed on the grains. A cover slip was placed over the microscope slide and then the stained pollen grains were counted under a stereomicroscope of 10x magnification. A minimum of 100 pollen grains were counted. Grains from different stages of the control plant were also investigated to detect any differences in pollen viability, which depends on the age of the flower. The lines that were stained were Aglo 1, SF 15-1, Aglo 3, Aglo 8.3, SF 13-1, SF 28-1, Aglo 4 and ‘Blue Uniform’ as a control. The test was done with a number of repetitions.

2.4.2 Pollen tube germination test of the pollen

The pollen germination was done on newly opened flowers. The lines that were tested for pollen growth were Aglo 1, SF 15-1, Aglo 3, Aglo 8.3, SF 13-1, SF 28-1, Aglo 4 and a BU control. The test was done with a number of replicates. Medium for the pollen tube germination was made by mixing 0.2 g manganese sulphate ($MnSO_4$), 0.2g calcium nitrate ($Ca(NO_3)2.4H_2O$) and 0.2 g potassium nitrate (KNO_3) in 100 mL-distilled water (Kedar & Clyton, 1998). Samples of 10 mL solution were taken and 0.004 g boric acid (H_3BO_3), 1.5g sucrose and 3 g polyethylene glycol (with a molecular weight of 20000) were added (Kedar & Clyton, 1998) and the pH was adjusted to 5-8. Then, 50 μL medium was placed on a microscope slide and the anthers and stigma from newly-opened flowers were dipped into the medium. The slides were then incubated in a plastic box with wet paper to achieve high humidity. The pollen grains were grown for around 5h and then the slides were studied under a stereomicroscope. A drop of methylene blue was added to the samples and a cover slip was placed over the samples. A minimum of one hundred pollen grains was counted per slide. The pollen was said to be mature when the pollen tube was longer than the diameter of the pollen.

2.4.3 Crossing of transgenic lines

The purpose of the crossings was to see whether the transgenic plants had viable pollen grains and also whether the gene *etr1-1* could be transferred to the progeny. The crossings were done in the mornings, because this was the best time to get viable pollen. The pollen grains are dispersed from the stamens in the morning before the humidity changes from high to low. The stigma is most receptive in the early stages of flowering, when the flower has not yet opened up. Pollination started two days before the flower opened and continued until the stigma started to curl up. This was done in 14 days. The pollen was removed from the hairs under the style, stigmas and also from the stamens with a fine brush. The pollen colour was white or yellowish-white when it was mature. The pollen was placed on the tip of the stigma and a bag was placed over the flower so that the flower could not be disturbed (Figure 2.2). In addition, the stamens had to be removed from the flower that was being pollinated to avoid self-pollination. Watering had to be done by flooding and it was very important that the plants did not dry out and that they were in the best condition. Pollination was performed twice a day during a 14-day period. Self-fertilisations were also made from the control plant. The brushes that were used had to be made of natural material to avoid static electricity. The brush was cleaned with ethanol (70%) and rinsed with water and dried between every crossing. The

seeds were ready to be harvested after 3-4 months or when the capsules had started to become dry and brown.

Figure 2.2
Plants being crossed were covered with paper bags.

In the first crossings the following transgenic lines were crossed: SF 15-1, 8,3, Aglo 4, Aglo 3, Aglo 1, SF 11-2, SF 28-1, SF 13-1 and Aglo 2. In addition, the 'Blue Uniform' control plants were crossed and self-crossed. The crossings started on 2005-05-18 and pollination took place twice a day over a 14-day period.

In the second crossings, the transgenic lines SF 15-1de, SF 15-4, Trans 1, Aglo 2, Aglo 3 and Aglo 4 were crossed with the two varieties 'Thor-Pedo' and 'Dark Blue' (supplied by the Campanula grower Thoruplund in Odense SØ) (Figure 2.3). The crossings started on 2005-07-12 and pollination took place twice a day over a 14-day period.

Figure 2.3

The two varieties, ‘Thor-Pedo’ and ‘Dark Blue’ were used for the second crossing with the transgenic lines.

2.5 Molecular investigations

In the genetic molecular investigations known protocols and ready-to-use kits were used and calibrations were performed.

2.5.1 Extraction of DNA

To extract the DNA for the molecular investigation, the DNeasy Plant Mini kit from Qiagen was used. In the first step of the extraction, 400 µL of the buffer AP1 (puffer/buffer1) was added to the finely-ground sample of plant material (buds, flowers and leaves), maximum 100 mg, and samples were then centrifuged. In the next step, 4 µL of RNase A (100 mg mL^{-1}) solution was added to the sample, to degrade the RNA in the sample, and then centrifuged hard again. In the second step the samples were incubated in a water bath for 10 minutes at a temperature of 65°C. In the next step 130 µL of the buffer AP2 (puffer/buffer2) was added and then mixed. The samples were then incubated for 5 minutes in a refrigerator. After this the sample was centrifuged for two minutes at 14000 rpm. In the fourth step, the samples were inserted into a QlAshredder Mini Spin Column that was placed in a 2 mL collection tube. After this step, the liquid collected in the collection tubes was transferred into a new tube. The volume of this collected liquid was approximately 450 µL. A volume of 1.5 of buffer AP3/E (puffer/buffer3/ethanol) was added to the clear liquid and was then mixed, *e.g.*

if the volume of the liquid was 450 µL then 675 µL of the buffer AP3/E had to be added. A volume of 650 µL of the sample was then inserted into the DNeasy Mini Spin Column, which was placed in a 2 mL collection tube. The tube was placed in a centrifuge for 1 minute at 8000 rpm. The liquid that had passed through the column was then disposed of. This step was then repeated with the rest of the liquid and finally the collection tube was disposed of. The DNeasy Spin Mini Column was then placed in a new 2 mL collection tube and 500 µL of the buffer AW (wash) was added to the DNeasy Mini Spin Column and centrifuged for 1 minute at 8000 rpm. The liquid that had passed through the column was disposed of. A volume 500 µL of the buffer AW was then added on the DNeasy Mini Spin Column and centrifuged for 2 minutes at 14000 rpm (to dry the membrane). The DNeasy Mini Spin Column was then transferred to a 1.5 mL micro centrifuge tube and 100 µL of the buffer AE (elution) was added to the membrane. The membrane was then incubated at room temperature for 5 minutes and after this centrifuged for 1 minute at 8000 rpm. This step was repeated once with a new microcentrifuge tube (Figure 2.4) (Qiagen, 2004). Then a test was carried out to see if there was any extracted DNA in the sample. This was done by mixing 4 µL of the extracted DNA sample with 4 µL MiliQ water. The sample was then run for 10 cm in a gel and visualised in a UV transilluminator and photographs taken of the results. A lane with 0.1 µg of the New England Biolabs (NEB) 100 bp DNA ladder was also made.

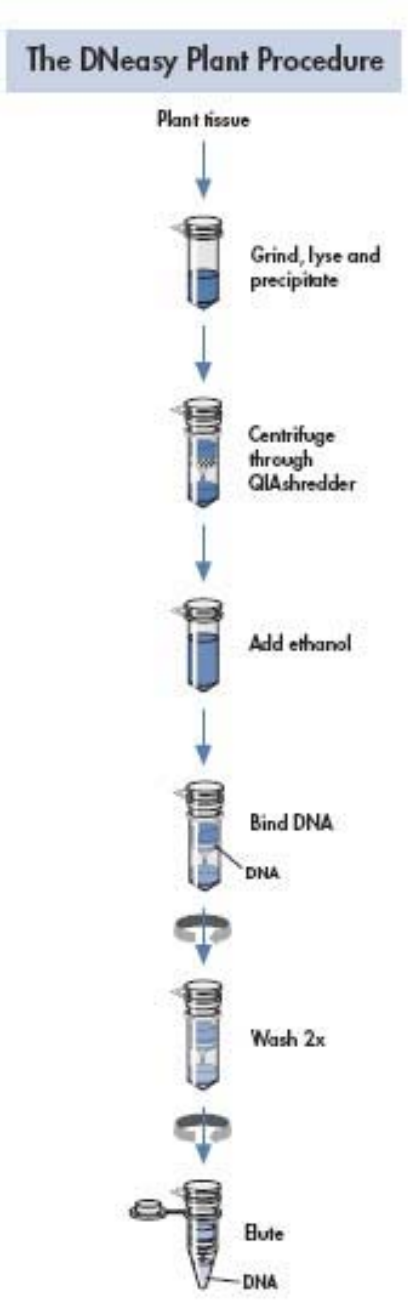


Figure 2.4

Schematic picture of the DNeasy procedure for extraction of plant DNA. In step 1, plant tissue was ground, lysed and precipitated. In step 2, it was centrifuged through a QIA shredder and in step 3, ethanol was added. In step 4, the DNA was bonded to the membrane. In step 5, the membrane was washed two times and in step 6, DNA could be eluted (Qiagen, 2004).

2.5.2 Polymerase Chain Reaction (PCR)

The presence of the *etr1-1* gene could be confirmed by PCR. Two different PCR reactions were done with different primers (see appendices A 8.5 and A 8.6 for more information). In the PCR, 2 µL of the DNA from the transgenic line of interest was used. The stock DNA was diluted 1:40, from the transgenic and control plants. The DNA was used as a template in a 20 µL volume reaction that contained the following components:

In the first PCR experiment, the primers that were used were: left primer 5'-GTG CCA ACT GGG AGT CAT TT-3' and right primer 5-CAC ACG TCC ATG AAG ACC AC-3'. The primers were mixed and 4 µL from the mixed primers was used. In addition, 4 µL of the GoTaq Reaction Buffer 5x, 1.5 µL of dNTP with concentration 2 mM, 0.2 µL of the GoTaq DNA polymerase with concentration 5u μL^{-1} and 9.7 µL of MiliQ water were added. The sample was then placed in a thermocycler (Biometra) and run with a programme that had the following cycles: 5 minutes at 95°C, then 40 cycles of 30 seconds at 94°C, 1 minute at 55°C and 1 minute and 30 seconds at 65°C. After the 40 cycles, the temperature was raised to 72°C for 20 minutes and then lowered to 4°C.

The dNTP was made by mixing 10 µL of each nucleotide, 100 mM deoxyadenosine-5'triphosphate (dATP), 100 mM deoxyguanosine-5'triphosphate (dGTP), 100 mM deoxythymidine-5'triphosphate (dTTP) and 100 mM deoxycytidine-5'triphosphate (dCTP) 100 mM (Hartl & Jones, 2001) with 460 µL MiliQ water so that the total volume was 500 µL (S. Topp, pers. comm.).

The following primers were used in the second PCR amplification by taking 50 µL from each stock solution of mixed primers, which produced the primers that were used in the second set-up of PCR. The primer mix was mixed with 900 µL of MiliQ water so that the total concentration was 100 pmol μL^{-1}. In the stock solution of primer, the concentration of primer Fbp1-etr1-1s 5'-GTT TTG GCC GTA AAC TTG GA-3' was 64 pmol μl^{-1} and 37 pmol μl^{-1} for primer Fbp1-etr1-1as 5'-GTT GAA AGC TCA GGC CAG TC-3'. These two primers were derived from the company MWG Biotech AG. A volume of 2 µL from the final mixed primers and 1.5 µL of dNTP with concentration 2mM were added to the PCR. In addition, 4 µL of the GoTaq Reaction Buffer 5x were added with 0.2 µL of the Taq DNA polymerase, with a concentration 5 u μL^{-1}. Finally, MiliQ water was added so that the total volume was 20 µL. The samples were placed in a thermocycler (Biometra) with a PCR programme that had

the following cycles: 5 minutes at 95°C and then 40 cycles of 30 seconds at 94°C, 1 minute at 55°C, 1 minute and 30 seconds at 65°C. After 40 cycles, the temperature was raised to 72°C for 20 minutes and then lowered to 4°C.

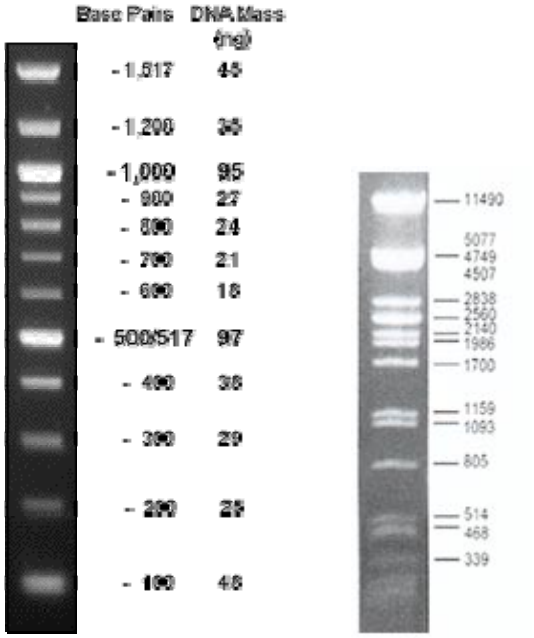


Figure 2.5

100 bp DNA ladder from New England Biolabs and λ *Pst*I DNA ladder from Microzone Limited. The ladders were used to detect the size of the amplified DNA fragment in the electrophoresis. Ladders were standard and manufactured by the suppliers.

The DNA molecules were then separated by gel electrophoresis in a flat gel of SeaKem 1% GTG Agarose in 1x TAE buffer. In addition, a sample of 10 µL of ladder was taken, either New England Biolabs (NEB) 100 bp DNA ladder 0.1 µg μL^{-1} or λ *Pst*I DNA 0.1 µg μL^{-1} (Figure 2.5). The samples were then electrophoresed at 80 V for 30 minutes and then visualised and photographed under UV-transilluminator.

2.5.3 Recipe for the gel

The gels that were used for visualisation of the amplified gene sequences were as follows: Smaller gels contained 25 mL of 1% agarose gel in 1x TAE buffer (40 mM Tris-Acetate, 1mM EDTA pH 8, (Mutui, 2005)) mixed with 0.5 µL ethidium bromide (10 mg/mL). For the medium-sized gels, 50 mL SeaKem 1% agarose gel in 1x TAE buffer was mixed with 3 µL ethidium bromide (10 mg/mL). The 1% agarose gel used was made by adding 2.5 g SeaKem

1% GTG agarose to 250 mL TAE buffer and then mixing and heating so that the GTG agarose was totally diluted (H. Zakizadeh, pers. comm.).

2.5.4 Neomycin phosphotransferase II enzyme-linked immunosorbent assay (*npt*II ELISA)

Tissue (leaf) samples from the different transgenic lines were collected and ground with the PEB1 extraction buffer. The total volume had to be 100 µL of diluted sample extract per well. The reconstruction control was divided into aliquots. Every aliquot had to be sufficient and be run with a small additional volume to assure easy dispensing. It is important not to thaw just before using. One control aliquot was taken out from storage and allowed to thaw. Extract samples were added to the wells and the same volume of the prepared control was added to the control well. The controls cannot be refrozen. By following the loading diagram 100 µL of the prepared samples were placed into the sample wells. A volume of 100 µL of each standard was placed in the standard wells and 100 µL of PEB1 extraction buffer added to the buffer wells. The plate was then placed in a humid box and incubated for 2 hours at room temperature (20°C). The enzyme was prepared by mixing MRS-2 components with 1x PBST buffer in the ratio 1 part MRS-2 component to 4 parts 1x PBST buffer. A few minutes before the incubation time was finished, the enzyme conjugate was prepared by taking enzyme conjugate diluted and from bottles A and B. When the first incubation was ready, the plate was washed by squeezing the long sides of the frame and using a quick flipping motion to get rid of contents in the wells without mixing the contents. All of the wells were then filled to overflowing with 1x PBST and then quickly emptied; a procedure that was repeated 4 to 6 times. After the washing, the frame was held upside down and tapped firmly on a paper towel to dry the wells. Then, 100 µL of the prepared enzyme conjugate was added to the wells. The plate was then incubated in a humid box for 2 hours at room temperature (20°C). The TMB substrate solution had to be warmed up to room temperature before it was used. The volume to be used was measured in a clean container just after addition of the enzyme conjugates. Then the plate was washed 4 to 6 times with 1x PBST as before. When washed, 100 µL of TMB substrate solution was added per well. They were then incubated for 15 minutes in a humid box. After this 50 µL of 3M sulphuric acid was added to each well. The colour changed from blue to yellow (Agdia, 2005). The absorbance of the samples was measured at 450 nm in an ELISA Spectrophotometer.

The concentration of protein in the samples was also determined to allow the total concentration of *npt*II to be calculated. The protein concentration was determined on a new plate with the same samples. Constant standard (bovine serum albumin, BSA) solution was added to the wells in the range of 1-10 µL and MiliQ water so that the total volume in the wells was 30 µL. Two µL of the ground tissue, with the buffer PEB1, was added to the other wells. Then 28 µL MiliQ water was added so that the total volume was 30 µL. The plate was then incubated for 10 minutes at room temperature (20°C), after which 260 µL of the Bradford Reagent (BR) was added to each well (Sigma, 2004). The absorbance of the samples was measured at 595 nm in an ELISA spectrophotometer.

3 Results

3.1 Ethylene sensitivity tests

In test 1, transgenic lines Aglo 2, Aglo 3, Aglo 4, SF 15-4, SF 15-1 and a 'Blue Uniform' control were tested. After two days, the stigma had grown out from the buds in the control plant. On the third day, the buds started to turn brown in the transgenic lines Aglo 2, Aglo 3, Aglo 4 and in the control. On the fourth day, the buds were completely brown in lines Aglo 2, Aglo 3, Aglo 4 and in the 'Blue Uniform' control. Lines SF 15-4 and SF 15-1 had healthy buds and also fine, new, open flowers (Figure 3.1).

In experiment 2, lines Aglo 1, Aglo 3, Aglo 8.3, SF 15-4 and a 'Blue Uniform' control were tested. After two days, Aglo 1, Aglo 3 and the control had brown buds. In line Aglo 8.3, the buds started to become slightly brown while line SF 15-4 had healthy buds and new, open flowers.

In the third experiment, lines Aglo 1, SF 11-2, SF 15-1 and a 'Blue Uniform' control lines were tested. After two days, very small buds in line SF 11-2 started to become brown and the stigma had grown out from the buds in the control plant. On the third, day Aglo 1 had brown buds and the control plant buds had started to become brown. After four days, the buds on SF 11-2 started to turn brown, and on the fifth and final day all buds were brown in all lines except SF 15-1, which had healthy buds and new, open flowers.

In the fourth experiment, lines SF 11-2, SF 15-4, SF 15-1 and a 'Blue Uniform' control were tested. After two days, the buds started to be brown in line SF 11-2 and in the control. The stigma had also grown out of the buds from line SF 15-4. After four days the buds were brown in lines SF 11-2 and in the 'Blue Uniform' control. Buds and new, open flowers were healthy in lines SF 15-4 and SF 15-1.

Test 5 consisted of lines Plant 8, SF 15-4, SF 15-1, 'White Uniform' control and 'Blue Uniform' control. After two days, the buds started to become brown in the 'White Uniform' control line and after three days the buds were brown in lines Plant 8, 'Blue Uniform' control

and ‘White Uniform’ control. In lines SF 15-4 and SF 15-1, the buds and new, open flowers were healthy.

Figure 3.1

Comparison between the line SF 15-1 (left) and a ‘Blue Uniform’ control plant (right). The plants had been tested for ethylene sensitivity for 5 days with an ethylene concentration of 2 ppm.

In experiment 6, lines SF 13-1, SF 13-1a, SF 15-1c and a ‘Blue Uniform’ control were tested. After two days, the stigma had started to grow out from the buds in lines SF 13-1 and SF 13-1a (Figure 3.2). On the third day, the stigma had grown out from the buds in the ‘Blue Uniform’ control plant and the buds were starting to brown in lines SF 13-1 and SF 13-1a. On the fourth and last day, the buds were brown in lines SF 13-1, SF 13-1a and in the ‘Blue Uniform’ control and the leaves had started to curl in these lines. In line SF 15-1c the buds and new, open flowers were healthy.

Test 7 consisted of lines Tran 1, Aglo 3, SF 13-1 and a ‘Blue Uniform’ control. After two days, the buds were brown in the line Trans 1 and after three days the buds were brown in all the lines and the leaves started to curl in line SF 13-1.

The eighth test was made with lines SF 13-1, GH 4 and a 'Blue Uniform' control. After three days, the stigma had started to grow out from the buds in the control plant. On the fourth day, the buds were brown in line SF 13-1 and in the control plant, and the stigma had grown out from the buds, while line GH 4 had healthy buds and new, open flowers.

Figure 3.2

A bud with stigma that have grown out from the buds in the line SF 13-1.

Table 3.1

Results from ethylene sensitivity tests in different transgenic lines of *Campanula carpatica.* The grades are classified: Low tolerance means, buds start to go brown after 1 day. Medium tolerance means, buds start to go brown after 3 days. High tolerance means, buds never go brown after a period up to 7 days.

	SF 15-1	SF 15-1c	SF 15-1de	SF 15-4	SF 11-2	SF 13-1	SF 13-1a	Trans 1	Aglo 1	Aglo 2	Aglo 3	Aglo 4	Aglo 8.3	GH 4	BU control	WU control
Low tolerance					●	●	●	●		●	●	●			●	●
Medium tolerance									●				●			
High tolerance	●	●	●	●										●		

3.2 Morphological studies

3.2.1 Description of the flower organs at different stages

Flowers from the control plant were investigated to see how the organs appeared in the different stages of flower development. When the buds were green, the stamens had not matured and they were bigger than the stigma. The stamens were closed, and did not release any pollen. The pollens were very sticky in this stage. In buds that had started to get some colour, the stamens were very tightly coiled around the stigma and the stigma had to grow through the stamens. When the stigma passed through the stamens, it became covered with pollen on the hairy side. In newly opened flowers, the stigma divided into three parts and at the back was totally covered with pollen. The stamens eventually started to shrink and dry out. In fully opened flowers, the stigma had grown out and curled at a later stage. The stamens had died off and were shrunken. (Figure 3.3).

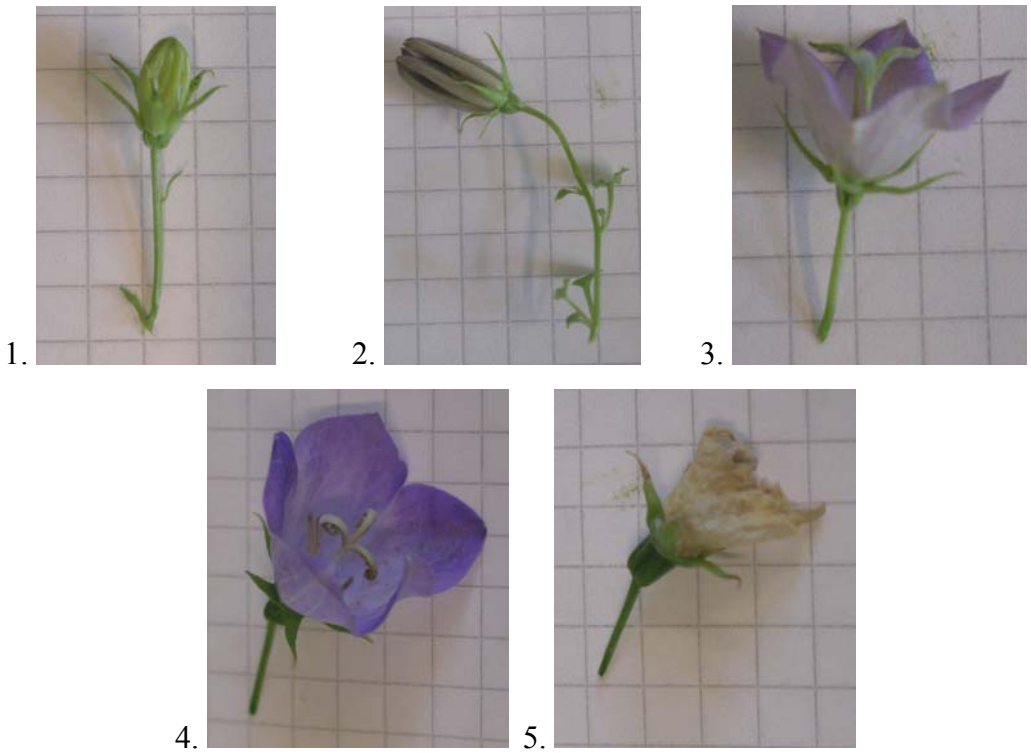


Figure 3.3

The different development stages of the flower. (1) The bud was still immature and green. (2) The bud started to mature and changed colour from green to blue. (3) The flower opened and the stigma was covered with pollen at the lower side and divided into three parts. (4) The stigma started to curve and the stamens had died off. (5) The flower senesced and petals had dried.

3.2.2 Description of the different transgenic lines

This section morphologically describes the different transgenic lines and the differences between them. There was a lack of information on some of the transgenic lines and due to this, a lack of good plants was available for the morphological investigation.

The lines have many common characters, but some differences could be seen. The plants have a compact growing behaviour (SF 11-2 have a pointier growing behaviour) and are 10-25 cm high (SF 13-1 and SF 13-1a up to 32 cm) and 6-20 cm in diameter. The petiole and stem re covered with stiff hairs, as well as the underside of the leaves. The lines SF 28-1, Aglo 2, Aglo 3, Aglo 4, Aglo 8.3 and Plant 8 have no hairs. Leaves are heart-shaped or kidney-shaped and the leaves are alternate (SF 11-2 have pointy sepals and pointed buds). The flowers are 3-6 cm in diameter, campanulate, actinomorphic and have a lilac-blue (white in 'White Uniform' control) colour. Petals are fused together and the flower is actinomorphic. The stigma is divided into 3 parts and there are 4 free stamens, in rare cases 5. The flower has a low-sitting ovary with 5 sepals. The sepals bend backwards as the buds get older. The fruit is a dry poricidal capsule with 5 openings and the seed case is centrally placed. The plant contains white latex. Pictures of some of the lines can be seen in Figure 3.4.

Table 3.2

Outline of the most recognised morphological characters in the different transgenic lines. Because of the lack of plants, some information is lacking about the morphological parts in the lines Aglo 1, Aglo 4, Aglo 8.3, GH 4 and Plant 8.

	SF 15-1	Sf 15-1c	SF 15-1de	SF 15-4	SF 11-2	SF 13-1	SF 13-1a	SF 28-1	Trans 1	Aglo 1	Aglo 2	Aglo 3	Aglo 4	Aglo 8.3	GH 4	Plant 8	BU control	WU control
Heart-shaped leaves	●	●	●	●	●	●	●		●			●					●	●
Oval/heart-shaped leaves											●							
Hairs on leaves	●	●	●	●		●	●					●					●	●
Hairs on petiole	●	●	●	●		●	●										●	●
Hairs on stem	●			●		●	●										●	
Blue-lilac flower colour	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
White flower colour																		●
Five petals	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Five sepals	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Pointy sepals					●													
Stigma divided in 3 parts	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Five stamens	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Ovary under placed	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Fruit dry capsule	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
White latex	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

SF 15-1

SF 15-1c

SF 15-1de

SF 15-4

SF 11-2

SF 13-1a

Trans 1

Aglo 2

Aglo 3

Plant 8 BU control WU control

Figure 3.4

Pictures of the different transgenic lines: SF 15-1, SF 15-1c, SF 15-de, SF 15-4, SF 11-2, SF 13-1a, Trans 1, Aglo 2, Aglo 3, Plant 8 and control plants 'Blue Uniform', 'White Uniform'.

3.3 Results of the pollen fertility tests

3.3.1 Results of the aniline blue staining test

Pollen grains were viable if they had absorbed the aniline blue staining after incubation in room temperature for 5 minutes (Figure 3.5). A mean value was calculated from the staining results and this value gave an indication of how good the pollen viability was. In the one-way ANOVA test it was seen that all lines except SF 13-1 were superior to the BU control ($P < 0.05$) (Appendices 8.8).

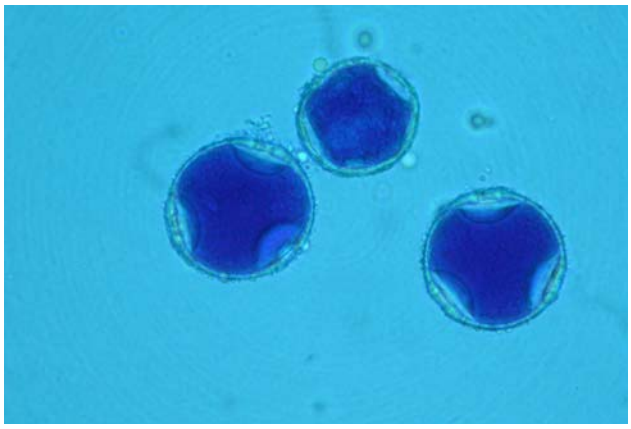


Figure 3.5

Aniline blue stained viable pollen.

Table 3.3

Results of the aniline blue staining test with mean values, P-values and groups.

	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1	SF 28-1	Aglo 4	BU control
Mean value	79.9%	59.7%	73.5%	70.2%	38.9%	75.3%	70.3%	60%
P-value	0.988	0.999	1.000	0.990	0.996	0.980	0.996	1.000
Notes	7 tests	7 tests	6 tests	6 tests	7 tests	3 test	4 tests	8 tests
Group	A	B	A	B	C	A	B	B

The results were placed in three groups. Group A included the lines that had over 70% viability (Aglo 1, Aglo 3, SF 28-1), group B had lines with between 50% and 70% viability (SF 15-1, Aglo 4 and Aglo 8.3 and 'Blue Uniform' control) and group C contained the lines that had a viability under 50% (SF 13-1) (Table 3.3). The results showed that Aglo 1 had the highest pollen viability Aglo 3 and SF 28-1 also had very high viability (all over 70%). More than half the pollen was viable in all lines except for SF 13-1, which had a low viability of only 38.9%. SF 15-1 had the closest viability (59.7%) compared to the 'Blue Uniform' control (60%).

3.3.2 Results from the pollen tube germination test

Pollen was grown when the pollen tube was longer than the diameter of the pollen (Figure 3.6). Mean value was calculated from the pollen tube growth results and gives an indication of the quality of the pollen viability. The pollen was grown at room temperature (20°C) and in light condition of 29-31 μmol m^{-2} s^{-1} daylight. In the one-way ANOVA test, it was seen that all lines except SF 13-1 were above the 'Blue Uniform' control ($P<0.05$) (Appendices 8.9).

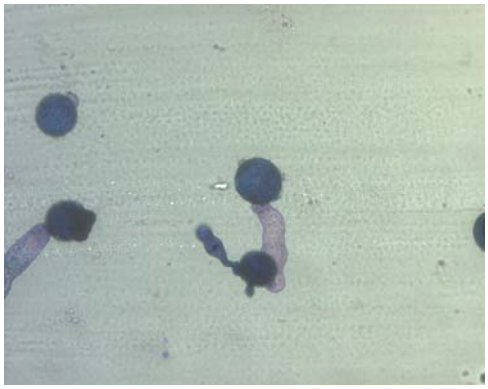


Figure 3.6

Pollen grown from the line Aglo 8.3. In the picture, the pollen tube is visible after staining with methylene blue.

Table 3.4

Results of the pollen tube germination test with mean values, P-values and groups.

	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1	SF 28-1	Aglo 4	BU control
Mean value	36.7%	16.6%	32.8%	24.5%	2.0%	41.0%	30.3%	38.7%
P-value	0.992	0.997	0.994	0.997	0.981	1.000	0.996	0.999
Notes	5 tests	5 tests	5 tests	5 tests	4 tests	2 tests	3 tests	5 tests
Group	A	B	A	B	C	A	A	A

The results indicate that SF 28-1 had the highest pollen growth rate and Aglo 1 and Aglo 3 had over 30% of growth. SF 13-1 had the lowest growth (2%) and SF 28-1 had the closest value compared with the control (Table 3.4). The results indicate that there are three groups. Group A contains the lines that have pollen growth of over 30%: Aglo 1, Aglo 3, Aglo 4, SF 28-1 and 'Blue Uniform' control. Group B line SF 15-1 has a pollen growth of 10–30%, while group C line SF 13-1 has pollen growth under 10%.

3.3.3 Results of the crossings

The crossing of different transgenic lines was very difficult and therefore the results of the two different crossings were not very good. It is very normal that it can be difficult to cross these plants (J. Andersen, pers. comm.)

In the first crossing, seeds were harvested after 4 weeks in the crossings between SF 15-1 and SF 28-1, SF 15-1 and Aglo 3, SF 15-1 and Aglo 1. In the second crossing, seeds were harvested in the crossings between SF 15-4 and Thor-Pedo, Aglo 1 and Dark Blue, Aglo 4 and Dark Blue (Table 3.7). The seeds were used for further investigations. The seeds were viable and seedlings were generated from these seeds (S. Sriskandarajah per. comm.).

Table 3.5

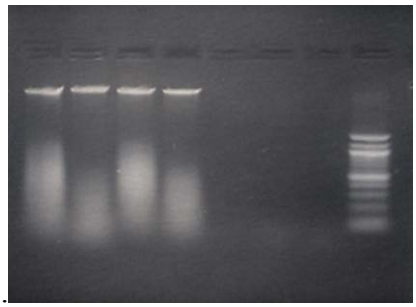
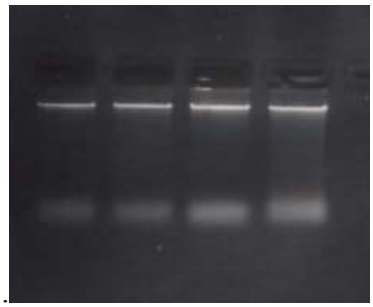
Results from the second crossing.

	SF 15-1	SF 15-4	Aglo 1	Aglo 2	Aglo 3	Aglo 4	Aglo 8.3
Dark Blue		●					
Thor Pedo			●			●	

3.4 Results from the PCR analyses

3.4.1 Extraction of DNA

Forty-five ng of DNA was successfully extracted from the following lines: SF 15-1, SF 15-1c, SF 15-1de, SF 15-4, SF 11-2, SF 13-1a, SF 13-1, Aglo 1, Aglo 2, Aglo 3, GH 4, Trans 1, Plant 8, 'Blue Uniform' control and 'White Uniform' control (Figure 3.7).

1.  2.  3. 

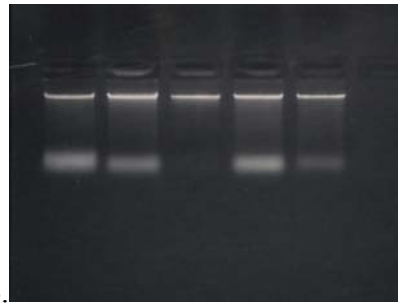
4. 

Figure 3.7

Results of DNA extraction from the lines. The lines were: 1. 'Blue Uniform' control and SF 15-1. 2. SF 15-4, Aglo 1, Aglo 3 and SF 15-1c. 3. SF 15-1de, Aglo 2, SF 11-2 and SF 13-1a. 4. SF 13-1, GH 4, Trans 1, Plant 8 and 'White Uniform' control. In picture 2, a lot of RNA under the DNA bands can be seen because RNase A was missing in the extraction of the sample's DNA, but this did not influence the PCR. Also, the lower bands in fig. 3 and 4 are rRNA.

3.4.2 Results from the first PCR set-up

The PCR was set up as described before with the following primers that were used are: left primer 5'-GTG CCA ACT GGG AGT CAT TT-3' and right primer 5-CAC ACG TCC ATG AAG ACC AC-3'. Amplification of the desired sequence for *etr1-1* in the lines that had a high tolerance to ethylene, with size 800 bp, was successful. Amplification of the *etr1-1* gene in lines that were poorly or moderately tolerant to ethylene was negative. However, there were unexpected results in the transgenic lines SF 13-1 and SF 13-1a, where the gene sequence was also amplified in the PCR, but was poorly tolerant to ethylene in the ethylene

sensitivity test. There was positive amplification of the gene *etr1-1* in the transgenic lines: SF 15-1, SF 15-1c, SF 15-1de, SF 15-4, SF 13-1, SF 13-1a and GH 4. It was not expected to see the gene amplified in the lines SF 13-1 and SF 13-1a because they were very sensitive to ethylene in the ethylene tests. The gene must be inactive, perhaps because of being incorporated in an inactive chromatin region in the lines (B. Stummann, pers. comm.). In the lines SF 11-2, Trans 1, Aglo 1, Aglo 2, Aglo3 and Plant 8 the gene was not amplified, as expected, as they were sensitive to ethylene. Further, the two controls did not amplify the gene (Figure 3.8).

Table 3.6

The symbol ● indicates the result of the amplification test.

	SF 15-1	SF 15-1c	SF 15-1de	SF 15-4	SF 11-2	SF 13-1	SF 13-1a	Trans 1	Aglo 1	Aglo 2	Aglo 3	Plant 8	GH 4	BU control	WU control
Amplification of the gene	●	●	●	●		●	●						●		
Non-amplification of the gene					●			●	●	●	●	●		●	●

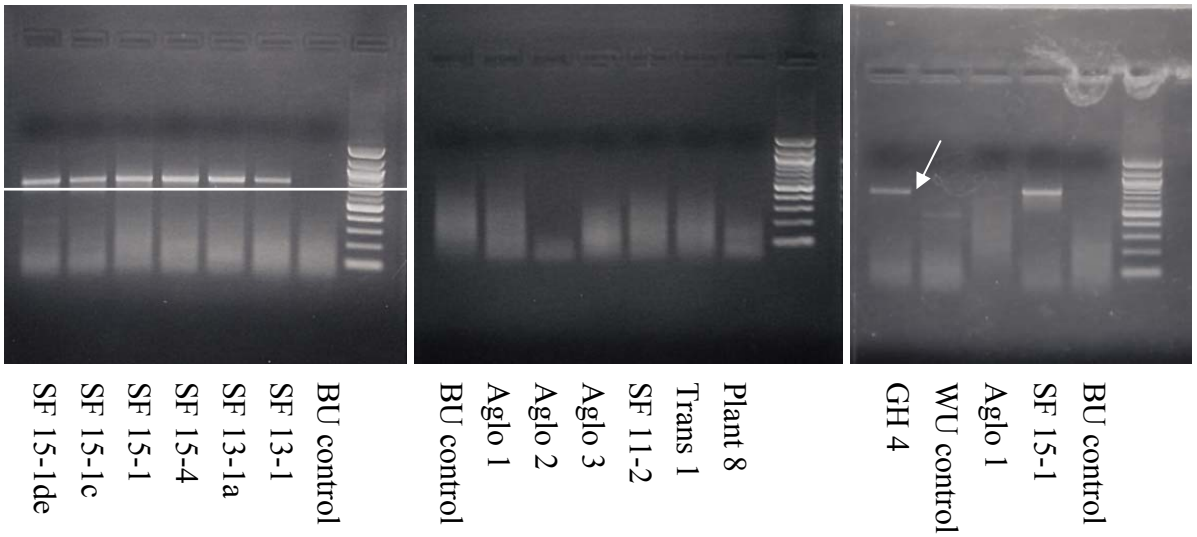


Figure 3.8

Results of the first PCR analysis. Starting from the left picture, the positive result of the amplification of the gene *etr1-1* is seen in lines SF 15-1de, SF 15-1c, SF 15-1, SF 15-4, SF 13-1a and SF 13-1. The plant 'Blue Uniform' control was negative. In picture 2, all lines were negative starting from the left, control, Aglo 1, Aglo 2, Aglo 3, SF 11-2, Trans 1 and Plant 8. In the third and last picture, a positive result, indicated with an arrow, was obtained from line GH 4 and a negative result from the 'White Uniform' control. Aglo 1, SF 15-1 and the control were tested again to confirm the results of PCR and produced the same results.

3.4.3 Results from the second PCR set-up

The PCR was set up as described before and the primers used were Fbp1-etr1-1s 5'-GTTTTGGCCGTAAACTTGGA-3' and Fbp1-etr1-1as 5'-GTTGAAAGCTCAGGCCAGTC-3'. In the PCR, there were some problems in that a lot of unknown bands were amplified because there was an unknown part of the sequence of this gene (Appendices 8.6), but now this unknown part is known (B. Stummann, pers. comm.) (Appendices 8.7). There was one band that was visual only in the transgenic lines that are highly tolerant to ethylene. By using this band they could be separated from the other lines with low tolerance to ethylene. The expected lines that had a very high tolerance to ethylene all amplified the desired sequence for *etr1-1* in the gene at 1700 bp. There was an unexpected result in the transgenic lines SF 13-1 and SF 13-1a, which amplified the gene sequence in the PCR but was poorly tolerant to ethylene in the ethylene sensitivity test. The other lines that were poorly or moderately tolerant to ethylene were not amplified in the gene sequence. There were positive amplifications of the gene *etr1-1* in the transgenic lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4, SF 13-1, SF 13-1a and GH 4. It was not expected to see the gene amplified in the lines SF 13-1 and SF 13-1a because these lines were very sensitive to ethylene in the ethylene tests. The gene must be inactive, perhaps because of being incorporated in an inactive chromatin region, in the lines (B. Stummann, pers. comm.) In the lines SF 11-2, Trans 1, Aglo 1, Aglo 2, Aglo 3 and Plant 8 the gene was not amplified, which was expected since they were sensitive to ethylene. Further, the two controls did not amplify the gene (Figure 3.9).

Table 3.9

The symbol ● indicates the result of the amplification test.

	SF 15-1	SF 15-1c	SF 15-1de	SF 15-4	SF 11-2	SF 13-1	SF 13-1a	Trans 1	Aglo 1	Aglo 2	Aglo 3	Plant 8	GH 4	BU control	WU control
Amplification of the gene	●	●	●	●		●	●						●		
Non-amplification of the gene					●			●	●	●	●	●		●	●

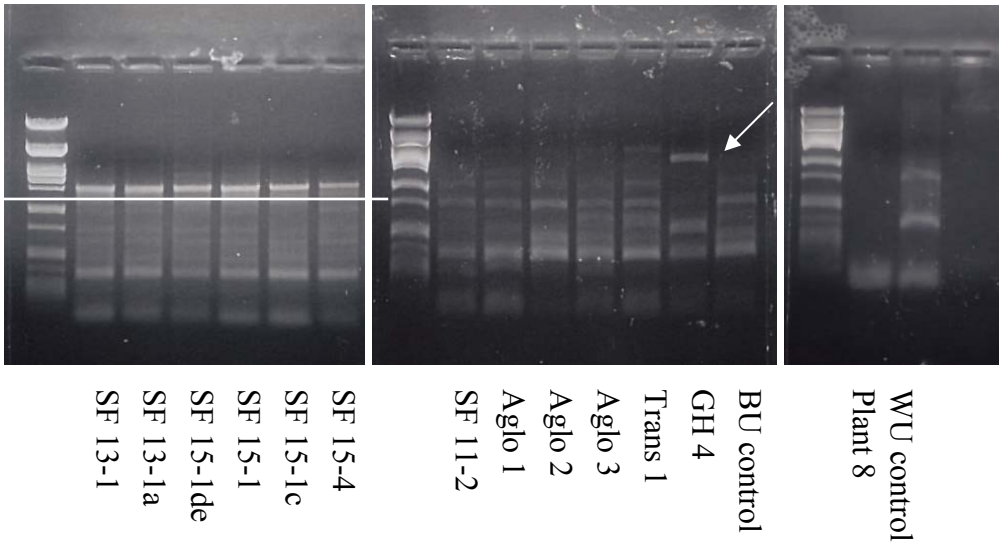


Figure 3.9

Results of the first PCR. Starting from the left picture, the positive result of amplification of the gene *etr1-1* is shown in the lines SF 13-1, SF 13-1a, SF 15-1de, SF 15-1, SF 15-1c and SF 15-4. In picture 2 all lines were negative, starting from the left, SF 11-2, Aglo 1, Aglo 2, Aglo 3, Trans 1 and GH 4 and 'Blue Uniform' control. GH 4 was the only line that was positive (see arrow). In the third and last picture, lines Plant 8 and 'White Uniform' control was tested and proved negative.

3.5 Results from *npt*II ELISA-test

The total protein was calculated by plotting a standard curve for the concentrations of the BSA standard solutions compared with the corresponding absorbance values (Table 3.8 and Figure 3.10 in Appendices 8.10). By using the known equation from the standard curve, the following second order equation. $-0.0019x^2 + 0.092x + (0.3499 + y) = 0$, was derived and used for calculation of the total amount of protein in the transgenic lines and in control lines. The total protein was divided twice because 2 µL of the plant sample was added to the measurements (Table 3.9 in Appendices 8.10). The total *npt*II was calculated by plotting a standard curve for the concentrations of the *npt*II compared with the corresponding absorbance values. These values were taken from the Agdia product documentation that can be found at http://www.agdia.com (Table 3.10 and Figure 3.11 in Appendices 8.10).

Using the known equation from the standard curve, the following second order equation $0.1233x^2 - 0.5702x + (0.6183 - y) = 0$, was obtained and used for calculation of total amount of *npt*II in the transgenic lines and in control lines. Then by dividing the *npt*II amount by the

total protein content in the samples, the pentagram of *npt*II per milligram total protein in plant leaves and buds could be calculated (Table 3.11 in Appendices 8.10). It could be seen that the transgenic plants from the lines that had amplified the gene sequence from the PCR had an *npt*II content of 0.32 pg mg^{-1} of pentagram of *npt*II for the line SF 15-1 to 0.279 pg mg^{-1} of pentagram of *npt*II for the line GH4 of total protein content. The transgenic lines Aglo 2, Aglo 3 and control plants had an *npt*II pentagram content of around 0.22-0.28 pg mg^{-1}. The lowest content was present in the line SF 11-2 with a pentagram of 0.214 pg mg^{-1}.

4 Discussions

4.1 Methods

A detailed description of the ethylene sensitivity test is found in Section 2.2. In the test, buds and flowers sensitive to ethylene from different transgenic lines could be compared. Selection of the transgenic lines with the lowest sensitivity for ethylene expression can be done with this method. The selected transgenic lines have to undergo further molecular investigations to prove that it is the gene *etr1-1* that is responsible for their tolerance to ethylene expression.

Pollen viability, pollen tube growth and crossing tests were chosen to show whether the pollen had changed when the transgene was inserted in to the plants. The three tests are described in Section 2.3. The construction of the viability tests and pollen tube growth tests was possible with the information from several scientific articles. Later in the season it was very difficult to perform these tests due to the fact that the viability of the pollen decreased significantly. The conclusion is that the best time to do these types of tests is in the spring and/or in the beginning of the summer. Campanula plants are known to be very hard to cross and because of this fact the results of crossing were poor. To get a highly quantity of seeds, high numbers of crossings and repetitions have to be done.

A detailed description of the PCR process is given in Section 2.4. The DNeasy Plant Mini Kit was employed in the extraction of the DNA. The DNA was easily and quickly isolated with this method. The PCR method is known to be very sensitive and could detect down to a single DNA molecule, but there can be problems such as amplifying non-specific products due to the presence of other nucleotide sequences. The most probable cause of this is that the binding of primers to the DNA matrix does not exactly match the complementary base sequences, a phenomenon known as misprinting (Mutui, 2005).

The ELISA (Section 2.4.4) indicates the concentration of *npt*II in the plants. The *npt*II is involved in the insertion of the *etr1-1* gene (Section 1.5.1.4). The concentration of *npt*II could be measured and compared to total protein concentration. The test is very sensitive to any disturbances and to get an optimal result it must be done more than once. In this work it was done just once because of the time limitation.

4.2 Results

4.2.1 Ethylene sensitivity tests

The transgenic lines that had the highest tolerance when exposed to ethylene were SF 15-1, SF 15-1de and SF 15-4 and GH 4 (Table 3.1). Medium tolerance was seen in the lines Aglo 1 and Aglo 8.3 and the lines that had low tolerance were Aglo 2, Aglo 3, Aglo 4, SF 11-2, SF 13-1 and SF 13-1a. There were some morphological changes while testing for ethylene sensitivity, in the lines that had a low tolerance to ethylene. The most obvious change was the growth of stigma out from the buds (Figure 3.1). It could be seen that the leaves started to curl in some plant lines. After the plants had been exposed to ethylene (2 ppm), growth was slowed down, which resulted in the plant being more compact. This was mostly an effect of the ethylene treatment.

4.2.2 Morphological studies

There were some changes in the transgenic lines, the most common being a lack of hairs. The lines Aglo 1, Aglo 2, Aglo 3, Aglo 4 and Aglo 8.3 all lacked hairs, the line SF 11-2 had a more pointed growth pattern lines SF 13-1 and SF 13-1a grew much taller than the other lines (Table 3.2). It was also seen that some flowers had more than 5 petals, sometimes up to 7, but this is a natural variation and has nothing to do with the gene transformation. The shape of the leaves was generally pointier than normal, and this could be due to problems with potassium availability for the plants (J. Andersen per. comm.). The other lines had a normal morphological appearance overall.

4.2.3 Pollen fertility tests

In the pollen viability tests, only some lines were tested to get an indication of the viability between the lines. The lines Aglo 1, Aglo 3, Aglo 4, Aglo 8.3, SF 15-1, SF 13-1, SF 28-1 and control plants were tested. When the pollen quality was low due to the seasonality of the pollen (discussed earlier) the studies had to stop even though more results were wanted.

- *Pollen staining for viability*

In the results of the pollen staining, it could be seen that Aglo 1 had the highest mean value and SF 13-1 had the lowest mean value. The control plants and SF 15-1 had the same mean value. This indicates that the line SF 15-1 has the same pollen viability as the control plants.

- *Pollen tube growth*

Results from the pollen tube growth tests show that line SF 28-1 had the highest mean value and line SF 13-1 had the lowest mean value. The control plants had a mean value that was nearest the mean value of SF 28-1. The best time for growth was in the beginning of the season (April to June), while later in the season (August to March) the pollen grains are not viable (J. Andersen, pers. comm.).

- *Crossings*

There were two rounds of crossings. In the first one, different transgenic lines were crossed with each other and in the second, the two varieties 'Thor-Pedo' and 'Dark Blue' were crossed with the transgenic lines. The results from these crossings were favourable. In the first and second set-up, only three crosses succeeded. It is known that crossings in this species can be problematic and that can explain the low degree of success.

4.2.4 Concentration of *npt*II using the ELISA method

In the results, the transgenic plants from the lines that amplified the gene sequence from the PCR had an *npt*II content around 0.3 pg mg^{-1} of total protein content. Because the values do not differ very much between the samples (highest value was 0.311 pg mg^{-1} in the line SF 15-1 and the lowest value was 0.214 pg mg^{-1} in the line SF 11-2) this method is not favoured to prove if the gene *etr1-1* was inserted, but it can give an indication. The results could be influenced by the fact that the test was only done once.

4.2.5 Amplification of *etr1-1* using the PCR method

The amplification of the gene *ert1-1* with the PCR method resulted in the two first primers that could be clearly seen in the lines SF 15-1, SF 15-1de, SF 15-1c, SF 15-4, SF 13-1, SF 13-1a and GH 4. The same result was obtained with the second two primers. But when using the two last primers, problems arose with amplifying non-specific products. This indicates that the gene is present in these lines and with the exception of lines SF 13-1 and SF 13-1a, the ethylene mutated receptor gene works in agreement with the ethylene sensitivity tests. If etr1-1 is not expressed in lines SF 13-1 and SF 13-1a it could be because the T-DNA has been incorporated into "inactive" chromatin. But if that were true we would also expect the Kan gene to be unexpressed (or expressed only slightly). So is the Kan protein level lower (or undetectable) in the lines compared to the ethylene tolerant lines?

5 Conclusions

Plants from the lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 and GH 4 had a high tolerance to ethylene. Lines Aglo 1 and Aglo 8.3 had medium tolerance and the lines SF 11-2, SF 13-1, SF 13-1a, Trans 1, Aglo 2, Aglo 3 and Aglo 4 had low tolerance. In the morphology studies, the lines Aglo 1, Aglo 2, Aglo 3, Aglo 4 and Aglo 8.3 had a wide variation. The line SF 11-2 had a pointy growing behaviour. The two lines SF 13-1 and SF 13-1a grew much higher than the other lines and the lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 and GH 4 had no variation in the morphology. The pollen viability tests showed that the line SF 15-1 had the closest value to control plants. In the pollen tube growth test, line SF 28-1 had the closest value to the control plant. However, the line SF 28-1 was not tested in the ethylene sensitivity or molecular tests. The molecular tests showed the amplification of the gene *etr1-1* in the lines SF 15-1, SF 15-1c, SF 15-1de, SF 15-4 and GH 4 with a high expression of *npt*II. Amplification of the gene *etr1-1* could also be seen in the lines SF 13-1 and SF 13-1a but these lines had a low tolerance to ethylene.

The conclusions are that the lines that have a high tolerance to ethylene have no morphological variations, do have high pollen viability but have low pollen tube growth. Lines that have a low tolerance to ethylene have morphological variation (*e.g.* lack of hairs), low pollen viability but high pollen tube growth (except SF 13-1 that had the lowest pollen tube growth).

More tests, such as RT-PCR, have to be carried out to determine whether the *etr1-1* gene is active in the flower or in other plant parts. Southern blotting has to be carried out to confirm the indications of the gene that was shown in the PCR and ELISA.

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8 Appendices

8.1 Crossings of transgenic plants

Crossing scheme number one

Lines	SF 15-4	SF 15-1	Aglo 8,3	Aglo 4	Aglo 3	Aglo 1	SF 11-2	SF 28-1	SF 13-1*	Aglo 2
	SF 15-1	Aglo 8.3	Aglo 4	Aglo 3	Aglo 1	SF 11-2	SF 28-1	SF 13-1	Aglo 2	
	Aglo 8.3	Aglo 4	Aglo 3	Aglo 1	SF 11-2	SF 28-1	SF 13-1	Aglo 2		
	Aglo 4	Aglo 3	Aglo 1	SF 11-2	SF 28-1	SF 13-1	Aglo 2			
	Aglo 3	Aglo 1	SF 11-2	SF 28-1	SF 13-1	Aglo 2				
	Aglo 1	SF 11-2	SF 28-1	SF 13-1	Aglo 2					
	SF 11-2	SF 28-1	SF 13-1	Aglo 2						
	SF 28-1	SF 13-1	Aglo 2							
	SF 13-1	Aglo 2								
	Aglo 2									

*This plant had a very low pollen quantity

Results from crossing number one

Results									
		SF 28-1	Aglo 3	Aglo 1	BU Control	Aglo 8.3	SF 13-1	Self cont.	-
	SF 15-1	+	+	+		-	-		
	Aglo 8.3	-	-	-					
	BU Control				-				
	Aglo 1	-	-						
	SF 13-1	-	-	-		-			
	Aglo 4	-	-	-		-	-		
	Aglo 3	-							

- = no results and + = results

Crossing scheme number two

Crossing number 2	**Start 080712**							
		SF 15-1	SF 15-4	Aglo 1	Aglo 2	Aglo 3	Aglo 4	Aglo 8.3
Thor-Pedo	-	+	-	-	-	-	-	
Dark Blue	-	-	+	-	-	+	-	

- = no results and + = results

8.2 Results of the staining test of pollen

Lines	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1*	BU control		Date: 2005-05-24	
Stained pollen	137.00	74.00	90.00	107.00	23.00	136.00			
Non stained	36.00	143.00	1.00	28.00	66.00	113.00			
Total pollen	173.00	217.00	91.00	135.00	89.00	249.00			
Fraction %	0.79	0.34	0.99	0.79	0.26	0.55			
% viability	**79.00**	**34.00**	**99.00**	**79.00**	**26.00**	**55.00**			
Lines	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1*	BU control		Date: 2005-05-26	
Stained pollen	166.00	59.00	202.00	214.00	29.00	73.00			
Non stained	35.00	83.00	61.00	105.00	193.00	61.00		*This plant had only old flowers	
Total pollen	201.00	142.00	263.00	319.00	222.00	134.00			
Fraction %	0.83	0.42	0.77	0.67	0.13	0.54			
% viability	**83.00**	**42.00**	**77.00**	**67.00**	**13.00**	**54.00**			
Lines	Aglo 1	SF 15-1	Aglo 3	SF 8.3	SF 13-1*	BU control		Date: 2005-05-27	
Stained pollen	204.00	250.00	149.00	33.00	3.00	138.00			
Non stained	95.00	99.00	110.00	16.00	3.00	116.00		*This plant had only old flowers	
Total pollen	299.00	349.00	259,00	49.00	6.00	254.00			
Fraction %	0.68	0.72	0.58	0.67	0.50	0.54			
% viability	**68.00**	**72.00**	**58.00**	**67.00**	**50.00**	**54.00**			
Lines	Aglo 1	SF 15-1	Aglo 3	SF 8.3	SF 13-1	BU control	SF 28-1	Aglo 4	Date: 2005-06-02
Stained pollen	202.00	442.00	21.00	94.00	35.00	38.00	43.00	16.00	
Non stained	17.00	9.00	31.00	40.00	6.00	11.00	16.00	15.00	
Total stained	219.00	451.00	52.00	134.00	41.00	49.00	59.00	31.00	
Fraction %	0.92	0.98	0.40	0.70	0.85	0.78	0.73	0.52	
% viability	**92.00**	**98.00**	**58.00**	**70.00**	**85.00**	**78.00**	**73.00**	**52.00**	
Lines				Aglo 8.3	SF 13-1	BU control		Aglo 4	Date: 2005-06-27
Stained pollen				151.00	14.00	62.00		28.00	
Non stained				91.00	32.00	69.00		6.00	
Total stained				242.00	46.00	131.00		34.00	
Fraction %				0.62	0.30	0.47		0.82	
% viability				**62.00**	**30.00**	**47.00**		**82.00**	

Lines	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1	BU control	SF 28-1	Aglo 4	Date: 2005-06-27
Stained pollen	134.00	51.00	89.00		37.00	215.00	262.00	155.00	
Non stained	37.00	37.00	29.00		120.00	102.00	77.00	116.00	
Total stained	171.00	88.00	118.00		157.00	317.00	339.00	271.00	
Fraction %	0.78	0.58	0.75		0.24	0.68	0.77	0.57	
% viability	**78.00**	**58.00**	**75.00**		**24.00**	**68.00**	**77.00**	**57.00**	
Lines	Aglo 1	SF 15-1	Aglo 3	Aglo 8.3	SF 13-1	BU control	SF 28-1	Aglo 4	Date: 2005-07-13
Stained pollen	87.00	109.00	145.00	41.00	59.00	107.00	78.00	36.00	
Non stained	28.00	69.00	52.00	13.00	74.00	59.00	24.00	4.00	
Total stained	115.00	178.00	197.00	54.00	133.00	166.00	102.00	40.00	
Fraction %	0.76	0.61	0.74	0.76	0.44	0.64	0.76	0.90	
% viability	**76.00**	**61.00**	**74.00**	**76.00**	**44.00**	**64.00**	**76.00**	**90.00**	
Lines	Aglo 1	SF 15-1				BU control			Date: 2005-07-13
Stained pollen	34.00	108.00				201.00			
Non stained	7.00	95.00				134.00			
Total stained	41.00	203.00				336.00			
Fraction %	0.83	0.53				0.60			
% viability	**83.00**	**53.00**				**60.00**			

8.3 Results of the pollen-growing test

Lines	**Aglo 1**	**SF 15-1**	**Aglo 3**	**Aglo 8.3**	**SF 13-1***	**BU control**	Date: 2005-05-25
Grown pollen	189	23	96	125	1	142	*This plant had only old flowers
Non grown	42	204	165	81	92	163	
Total pollen	231	227	261	206	93	305	
Fraction %	0.818182	0.101322	0.367816	0.606796	0.010753	0.465574	
% growth	**81.8**	**10.1**	**36.8**	**60.7**	**1.1**	**46.6**	

Lines	**Aglo 1**	**SF 15-1**	**Aglo 3**	**Aglo 8.3**	**SF 13-1***	**BU control**	Date: 2005-05-26
Grown pollen	83.00	75.00	38.00	153.00	6.00	46,00	
Non grown	235.00	290.00	235.00	214.00	121.00	120,00	
Total pollen	318.00	365.00	273.00	367.00	127.00	166,00	
Fraction %	0.26	0.21	0.14	0.42	0.05	0.28	
% growth	**26.00**	**21.00**	**14.00**	**42.00**	**5.00**	**28.00**	

Lines	**Aglo 1**	**SF 15-1**	**Aglo 3**	**Aglo 8.3**	**SF 13-1**	**BU control**	**SF 28-1**	**Aglo 4**	Date: 2005-06-02
Grown pollen	6.00	29.00	48.00	26.00	2.00	15.00	170.00	29.00	
Non grown	34.00	154.00	54.00	171.00	288.00	181.00	78.00	118.00	
Total pollen	40.00	183.00	102.00	197.00	290.00	196.00	248.00	147.00	
Fraction %	0.15	0.16	0.47	0.13	0.01	0.08	0.69	0.20	
% growth	**15.00**	**16.00**	**47.00**	**13.00**	**1.00**	**8.00**	**69.00**	**20.00**	

Lines	**Aglo 1**	**SF 15-1**	**Aglo 3**	**Aglo 8.3**	**SF 13-1**	**BU control**	**SF 28-1**	**Aglo 4**	Date: 2005-06-03 (in dark)
Grown pollen	20.00	22.00	105.00	3.00	2.00	82.00	50.00	104.00	
Non grown	272.00	398.00	183.00	81.00	262.00	199.00	347.00	152.00	
Total pollen	292.00	420.00	288.00	84.00	264.00	281.00	397.00	256.00	
Fraction %	0.07	0.05	0.36	0.04	0.01	0.29	0.13	0.41	
% growth	**7.00**	**5.00**	**36.00**	**4.00**	**1.00**	**29.00**	**13.00**	**41.00**	

Lines	**Aglo 1**	**SF 15-1**	**Aglo 3**	**Aglo 8.3**		**BU control**		**Aglo 4**	Date: 2005-07-18
Grown pollen	62	54	41	6		144		42	
Non grown	55	122	94	179		31		96	
Total pollen	117	176	135	185		175		1380	
Fraction %	0.53	0.31	0.30	0.03		0.82		0.30	
% growth	**53.00**	**31.00**	**30.00**	**3.00**		**82.00**		**30.00**	

8.4 Ethylene tests

Test number one (2005-05-24)

Plants	Aglo 3	Aglo 2	BU control	Aglo 4	SF 15-4	SF 15-1
Buds/flowers	1-1	2-1	3-1	4-1	5-1	6-1
	1-2			4-2		6-2
	1-3			4-3		6-3
						6-4
						6-5
						6-5

Notes to the test:

Plants of the lines SF 15-1 and Aglo 4 had open flowers, number 6-3, 6-2 and 4-2 when they were placed in the aquarium. After one day flowers had opened in all the buds of the plants SF 15-1, plant Aglo 4 (bud number 4-2), SF 15-1 (number 5-1) and Aglo 3 (number 1-2).

After two days all flowers were open in the plant of the line SF 15-1 and they were healthy Aglo 4 the buds and flowers were healthy, SF 15-4 the flowers were healthy, in BU control the bud numbers 3-1 were healthy but the stigma had started to grow out, in line Aglo 2 the buds were healthy, in Aglo 3 the flowers 1-2 were healthy, bud 1-1 was healthy and bud 1-3 was healthy and starting to open, and the other buds were healthy.

On the third day the plant of the line SF 15-1 had buds that were opened and the flowers were healthy. In the plant of Aglo 4 the buds were little brown in the top and the flowers also started to be a little brown. In the plant of line SF 15-4 the buds and flowers were healthy, in BU control the buds were starting to go brown. In the plant of Aglo 2 the buds were starting to go brown and in Aglo 3 the flowers and buds were starting to go brown but bud 1-3 was healthy.

After four days the buds were totally dried in the plant of Aglo 3. In Aglo 2 the buds were dried, in BU control the buds were dried. In Aglo 4 the buds were totally dried and in SF 15-4

the flowers were healthy but a little pale. In SF 15-1 the flowers looked healthy and all the buds were open

On the fifth day and last, the Aglo 3 plant had six brown and dried buds. In Aglo 2 there were two brown and dried buds and some leaves were brown. In BU control there were two brown buds. Aglo 4 had six brown buds and some leaves starting to go brown. SF 15-4 had six open flowers and SF 15-1 had 18 open flowers and the buds 6-3 and 6-4 were starting to go brown.

Test number two (2005-06-01)

Plants	SF 15-4	Aglo 3	BU control (1)	Aglo 1	BU control (2)	Aglo 8.3
Buds/Flowers	1-1	2-1	3-1	4-1	5-1	6-1
	1-2	2-2	3-2	4-2	5-2	6-2
	1-3	2-3	3-3	4-3	5-3	6-3
		3-4	3-4	4-4	5-4	
				4-5		
				4-6		
				4-7		

Notes to the test:

On the first day all flowers and buds were healthy in all plants.
On the second day, plant from lines SF 15-4, the buds 1-1 and 1-2 are open and flowering nicely. In Aglo 3, the flowers and buds were healthy but buds number 2-3 was brown. In plant from BU control (1) buds 3-1, 3-2 were brown and buds 3-3, 3-4 healthy. In Aglo 1, all the flowers and buds were brown or going brown but bud 4-4 was healthy. In the plant from line BU control (2) the flowers and buds were healthy. In the Aglo 8.3 plant bud 6-1 was healthy and bud 6-2 browning in the top, bud 6-3 was brown.

Test number three (2005-06-13)

Plants	SF 11-2	BU control (1)	Aglo 1	SF 15-1	BU control (2)
Buds/Flowers	1-1	2-1	3-1	1-4	5-1
	1-2	2-2	3-2	2-4	5-2
	1-3	2-3		3-4	5-3
	1-4	2-4		4-4	5-4
					5-5

Notes to the test:

On day one all the flowers and buds were healthy on all plants. On the second day too, all the flowers and buds were healthy on all plants.

On the third day, the buds were healthy on the plant from line SF 11-2, but very small buds were brown. In the BU control (1) plant, bud 2-1 was flowering nicely and rest of the buds were healthy. In Aglo 1 the bud 3-1 were flowering nicely and the rest of the buds were healthy. In SF 15-1 all the buds had started to flower. In the BU control (2), the buds 5-1, 5-3 and 5-4 were flowering nicely and in the bud 5-2 the stigma had grown out from the buds.

After four days the plant of SF 11-2 the buds were healthy, but bud 1-4 had just started to go brown, some other buds had the same tendency. In the plant of BU control (1) the flowers and buds were healthy. The stigma had grown out in the buds 2-2, 2-3 and 2-4. In the plant of line Aglo 1, the bud 3-1 was totally brown, 3-2 was healthy and the other buds were just starting to go brown. In the plant of SF 15-1, the flowers and buds were healthy. In line BU control (2), the buds 5-1 and 5-3 were flowering nicely and in other buds the stigma had grown out and bud 5-2 were starting to get brown areas.

At the fifth day the plant of the line SF 15-1 had flowers that were healthy, but some were pale because of the bad light. Also, one unlabelled flower was a little brown. In the plant from line BU control (1), the buds 2-2 and 2-3 were starting to go brown, 2-1 flowers looked nice and the other buds were healthy. In line Aglo 1, the buds were starting to go brown and bud 4-1 was totally brown. In the plant from line SF 11-2, buds were just starting to go brown. In BU control (2), the buds 5-2 and 5-5 and flower 5-3 were starting to go brown, other looked healthy although some buds were starting to go brown.

After seven days, all buds are brown on the plant from the line SF 11-2. In the plant from BU control (1), all flowers and buds were brown. In the plant from the line Aglo 1, all flowers and buds were brown. In the plant from line SF 15-1, flowers and buds looked healthy. In the plant from the line BU control (2), all buds were brown.

Test number four (2005-06-30)

Plants	SF 11-2	SF 15-4	SF 15-1de	BU control (1)	BU control (2)
Bud number	1-1	2-1	3-1	4-1	5-1
	1-2	2-2-		4-2	5-2
	1-3	2-3		4-3	5-3
	1-4	2-4		4-4	
		2-5		4-5	
		2-6		4-6	
		2-7		4-7	

Notes to the test:

After one day, on the plant from SF 15-4, the buds 2-1, 2-5 and 2-7 were flowering nicely and the other buds were healthy. In the plant from the line SF 11-2, the bud 1-2 was flowering nicely and the other buds were healthy. In the plant from line BU control (1), the bud 4-6 was flowering nicely and the other buds were healthy. In SF 15-1de, the bud 3-1 was flowering nicely and the others were healthy. In the plant from the line BU control (2), all the buds were healthy.

On the second day the plant from line SF 11-2 had buds 1-1, 1-5, 1-4 and 1-3 starting to go brown at the top, bud 1-2 flowers were starting to go brown and other buds were also starting to go brown. In the plant from the line BU control (1), the buds 4-2, 4-3, 4-4, 4-7, 4-8, 4-1 and 4-5 were starting to go brown and 4-6 was flowering nicely. In the plant from the line BU control (2), the flowers were healthy and the stigma had grown out from the buds. In the plant from the line SF 15-1de, the buds and flowers were healthy.

On the third day the plant from line BU control (1) had all the buds brown and the stigma had grown out. In the plant from the line SF 15-4, all flowers looked nice. In the plant from line SF 11-21, the flowers and buds were brown. In the plant from line BU control (2), all the buds

were brown. In the plant SF 15-1de, the bud 3-1 was flowering nicely and the other buds were healthy.

After four days the plant from line BU control (1) had all the buds brown. In the plant from line SF 11-2 all the buds were brown. In SF 15-4, the bud 2-5 was brown and the other buds were flowering nicely. In the plant from line BU control (2), all the buds were totally brown. In SF 15-1de, bud 3-1 was flowering nicely and the rest of the buds were healthy.

Test number five (2005-07-13)

Plants	Plant 8	BU control	SF 15-4	SF 15-1	WU control
Bud number	1-1	2-1	3-1	4-1	5-1
	1-2	2-2-	3-2	4-2	5-2
	1-3		3-3	4-3	5-3
			3-4	4-4	
		2-5	3-5	4-5	
		2-6	3-6	4-6	
		2-7		4-7	

Notes to the tests:

After one day the plant from the line SF 15-1 had buds 4-1, 4-2, 4-3 and 4-5 flowering nicely and the other buds were healthy. In the plant BU control, the buds were healthy and in the plant from the line SF 15-4 bud 3-4 was flowering nicely and the other buds were healthy. In the plant from the line Plant 8 the buds were healthy. In the line WU control plant the buds 5-2 and 5-3 were flowering nicely and the rest of the buds were healthy.

In the second day the plant from the line Plant 8 had buds that were healthy. In the plant from the line BU control the bud 2-2 had a tendency to be pale but the other buds were healthy. In the plant from the line SF 15-4 the buds, 3-1 and 3-4 were flowering nicely and the rest of the buds were healthy. In the plant from the line SF 15-1 the buds, 4-6, 4-1, 4-2, 4-3 and 4-5 were flowering nice and rest of the buds looked healthy. In the plant from line WU control the buds 5-2 and 5-3 were brown and the rest of the buds look healthy.

After three days the plant from the line SF 15-4 had flowers that were healthy and bud 3-4 was starting to go brown. In the plant from the line BU control, all the buds were brown. In the plant from the line SF 15-1, all the buds were flowering nicely and the buds 4-3 and 4-5 starting to go brown. In the plant from the line Plant 8 all buds were brown. In the plant from the line WU control, all the flowers and buds were brown.

Test number six (2005-08-30)

Plants	SF 15-1c	BU control	SF 13-1a	SF 13-1
Bud number	1-1	2-1	3-1	4-1
	1-2	2-2	3-2	4-2
	1-3	2-3	3-3	4-3
	1-4		3-4	4-4
	1-5		3-5	
	1-6		3-6	
	1-7		3-7	
	1-8		3-8	
			3-9	

Notes to the tests:

After one day the buds and flowers looked healthy on all the plants. On the second day the plant from the line SF 13-1 the buds, 4-1 and 4-2 were flowering nicely. In the buds 4-3 and 4-4 the stigma had grown out from the bud. In the plant from the line SF 15-1 the buds, 1-1, 1-2, 1-3, 1-4, 1-6 were flowering nicely and the rest of buds were healthy too. In the plant from the line SF 13-1a buds 3-5, 3-7 and 3-9 were flowering nicely and the rest of buds were looking healthy, but the stigma had grown out from the buds. In the plant from the line BU control, the buds looked healthy.

After three days, the BU control plant had the stigma grown out in bud 2-3 and the rest of the buds were looking healthy. In the plant from the line SF 15-1, all the buds and flowers were nice and the rest of the buds looked healthy. In the plant from the line SF 13-1a the buds, 3-5, 3-7 and 3-9 had started to go brown. The buds 3-3 and 3-4 had a tendency to go brown. The rest of the buds looked nice and the stigma had grown out from all the buds. In the plant from

the line SF 13-1 the buds, 4-1 and 4-4 were starting to go brown and 4-2 was brown. In all the buds the stigma had grown out.

On the fourth day in the plant from the line SF 13-1, all the buds and flowers were brown. In the plant from the line SF 13-1, all the buds and flowers were brown and also the leaves were starting to curl. In the plant from the line BU control, all the buds and flowers were brown and the leaves were starting to curl. In SF 15-1, all the flowers were flowering nicely but the buds 1-2, 1-3 and 1-4 had started to senescence.

Test number seven (2005-09-07)

Plants	Aglo 3	BU control	SF 13-1	Trans 1
Bud number	1-1	2-1	3-1	4-1
	1-2	2-2	3-2	
	1-3	2-3	3-3	
		2-4	3-4	
		2-5	3-5	
		2-6	3-6	

Notes to the tests:

In the first day in the Aglo 3 plant, the bud 1-2 was flowering nicely and rest of the buds looked healthy. In the plant from the line BU control the buds, 2-1 and 2-2 had started to flower and the rest of the buds looked healthy. In the plant from the line SF 13-1 buds, 3-1, 3-2 and 3-3 were flowering nicely but bud 3-4 started to be brown. The rest of buds looked nice. In the plant from the line Trans 1 the bud 4-1 looked nice and the rest of buds had started to go brown.

In the second day in the plant from the line Aglo 3, all the flowers were brown. In the plant from the line BU control, all flowers and buds were brown and leaves had also started to go brown. In the plant from the line SF 13-1, all the flowers and buds were brown and the leaves started to curl. In the plant from the line Trans 1 bud 4-1 was brown and rests of the buds were brown.

Test number eight

Plants	SF 13-1	BU control	GH 4
Buds number	1-1	2-1	3-1
	1-2	2-2	
	1-3		
	1-4		
	1-5		

Notes to the tests:

On the first day, bud 3-1 in the plant from the line GH 4 looked nice and the rest of the buds also looked healthy. In the plant from the line BU control the bud 2-1 petals had started to bend backwards. The bud 2-2 looked nice and the rest of the buds also looked healthy. In the plant from the line SF 13-1, all the buds looked nice.

After two days in the plant from the line GH 4 the bud 3-1 looked nice and the rest of the buds were healthy. In the plant from the line BU control the bud 2-1 was flowering nicely and the bud 2-2 had started to open and the stigma had grown out. In bud 1-1 and 1-3 in the plant from the line SF 13-1 the stigma had grown out and rest of buds looked healthy.

On the third day in the plant from the line BU control, the bud the 2-2 was brown and the stigma had grown out. The other buds were brown. In the plant from the line SF 13-1 1, all the buds were brown and the stigma had grown out. In the plant from the line GH 4, bud 3-1 was flowering nicely and the other buds looked nice.

8.5 Gene sequences obtained from gene bank and used for the construction of gene-specific primer pairs in the first PCR set-up

L24119. Arabidopsis thali...[gi:409704]

Arabidopsis thaliana ETR1 gene

AAAGATAGTATTTGTTGATAAATATGGGGATATTTATCCTATATTATCTGTATTTTTCTTACCATTTTTACTCTA
TTCCTTTATCTACATTACGTCATTACACTATCATAAGATATTTGAATGAACAAATTCATGCACCCACCAGCTATA
TTACCCTTTTTTATTAAAAAAAAACATCTGATAATAATAACAAAAAAATTAGAGAAATGACGTCGAAAAAAAAAG
TAAGAACGAAGAAGAAGTGTTAAACCCAACCAATTTTGACTTGAAAAAAAGCTTCAACGCTCCCCTTTTCTCCTT
CTCCGTCGCTCTCCGCCGCGTCCCAAATCCCCAATTCCTCCTCTTCTCCGATCAATTCTTCCCAAGTAAGCTTCT
TCTTCCTCGATTCTCTCCTCAGATTGTTTCGTGACTTCTTTATATATATTCTTCACTTCCACAGTTTTCTTCTGT
TGTTGTCGTCGATCTCAAATCATAGAGATTGATTAACCTAATTGGTCTTTATCTAGTGTAATGCATCGTTATTAG
GAACTTTAAATTAAGATTTAATCGTTAATTTCATGATTCGGATTCGAATTTTACTGTTCTCGAGACTGAAATATG
CAACCTATTTTTTCGTAATCGTTGTGATCGAATTCGATTCTTCAGAATTTATAGCAATTTTGATGCTCATGATCT
GTCTACGCTACGTTCTCGTCGTAAATCGAAGTTGATAATGCTATGTGTTTGTTACACAGGTGTGTGTATGTGTGA
GAGAGGAACTATAGTGTAAAAAATTCATAATGGAAGTCTGCAATTGTATTGAACCGCAATGGCCAGCGGATGAAT
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CACTTGTTGAGCTTGGTAGGACATTAGCTTTGGAGGAGTGTGCATTGTGGATGCCTACTAGAACTGGGTTAGAGC
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AATATATGCTAGGGGAGGTGGTCGCTGTGAGGGTTCCGCTTCTCCACCTTTCTAATTTTCAGATTAATGACTGGC
CTGAGCTTTCAACAAAGAGATATGCTTTGATGGTTTTGATGCTTCCTTCAGATAGTGCAAGGCAATGGCATGTCC
ATGAGTTGGAACTCGTTGAAGTCGTCGCTGATCAGGTTTTACATTGCTGAGAATTTCTCTTCTTTGCTATGTTCA
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TCCGTGCCCGCAATGATTTCCTAGCGGTTATGAACCATGAAATGCGAACACCGATGCATGCGATTATTGCACTCT
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CATTCAATCTTCATACATTATTTAGAGAGGGTAACTTTTGAACAGCTCTATGTTTCATAAGTTTATACTATTTGTG
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CATCACACTAAATCTTGCACCAGATTTGCCAGAATTTGTTGTTGGGGATGAGAAACGGCTAATGCAGATAATATT
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CACACGAGCTGCTGACTTTTTTGTCGTGCCAACTGGGAGTCATTTCTACTTGAGAGTGAAGGTTATTATCTTGTA
TCTTGGGATCTTATACCATAGCTGAAAGTATTTCTTAGGTCTTAATTTTGATGATTATTCAAATATAGGTAAAAG
ACTCTGGAGCAGGAATAAATCCTCAAGACATTCCAAAGATTTTCACTAAATTTGCTCAAACACAATCTTTAGCGA
CGAGAAGCTCGGGTGGTAGTGGGCTTGGCCTCGCCATCTCCAAGAGGTTTGAGCCTTATTAAAAGACGTTTTTTT
CCAACTTTTTCTTGTCTTCTGTGTTGTTAAAAGTTTACTCATAAGCGTTTAATATGACAAGGTTTGTGAATCTGA
TGGAGGGTAACATTTGGATTGAGAGCGATGGTCTTGGAAAAGGATGCACGGCTATCTTTGATGTTAAACTTGGGA
TCTCAGAACGTTCAAACGAATCTAAACAGTCGGGCATACCGAAAGTTCCAGCCATTCCCCGACATTCAAATTTCA
CTGGACTTAAGGTTCTTGTCATGGATGAGAACGGGTTAGTATAAGCTTCTCACCTTTCTCTTTGCAAAATCTCTC
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CGTATCACTAGACAACATAAGAGATGTTCTGTCTGATCTTCTCGAGCCCCGGGTACTGTACGAGGGCATGTAAAG
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TCATACCATTTCAGATTACATGTTTGACTAATGTTGTATCCTTATATATGTAGTTACATTCTTATAAGAATTTGG
ATCGAGTTATGGATGCTTGTTGCGTGCATGTATGACATTGATGCAGTATTATGGCGTCAGCTTTGCGCCGCTTAG
TAGAACAACAACAATGGCGTTACTTAGTTTCTCAATCAACCCGATCTCCAAAA

ATGGAAGTCTGCAATTGTATTGAACCGCAATGGCCAGCGGATGAATTGTTAATGAAATACCAATACATCTCCGAT
TTCTTCATTGCGATTGCGTATTTTTCGATTCCTCTTGAGTTGATTTACTTTGTGAAGAAATCAGCCGTGTTTCCG
TATAGATGGGTACTTGTTCAGTTTGGTGCTTTTATCGTTCTTTGTGGAGCAACTCATCTTATTAACTTATGGACT
TTCACTACGCATTCGAGAACCGTGGCGCTTGTGATGACTACCGCGAAGGTGTTAACCGCTGTTGTCTCGTGTGCT
ACTGCGTTGATGCTTGTTCATATTATTCCTGATCTTTTGAGTGTTAAGACTCGGGAGCTTTTCTTGAAAAATAAA
GCTGCTGAGCTCGATAGAGAAATGGGATTGATTCGAACTCAGGAAGAAACCGGAAGGCATGTGAGAATGTTGACT
CATGAGATTAGAAGCACTTTAGATAGACATACTATTTTAAAGACTACACTTGTTGAGCTTGGTAGGACATTAGCT
TTGGAGGAGTGTGCATTGTGGATGCCTACTAGAACTGGGTTAGAGCTACAGCTTTCTTATACACTTCGTCATCAA
CATCCCGTGGAGTATACGGTTCCTATTCAATTACCGGTGATTAACCAAGTGTTTGGTACTAGTAGGGCTGTAAAA
ATATCTCCTAATTCTCCTGTGGCTAGGTTGAGACCTGTTTCTGGGAAATATATGCTAGGGGAGGTGGTCGCTGTG
AGGGTTCCGCTTCTCCACCTTTCTAATTTTCAGATTAATGACTGGCCTGAGCTTTCAACAAAGAGATATGCTTTG
ATGGTTTTGATGCTTCCTTCAGATAGTGCAAGGCAATGGCATGTCCATGAGTTGGAACTCGTTGAAGTCGTCGCT
GATCAGGTGGCTGTAGCTCTCTCACATGCTGCGATCCTAGAAGAGTCGATGCGAGCTAGGGACCTTCTCATGGAG
CAGAATGTTGCTCTTGATCTAGCTAGACGAGAAGCAGAAACAGCAATCCGTGCCCGCAATGATTTCCTAGCGGTT
ATGAACCATGAAATGCGAACACCGATGCATGCGATTATTGCACTCTCTTCCTTACTCCAAGAAACGGAACTAACC
CCTGAACAAAGACTGATGGTGGAAACAATACTTAAAAGTAGTAACCTTTTGGCAACTTTGATGAATGATGTCTTA
GATCTTTCAAGGTTAGAAGATGGAAGTCTTCAACTTGAACTTGGGACATTCAATCTTCATACATTATTTAGAGAG
GTCCTCAATCTGATAAAGCCTATAGCGGTTGTTAAGAAATTACCCATCACACTAAATCTTGCACCAGATTTGCCA
GAATTTGTTGTTGGGGATGAGAAACGGCTAATGCAGATAATATTAAATATAGTTGGTAATGCTGTGAAATTCTCC
AAACAAGGTAGTATCTCCGTAACCGCTCTTGTCACCAAGTCAGACACACGAGCTGCTGACTTTTTTGTCGTGCCA
ACTGGGAGTCATTTCTACTTGAGAGTGAAGGTAAAAGACTCTGGAGCAGGAATAAATCCTCAAGACATTCCAAAG
ATTTTCACTAAATTTGCTCAAACACAATCTTTAGCGACGAGAAGCTCGGGTGGTAGTGGGCTTGGCCTCGCCATC
TCCAAGAGGTTTGTGAATCTGATGGAGGGTAACATTTGGATTGAGAGCGATGGTCTTGGAAAAGGATGCACGGCT
ATCTTTGATGTTAAACTTGGGATCTCAGAACGTTCAAACGAATCTAAACAGTCGGGCATACCGAAAGTTCCAGCC
ATTCCCCGACATTCAAATTTCACTGGACTTAAGGTTCTTGTCATGGATGAGAACGGGGTAAGTAGAATGGTGACG
AAGGGACTTCTTGTACACCTTGGGTGCGAAGTGACCACGGTGAGTTCAAACGAGGAGTGTCTCCGAGTTGTGTCC
CATGAGCACAAAGTGGTCTTCATGGACGTGTGCATGCCCGGGGTCGAAAACTACCAAATCGCTCTCCGTATTCAC
GAGAAATTCACAAAACAACGCCACCAACGGCCACTACTTGTGGCACTCAGTGGTAACACTGACAAATCCACAAAA
GAGAAATGCATGAGCTTTGGTCTAGACGGTGTGTTGCTCAAACCCGTATCACTAGACAACATAAGAGATGTTCTG
TCTGATCTTCTCGAGCCCCGGGTACTGTACGAGGGCATGTAA

MEVCNCIEPQWPADELLMKYQYISDFFIAIAYFSIPLELIYFVKKSAVFPYRWVLVQFGAFIVLCGATHLINLWT
FTTHSRTVALVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQEETGRHVRMLT
HEIRSTLDRHTILKTTLVELGRTLALEECALWMPTRTGLELQLSYTLRHQHPVEYTVPIQLPVINQVFGTSRAVK
ISPNSPVARLRPVSGKYMLGEVVAVRVPLLHLSNFQINDWPELSTKRYALMVLMLPSDSARQWHVHELELVEVVA
DQVAVALSHAAILEESMRARDLLMEQNVALDLARREAETAIRARNDFLAVMNHEMRTPMHAIIALSSLLQETELT
PEQRLMVETILKSSNLLATLMNDVLDLSRLEDGSLQLELGTFNLHTLFREVLNLIKPIAVVKKLPITLNLAPDLP
EFVVGDEKRLMQIILNIVGNAVKFSKQGSISVTALVTKSDTRAADFFVVPTGSHFYLRVKVKDSGAGINPQDIPK
IFTKFAQTQSLATRSSGGSGLGLAISKRFVNLMEGNIWIESDGLGKGCTAIFDVKLGISERSNESKQSGIPKVPA
IPRHSNFTGLKVLVMDENGVSRMVTKGLLVHLGCEVTTVSSNEECLRVVSHEHKVVFMDVCMPGVENYQIALRIH
EKFTKQRHQRPLLVALSGNTDKSTKEKCMSFGLDGVLLKPVSLDNIRDVLSDLLEPRVLYEGM

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        259   20   59.97   50.00  4.00  1.00 GTGCCAACTGGGAGTCATTT
RIGHT PRIMER       746   20   60.00   55.00  4.00  0.00 CACACGTCCATGAAGACCAC
SEQUENCE SIZE: 981
INCLUDED REGION SIZE: 981

PRODUCT SIZE: 488, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 GAACTTGGGACATTCAATCTTCATACATTATTTAGAGAGGTCCTCAATCTGATAAAGCCT

   61 ATAGCGGTTGTTAAGAAATTACCCATCACACTAAATCTTGCACCAGATTTGCCAGAATTT

  121 GTTGTTGGGGATGAGAAACGGCTAATGCAGATAATATTAAATATAGTTGGTAATGCTGTG

  181 AAATTCTCCAAACAAGGTAGTATCTCCGTAACCGCTCTTGTCACCAAGTCAGACACACGA

  241 GCTGCTGACTTTTTTGTCGTGCCAACTGGGAGTCATTTCTACTTGAGAGTGAAGGTAAAA
                      >>>>>>>>>>>>>>>>>>>>

  301 GACTCTGGAGCAGGAATAAATCCTCAAGACATTCCAAAGATTTTCACTAAATTTGCTCAA

  361 ACACAATCTTTAGCGACGAGAAGCTCGGGTGGTAGTGGGCTTGGCCTCGCCATCTCCAAG

  421 AGGTTTGTGAATCTGATGGAGGGTAACATTTGGATTGAGAGCGATGGTCTTGGAAAAGGA

  481 TGCACGGCTATCTTTGATGTTAAACTTGGGATCTCAGAACGTTCAAACGAATCTAAACAG

  541 TCGGGCATACCGAAAGTTCCAGCCATTCCCCGACATTCAAATTTCACTGGACTTAAGGTT

  601 CTTGTCATGGATGAGAACGGGGTAAGTAGAATGGTGACGAAGGGACTTCTTGTACACCTT

  661 GGGTGCGAAGTGACCACGGTGAGTTCAAACGAGGAGTGTCTCCGAGTTGTGTCCCATGAG

  721 CACAAAGTGGTCTTCATGGACGTGTGCATGCCCGGGGTCGAAAACTACCAAATCGCTCTC
            <<<<<<<<<<<<<<<<<<<

  781 CGTATTCACGAGAAATTCACAAAACAACGCCACCAACGGCCACTACTTGTGGCACTCAGT

  841 GGTAACACTGACAAATCCACAAAAGAGAAATGCATGAGCTTTGGTCTAGACGGTGTGTTG

  901 CTCAAACCCGTATCACTAGACAACATAAGAGATGTTCTGTCTGATCTTCTCGAGCCCCGG

  961 GTACTGTACGAGGGCATGTAA
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8.6 Gene sequences obtained from gene bank and used for the construction of gene-specific primer pairs in the second PCR set-up (with the unknown part)

fbp1:etr1-1s GTTTTGGCCGTAAACTTGGA
fbp1:etr1-1as GTTGAAAGCTCAGGCCAGTC

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	500	20	59.97	45.00	6.00	0.00	GTTTTGGCCGTAAACTTGGA
RIGHT PRIMER	2216	20	60.00	55.00	4.00	1.00	GTTGAAAGCTCAGGCCAGTC

SEQUENCE SIZE: 2313
INCLUDED REGION SIZE: 2313

PRODUCT SIZE: 1717, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 2.00

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    1 GAGAAGTAATAATAATGATACTTCTAATAATAATAATATAGAAATAATTATCGATTGTTT

   61 ACCAGCATAACTCTGTTTGTGAGCTTACTTACATTGTCAGCCCAAACTCGACTAAAGGAG

  121 GAGGATTGCAGTAGGCTGGCTTGCCAGCGTAAAATTTTACCAATCATGACGAATCCTCAT

  181 AATACAGATATTATTTATCACATGTATAGATATTTTTCTTCCATCTTATGATATTTTTAG

  241 CTGAATTTGCATTAATTCTAGGTATTTGTAGGTCTTCAAGCTAATTTATGTCCATGTGAT

  301 TTTAAGTCTACATAGTTCAATTTTAACATATTTTCACTAGTTTCATACCTAGGTCAATGT

  361 CAAATATGACTAAATCATCCCAACCGATATTCCCTTATTTGAGCCTCAACGTGTGCTACT

  421 TCGACCTTCTAGAAAATGTAATATTCCCTTCGTTCTACAAAAGATGGCACAATTCGGACT

  481 TCAAGAGTCACACGTTTTAGTTTTGGCCGTAAACTTGGACATAAAATCATTAAATTTCTT
                        >>>>>>>>>>>>>>>>>>>>

  541 AATATAAAGTTTATATATTCGGACACTATATAAAAAGTAACATATATAAGTCATAATAGT

  601 TAATAATTTAAAACATCTAACATGAAATTATTGTGGTGAAAGAAAAACTCGACTCTTCAA

  661 ATTCGACTTGTGTCATCTTTTGTAGAAATGATGGAGTAATTCTTTAGTACCACAATTATC

  721 CACCTTTATGTTGAAGTAGAATGAGATCTTTAACACCAGCCGGTTAAAAAAATCACTAAT

  781 TTAAGCTTTTAGATTACACAATTCAACTAGAGAGAGATGAGAAGATGGAGAAAAAACCAA

  841 AGGATAAGAAGGTGAAAGAAAAGGGTTAAGAGGAGGGGCTTTAATGCAGATGGAGTTGGC

  901 TTTGTCAAATACCTTTAAAACAAACCAACTTTTTCTGAAACTTAAGAAAGTTGATTGTCT
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 961 GTCAATCCTTAAATAGATATTCCCATTAATTCATTTCTTCTCCTTTTGCAACTCTATCAT

1021 TATATACTTGTTCTTCTCAAGCAAAAGAATAGTCCAACAAGAGAAAGGGAAAAT??????

1081 ??????????????????????????TTGATTAACCTAATTGGTCTTTATCTAGTGTAAT

1141 GCATCGTTATTAGGAACTTTAAATTAAGATTTAATCGTTAATTTCATGATTCGGATTCGA

1201 ATTTTACTGTTCTCGAGACTGAAATATGCAACCTATTTTTTCGTAATCGTTGTGATCGAA

1261 TTCGATTCTTCAGAATTTATAGCAATTTTGATGCTCATGATCTGTCTACGCTACGTTCTC

1321 GTCGTAAATCGAAGTTGATAATGCTATGTGTTTGTTACACAGGTGTGTGTATGTGTGAGA

1381 GAGGAACTATAGTGTAAAAAATTCATAATGGAAGTCTGCAATTGTATTGAACCGCAATGG

1441 CCAGCGGATGAATTGTTAATGAAATACCAATACATCTCCGATTTCTTCATTGCGATTGCG

1501 TATTTTTCGATTCCTCTTGAGTTGATTTACTTTGTGAAGAAATCAGCCGTGTTTCCGTAT

1561 AGATGGGTACTTGTTCAGTTTGGTGCTTTTATCGTTCTTTGTGGAGCAACTCATCTTATT

1621 AACTTATGGACTTTCACTACGCATTCGAGAACCGTGGCGCTTGTGATGACTACCGCGAAG

1681 GTGTTAACCGCTGTTGTCTCGTGTGCTACTGCGTTGATGCTTGTTCATATTATTCCTGAT

1741 CTTTTGAGTGTTAAGACTCGGGAGCTTTTCTTGAAAAATAAAGCTGCTGAGCTCGATAGA

1801 GAAATGGGATTGATTCGAACTCAGGAAGAAACCGGAAGGCATGTGAGAATGTTGACTCAT

1861 GAGATTAGAAGCACTTTAGATAGACATACTATTTTAAAGACTACACTTGTTGAGCTTGGT

1921 AGGACATTAGCTTTGGAGGAGTGTGCATTGTGGATGCCTACTAGAACTGGGTTAGAGCTA

1981 CAGCTTTCTTATACACTTCGTCATCAACATCCCGTGGAGTATACGGTTCCTATTCAATTA

2041 CCGGTGATTAACCAAGTGTTTGGTACTAGTAGGGCTGTAAAAATATCTCCTAATTCTCCT

2101 GTGGCTAGGTTGAGACCTGTTTCTGGGAAATATATGCTAGGGGAGGTGGTCGCTGTGAGG

2161 GTTCCGCTTCTCCACCTTTCTAATTTTCAGATTAATGACTGGCCTGAGCTTTCAACAAAAG
                                        <<<<<<<<<<<<<<<<<<<<

2221 AGATATGCTTTGATGGTTTTGATGCTTCCTTCAGATAGTGCAAGGCAATGGCATGTCCAT

2281 GAGTTGGAACTCGTTGAAGTCGTCGCTGATCAG
```

fbp1 ACCESSION NO L10115..

GAGAAGTAATAATAATGATACTTCTAATAATAATAATATAGAAATAATTATCGATTGTTTACCAGCATAACTCTG
TTTGTGAGCTTACTTACATTGTCAGCCCAAACTCGACTAAAGGAGGAGGATTGCAGTAGGCTGGCTTGCCAGCGT
AAAATTTTACCAATCATGACGAATCCTCATAATACAGATATTATTTATCACATGTATAGATATTTTTCTTCCATC
TTATGATATTTTTAGCTGAATTTGCATTAATTCTAGGTATTTGTAGGTCTTCAAGCTAATTTATGTCCATGTGAT
TTTAAGTCTACATAGTTCAATTTTAACATATTTTCACTAGTTTCATACCTAGGTCAATGTCAAATATGACTAAAT
CATCCCAACCGATATTCCCTTATTTGAGCCTCAACGTGTGCTACTTCGACCTTCTAGAAAATGTAATATTCCCTT
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TAATAATTTAAAACATCTAACATGAAATTATTGTGGTGAAAGAAAAACTCGACTCTTCAAATTCGACTTGTGTCA
TCTTTTGTAGAAATGATGGAGTAATTCTTTAGTACCACAATTATCCACCTTTATGTTGAAGTAGAATGAGATCTT
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TGGAGAAAAAACCAAAGGATAAGAAGGTGAAAGAAAAGGGTTAAGAGGAGGGGCTTTAATGCAGATGGAGTTGGC
TTTGTCAAATACCTTTAAAACAAACCAACTTTTTCTGAAACTTAAGAAAGTTGATTGTCTGTCAATCCTTAAATA
GATATTCCCATTAATTCATTTCTTCTCCTTTTGCAACTCTATCATTATATACTTGTTCTTCTCAAGCAAAAGAAT
AGTCCAACAAGAGAAAGGGAAAAT??????????????????????????????????TTGATTAACCTAATTGGTC
TTTATCTAGTGTAATGCATCGTTATTAGGAACTTTAAATTAAGATTTAATCGTTAATTTCATGATTCGGATTCGA
ATTTTACTGTTCTCGAGACTGAAATATGCAACCTATTTTTTCGTAATCGTTGTGATCGAATTCGATTCTTCAGAA
TTTATAGCAATTTTGATGCTCATGATCTGTCTACGCTACGTTCTCGTCGTAAATCGAAGTTGATAATGCTATGTG
TTTGTTACACAGGTGTGTGTATGTGTGAGAGAGGAACTATAGTGTAAAAAATTCATA**ATGGAAGTCTGCAATTGT**
ATTGAACCGCAATGGCCAGCGGATGAATTGTTAATGAAATACCAATACATCTCCGATTTCTTCATTGCGATTGCG
TATTTTTCGATTCCTCTTGAGTTGATTTACTTTGTGAAGAAATCAGCCGTGTTTCCGTATAGATGGGTACTTGTT
CAGTTTGGTGCTTTTATCGTTCTTTGTGGAGCAACTCATCTTATTAACTTATGGACTTTCACTACGCATTCGAGA
ACCGTGGCGCTTGTGATGACTACCGCGAAGGTGTTAACCGCTGTTGTCTCGTGTGCTACTGCGTTGATGCTTGTT
CATATTATTCCTGATCTTTTGAGTGTTAAGACTCGGGAGCTTTTCTTGAAAAATAAAGCTGCTGAGCTCGATAGA
GAAATGGGATTGATTCGAACTCAGGAAGAAACCGGAAGGCATGTGAGAATGTTGACTCATGAGATTAGAAGCACT
TTAGATAGACATACTATTTTAAAGACTACACTTGTTGAGCTTGGTAGGACATTAGCTTTGGAGGAGTGTGCATTG
TGGATGCCTACTAGAACTGGGTTAGAGCTACAGCTTTCTTATACACTTCGTCATCAACATCCCGTGGAGTATACG
GTTCCTATTCAATTACCGGTGATTAACCAAGTGTTTGGTACTAGTAGGGCTGTAAAAATATCTCCTAATTCTCCT
GTGGCTAGGTTGAGACCTGTTTCTGGGAAATATATGCTAGGGGAGGTGGTCGCTGTGAGGGTTCCGCTTCTCCAC
CTTTCTAATTTTCAGATTAATGACTGGCCTGAGCTTTCAACAAAGAGATATGCTTTGATGGTTTTGATGCTTCCT
TCAGATAGTGCAAGGCAATGGCATGTCCATGAGTTGGAACTCGTTGAAGTCGTCGCTGATCAG

8.7 Gene sequences obtained from gene bank and used for the construction of gene-specific primer pairs in the second PCR set-up (the unknown part now known)

L10115 *Petunia hybrida* fbp1 gene (the blue part)
L24119. *Arabidopsis thaliana* ETR1 gene (the rest of the sequence; CDS yellow) (The codon TGT is changed in etr1-1)

GAGAAGTAATAATAATGATACTTCTAATAATAATAATATAGAAATAATTATCGATTGTTTACCAGCATAACTCTG
TTTGTGAGCTTACTTACATTGTCAGCCCAAACTCGACTAAAGGAGGAGGATTGCAGTAGGCTGGCTTGCCAGCGT
AAAATTTTACCAATCATGACGAATCCTCATAATACAGATATTATTTATCACATGTATAGATATTTTTCTTCCATC
TTATGATATTTTTAGCTGAATTTGCATTAATTCTAGGTATTTGTAGGTCTTCAAGCTAATTTATGTCCATGTGAT
TTTAAGTCTACATAGTTCAATTTTAACATATTTTCACTAGTTTCATACCTAGGTCAATGTCAAATATGACTAAAT
CATCCCAACCGATATTCCCTTATTTGAGCCTCAACGTGTGCTACTTCGACCTTCTAGAAAATGTAATATTCCCTT
CGTTCTACAAAAGATGGCACAATTCGGACTTCAAGAGTCACACGTTTTAGTTTTGGCCGTAAACTTGGACATAAA
ATCATTAAATTTCTTAATATAAAGTTTATATATTCGGACACTATATAAAAAGTAACATATATAAGTCATAATAGT
TAATAATTTAAAACATCTAACATGAAATTATTGTGGTGAAAGAAAAACTCGACTCTTCAAATTCGACTTGTGTCA
TCTTTTGTAGAAATGATGGAGTAATTCTTTAGTACCACAATTATCCACCTTTATGTTGAAGTAGAATGAGATCTT
TAACACCAGCCGGTTAAAAAAATCACTAATTTAAGCTTTTAGATTACACAATTCAACTAGAGAGAGATGAGAAGA
TGGAGAAAAAACCAAAGGATAAGAAGGTGAAAGAAAAGGGTTAAGAGGAGGGGCTTTAATGCAGATGGAGTTGGC
TTTGTCAAATACCTTTAAAACAAACCAACTTTTTCTGAAACTTAAGAAAGTTGATTGTCTGTCAATCCTTAAATA
GATATTCCCATTAATTCATTTCTTCTCCTTTTGCAACTCTATCATTATATACTTGTTCTTCTCAAGCAAAAGAAT
AGTCCAACAAGAGAAAGGGAAAGGATCCTCTTCTCCGATCAATTCTTCCCAAGTAAGCTTCTTCTTCCTCGATTC
TCTCCTCAGATTGTTTCGTGACTTCTTTATATATATTCTTCACTTCCACAGTTTTCTTCTGTTGTTGTCGTCGAT
CTCAAATCATAGAGATTGATTAACCTAATTGGTCTTTATCTAGTGTAATGCATCGTTATTAGGAACTTTAAATTA
AGATTTAATCGTTAATTTCATGATTCGGATTCGAATTTTACTGTTCTCGAGACTGAAATATGCAACCTATTTTTT
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TCTCGTCGTAAATCGAAGTTGATAATGCTATGTGTTTGTTACACAGGTGTGTGTATGTGTGAGAGAGGAACTATA
GTGTAAAAAATTCATAATGGAAGTCTGCAATTGTATTGAACCGCAATGGCCAGCGGATGAATTGTTAATGAAATA
CCAATACATCTCCGATTTCTTCATTGCGATTGCGTATTTTTCGATTCCTCTTGAGTTGATTTACTTTGTGAAGAA
ATCAGCCGTGTTTCCGTATAGATGGGTACTTGTTCAGTTTGGTGCTTTTATCGTTCTT**TGT**GGAGCAACTCATCT
TATTAACTTATGGACTTTCACTACGCATTCGAGAACCGTGGCGCTTGTGATGACTACCGCGAAGGTGTTAACCGC
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GACTTTTTTGTCGTGCCAACTGGGAGTCATTTCTACTTGAGAGTGAAGGTTATTATCTTGTATCTTGGGATCTTA
TACCATAGCTGAAAGTATTTCTTAGGTCTTAATTTTGATGATTATTCAAATATAGGTAAAAGACTCTGGAGCAGG
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AAACGAATCTAAACAGTCGGGCATACCGAAAGTTCCAGCCATTCCCCGACATTCAAATTTCACTGGACTTAAGGT
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CAAATGCAGATATTGGCGTTTAGAAAAAACGCAAATTTAATCTTATGAGAAACCGATGATTATTTTGGTTGCAGG
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ATCGCTCTCCGTATTCACGAGAAATTCACAAAACAACGCCACCAACGGCCACTACTTGTGGCACTCAGTGGTAAC
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AACATAAGAGATGTTCTGTCTGATCTTCTCGAGCCCCGGGTACTGTACGAGGGCATGTAAAGGCGATGGATGCCC
CATGCCCCAGAGGAGTAATTCCGCTCCCGCCTTCTTCTCCCGTAAAACATCGGAAGCTGATGTTCTCTGGTTTAA
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TCTAAACCGGTATGTGTCCGTGGCGATTTCGGTTATAGAGGAACAAGATGGTGGTGGTATAATCATACCATTTCA
GATTACATGTTTGACTAATGTTGTATCCTTATATATGTAGTTACATTCTTATAAGAATTTGGATCGAGTTATGGA
TGCTTGTTGCGTGCATGTATGACATTGATGCAGTATTATGGCGTCAGCTTTGCGCCGCTTAGTAGAACAACAACA
ATGGCGTTACTTAGTTTCTCAATCAACCCGATCTCCAAAA

ETR1 CDS in mRNA

ATGGAAGTCTGCAATTGTATTGAACCGCAATGGCCAGCGGATGAATTGTTAATGAAATACCAATACATCTCCGAT
TTCTTCATTGCGATTGCGTATTTTTCGATTCCTCTTGAGTTGATTTACTTTGTGAAGAAATCAGCCGTGTTTCCG
TATAGATGGGTACTTGTTCAGTTTGGTGCTTTTATCGTTCTTTGTGGAGCAACTCATCTTATTAACTTATGGACT
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ATGAACCATGAAATGCGAACACCGATGCATGCGATTATTGCACTCTCTTCCTTACTCCAAGAAACGGAACTAACC
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ATTTTCACTAAATTTGCTCAAACACAATCTTTAGCGACGAGAAGCTCGGGTGGTAGTGGGCTTGGCCTCGCCATC
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ATCTTTGATGTTAAACTTGGGATCTCAGAACGTTCAAACGAATCTAAACAGTCGGGCATACCGAAAGTTCCAGCC
ATTCCCCGACATTCAAATTTCACTGGACTTAAGGTTCTTGTCATGGATGAGAACGGGGTAAGTAGAATGGTGACG
AAGGGACTTCTTGTACACCTTGGGTGCGAAGTGACCACGGTGAGTTCAAACGAGGAGTGTCTCCGAGTTGTGTCC
CATGAGCACAAAGTGGTCTTCATGGACGTGTGCATGCCCGGGGTCGAAAACTACCAAATCGCTCTCCGTATTCAC
GAGAAATTCACAAAACAACGCCACCAACGGCCACTACTTGTGGCACTCAGTGGTAACACTGACAAATCCACAAAA
GAGAAATGCATGAGCTTTGGTCTAGACGGTGTGTTGCTCAAACCCGTATCACTAGACAACATAAGAGATGTTCTG
TCTGATCTTCTCGAGCCCCGGGTACTGTACGAGGGCATGTAA

ETR1 protein

MEVCNCIEPQWPADELLMKYQYISDFFIAIAYFSIPLELIYFVKKSAVFPYRWVLVQFGAFIVLCGATHLINLWT
FTTHSRTVALVMTTAKVLTAVVSCATALMLVHIIPDLLSVKTRELFLKNKAAELDREMGLIRTQEETGRHVRMLT
HEIRSTLDRHTILKTTLVELGRTLALEECALWMPTRTGLELQLSYTLRHQHPVEYTVPIQLPVINQVFGTSRAVK
ISPNSPVARLRPVSGKYMLGEVVAVRVPLLHLSNFQINDWPELSTKRYALMVLMLPSDSARQWHVHELELVEVVA
DQVAVALSHAAILEESMRARDLLMEQNVALDLARREAETAIRARNDFLAVMNHEMRTPMHAIIALSSLLQETELT
PEQRLMVETILKSSNLLATLMNDVLDLSRLEDGSLQLELGTFNLHTLFREVLNLIKPIAVVKKLPITLNLAPDLP
EFVVGDEKRLMQIILNIVGNAVKFSKQGSISVTALVTKSDTRAADFFVVPTGSHFYLRVKVKDSGAGINPQDIPK
IFTKFAQTQSLATRSSGGSGLGLAISKRFVNLMEGNIWIESDGLGKGCTAIFDVKLGISERSNESKQSGIPKVPA
IPRHSNFTGLKVLVMDENGVSRMVTKGLLVHLGCEVTTVSSNEECLRVVSHEHKVVFMDVCMPGVENYQIALRIH
EKFTKQRHQRPLLVALSGNTDKSTKEKCMSFGLDGVLLKPVSLDNIRDVLSDLLEPRVLYEGM

8.8 Calculations from the computer programme Minitab for the pollen staining tests

One-Sample T: Aglo1

```
Test of mu = 79,9 vs not = 79,9


Variable  N     Mean   StDev  SE Mean        95% CI            T      P
Aglo1     7  79,8571  7,3808   2,7897  (73,0310; 86,6832)  -0,02  0,988
```

One-Sample T: SF 15-1

```
Test of mu = 59,7 vs not = 59,7


Variable  N     Mean    StDev  SE Mean        95% CI           T      P
SF 15-1   7  59,7143  20,9819   7,9304  (40,3093; 79,1193)  0,00  0,999
```

One-Sample T: Aglo 3

```
Test of mu = 73,5 vs not = 73,5


Variable  N     Mean    StDev  SE Mean        95% CI           T      P
Aglo 3    6  73,5000  15,1360   6,1793  (57,6157; 89,3843)  0,00  1,000
```

One-Sample T: Aglo 8.3

```
Test of mu = 70,2 vs not = 70,2


Variable  N     Mean   StDev  SE Mean        95% CI            T      P
Aglo 8.3  6  70,1667  6,3061   2,5744  (63,5488; 76,7845)  -0,01  0,990
```

One-Sample T: SF 13-1

```
Test of mu = 38,9 vs not = 38,9


Variable  N     Mean    StDev  SE Mean        95% CI            T      P
SF 13-1   7  38,8571  23,8497   9,0143  (16,7998; 60,9145)  -0,00  0,996
```

One-Sample T: BU Control

```
Test of mu = 60 vs not = 60


Variable    N     Mean   StDev  SE Mean        95% CI           T      P
BU Control  8  60,0000  9,7834   3,4589  (51,8209; 68,1791)  0,00  1,000
```

One-Sample T: SF 28-1

```
Test of mu = 75,3 vs not = 75,3


Variable  N     Mean   StDev  SE Mean        95% CI           T      P
SF 28-1   3  75,3333  2,0817   1,2019  (70,1622; 80,5045)  0,03  0,980
```

One-Sample T: Aglo 4

```
Test of mu = 70,3 vs not = 70,3


Variable  N     Mean    StDev  SE Mean        95% CI            T      P
Aglo 4    4  70,2500  18,5899   9,2949  (40,6694; 99,8306)  -0,01  0,996
```

One-way ANOVA: Aglo1; SF 15-1; Aglo 3; Aglo 8.3; SF 13-1; BU Control; ...

```
Source  DF     SS    MS     F      P
Factor   7   7739  1106  4,68  0,001
Error   40   9441   236
Total   47  17180

S = 15,36   R-Sq = 45,05%   R-Sq(adj) = 35,43%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level       N   Mean  StDev  ------+---------+---------+---------+---
Aglo1       7  79,86   7,38                     (-----*-----)
SF 15-1     7  59,71  20,98           (-----*-----)
Aglo 3      6  73,50  15,14                 (------*-----)
Aglo 8.3    6  70,17   6,31               (-----*-----)
SF 13-1     7  38,86  23,85  (----*-----)
BU Control  8  60,00   9,78            (----*----)
SF 28-1     3  75,33   2,08                (--------*--------)
Aglo 4      4  70,25  18,59              (-------*-------)
                           ------+---------+---------+---------+---
                                40        60        80       100

Pooled StDev = 15,36
```

8.9 Calculations from the computer programme Minitab for the pollen tube growth tests

One-Sample T: Aglo 1

```
Test of mu = 36,7 vs not = 36,7


Variable  N     Mean    StDev  SE Mean        95% CI            T      P
Aglo 1    5  36,5600  30,6882  13,7242  (-1,5445; 74,6645)  -0,01  0,992
```

One-Sample T: SF 15-1

```
Test of mu = 16,6 vs not = 16,6


Variable  N     Mean    StDev  SE Mean        95% CI          T      P
SF 15-1   5  16,6200  10,0485   4,4938  (4,1432; 29,0968)  0,00  0,997
```

One-Sample T: Aglo 3

```
Test of mu = 32,8 vs not = 32,8


Variable  N     Mean    StDev  SE Mean        95% CI            T      P
Aglo 3    5  32,7600  12,1362   5,4275  (17,6909; 47,8291)  -0,01  0,994
```

One-Sample T: Aglo 8.3

```
Test of mu = 24,5 vs not = 24,5


Variable  N     Mean    StDev  SE Mean        95% CI           T      P
Aglo 8.3  5  24,5400  25,6487  11,4705  (-7,3071; 56,3871)  0,00  0,997
```

One-Sample T: SF 13-1

```
Test of mu = 2 vs not = 2


Variable  N     Mean    StDev  SE Mean         95% CI           T      P
SF 13-1   4  2,02500  1,98389  0,99195  (-1,13182; 5,18182)  0,03  0,981
```

One-Sample T: BU Control

```
Test of mu = 38,7 vs not = 38,7


Variable    N     Mean    StDev  SE Mean        95% CI          T      P
BU Control  5  38,7200  27,7865  12,4265  (4,2184; 73,2216)  0,00  0,999
```

One-Sample T: SF 28-1

```
Test of mu = 41 vs not = 41


Variable  N     Mean    StDev  SE Mean          95% CI            T      P
SF 28-1   2  41,0000  39,5980  28,0000  (-314,7737; 396,7737)  0,00  1,000
```

One-Sample T: Aglo 4

```
Test of mu = 30,3 vs not = 30,3


Variable  N     Mean    StDev  SE Mean        95% CI          T      P
Aglo 4    3  30,3333  10,5040   6,0645  (4,2400; 56,4266)  0,01  0,996
```

One-way ANOVA: Aglo 1; SF 15-1; Aglo 3; Aglo 8.3; SF 13-1; BU Control; ...

```
Source  DF     SS   MS     F      P
Factor   7   4797  685  1,45  0,228
Error   26  12280  472
Total   33  17077

S = 21,73   R-Sq = 28,09%   R-Sq(adj) = 8,73%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level       N   Mean  StDev  --------+---------+---------+---------+-
Aglo 1      5  36,56  30,69               (-------*-------)
SF 15-1     5  16,62  10,05       (-------*-------)
Aglo 3      5  32,76  12,14             (-------*-------)
Aglo 8.3    5  24,54  25,65          (-------*-------)
SF 13-1     4   2,03   1,98  (--------*--------)
BU Control  5  38,72  27,79               (-------*-------)
SF 28-1     2  41,00  39,60            (-----------*-----------)
Aglo 4      3  30,33  10,50          (---------*---------)
                           --------+---------+---------+---------+-
                                   0        25        50        75

Pooled StDev = 21,73
```

8.10 Figures and tables from the ELISA-results

Table 3.8

Absorbance values for the standard solutions of BSA

Standard solution BSA (µL)	Absorbance mean (595 nm)
1.00	0.453
2.00	0.533
3.00	0.568
4.00	0.658
5.00	0.810
6.00	0.856
7.00	0.895
8.00	0.957
9.00	1.009
10.00	1.056
11.00	1.145

Table 3.9

Total amount of protein in the transgenic lines and in control lines according to the method of Bradford.

Transgenic line	Absorbance (595 nm)	Total protein, (2mg mL^{-1})	Total protein (mg mL^{-1})
SF 15-1	0.920	28.387	14.193
SF 15-1c	0.873	28.021	14.010
SF 15-1de	0.975	28.939	14.469
SF 15-4	0.999	29.176	14.588
SF 11-2	1.128	30.416	15.208
Aglo 2	0.811	27.258	13.629
Aglo 3	0.920	28.387	14.193
GH 4	1.108	30.227	15.114
Trans 1	0.870	27.875	13.938
SF 13-1	1.069	29.856	14.928
SF 13-1a	0.966	28.849	14.425
White Uniform control	0.921	28.397	14.199
Blue Uniform control	0.805	27.194	13.597

Table 3.10

The absorbance values for the standard solutions of *nptII*

Standard solution of *nptII* (pg/ml)	Absorbance (450 nm)
3000	2.800
1500	1.509
750	0.693
375	0.324
188	0.166
94	0.081
47	0.046

Table 3.11

Immunodetection of transgenic expression by ELISA. The values in the last column are pentagram *nptII* per milligram total protein.

Transgenic lines	OD (450 nm)	NPTII (pg mL^{-1})	Total protein (mg mL^{-1})	(pg *nptII*) (mg^{-1} total protein)
SF 15-1	0.574	4.045	14.193	0.320
SF 15-1c	0.464	4.336	14.010	0.309
SF 15-1de	0.550	4.501	14.469	0.311
SF 15-4	0.455	4.318	14.588	0.296
SF 11-2	0.067	3.248	15.208	0.214
Aglo 2	0.265	3.887	13.629	0.285
Aglo 3	0.275	3.913	14.193	0.276
GH 4	0.408	4.220	15.114	0.279
Trans 1	0.214	3.750	13.938	0.269
SF 13-1	0.476	4.359	14.928	0.292
SF 13-1a	0.448	4.304	14.425	0.298
White Uniform control	0.053	3.185	14.199	0.224
Blue Uniform control	0.055	3.194	13.597	0.235

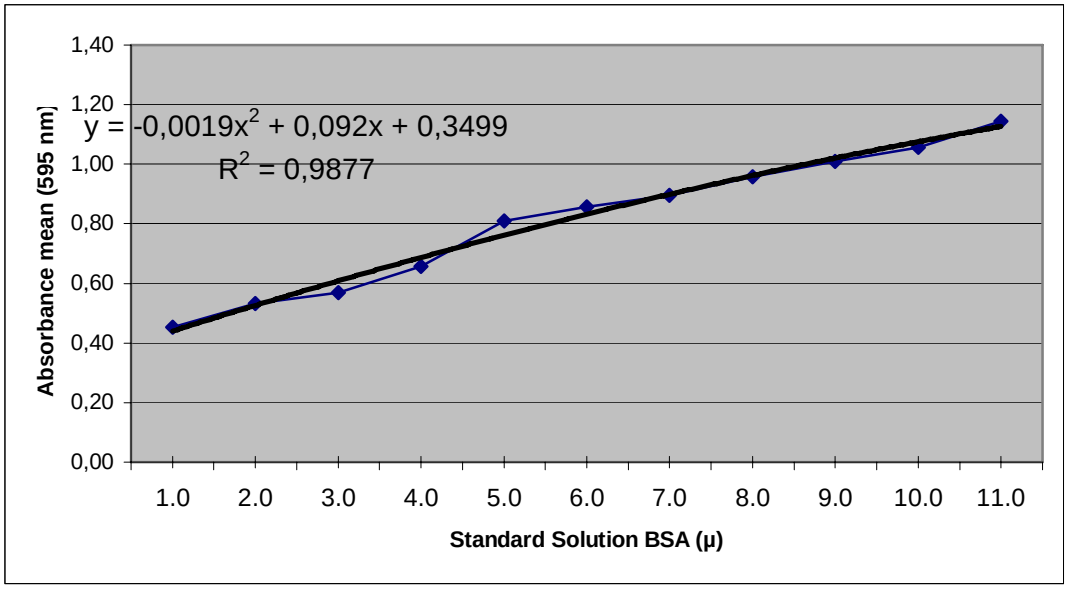

Figure 3.10

Standard curve used to calculate the total amount of protein in the transgenic lines and control line, and the equation used for calculation of the total amount of protein.

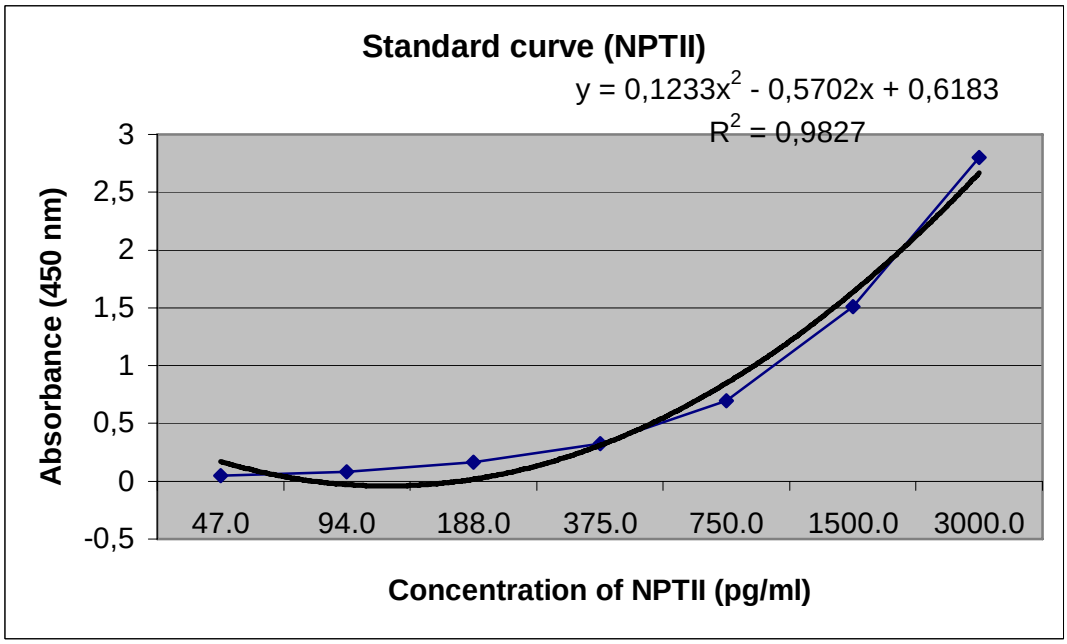

Figure 3.11

Standard curve used to calculate the total amount of *nptII* in the transgenic lines and control line.