# Using *Arabidopsis* TILLING alleles of COMATOSE to understand germination potential



by

**Langa Tembo**
**(B.Sc.)**

Thesis submitted to the University of Nottingham in partial fulfilment
of the requirements for the degree of
Master of Science in Crop Science and Management

Division of Agricultural and Environmental Sciences
School of Biosciences
University of Nottingham
Sutton Bonington Campus
LE12 5RD
UK



**September 2005**

To my Parents
For Unconditional Love

**ACKNOWLEDGEMENTS**

I am very grateful to my supervisor Prof. Michael Holdsworth for his guidance and help on the project. Special thanks to Dr Heike Schmuths for her assistance, patience and understanding during my learning of essential techniques used. Many thanks to Tara Holman for her assistance in this project especially on the hypocotyl and root elongation experiments.

I am highly indebted to Joint Chevening-Development Solutions, University of Nottingham for granting me the scholarship which enabled me to pursue the MSc programme. Thanks also go to the staff of the division of Agricultural and Environmental sciences.

I would further wish to extend my thanks to friends, Manda Konjashili and Fatau Adams for the good relationship we've had and for making my stay at Sutton Bonington a memorable experience. Above all, I thank God for bringing me so far.

## CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## ABBREVIATIONS

| | |
|---|---|
| 2,4 D | Dichlorophenoxyacetic acid |
| 2,4 DB | Dichlorophenoxybutyric acid |
| A | Adenine |
| ABI | ABSCISIC ACID INSENSITIVE |
| C | Cytosine |
| CAPS | Cleaved Amplified Polymorphic Sequences |
| Col | Columbia |
| Conc. | Concentration |
| CTS | COMATOSE |
| D | Aspartic acid |
| DNA | Deoxyribonucleic acid |
| E | Glutamic acid |
| F | Phenylalanine |
| FUS-3 | FUSCA-3 |
| FSL | Fisher Scientific Limited |
| G | Guanine |
| Hd | Heading |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| K | Lysine |
| L | Leucine |
| LEC-1 | LEAF COTYLEDON 1 |
| Ler | Landsberg *erecta* |
| MS | Murashige and Skoog |
| N | Asparagine |
| No. | Number |
| NAA | Naphtalene-1 acetic acid |
| PCR | Polymerase Chain Reaction |
| PN | Plant nutrient medium without sucrose |
| PNS | Plant nutrient medium with sucrose |
| S | Serine |
| T | Thymine |

| | |
|---|---|
| TILLING | Targeting Induced Local Lesions IN Genomes |
| VRN | Vernalisation |
| Ws | Wassilewskija |

**ABSTRACT**

The *CTS* locus in *Arabidopsis thaliana* was identified to promote the transition from embryo development to germination. Mutation of this locus results in reduction of germination potential. This is caused by the disruption of the β-oxidation pathway which is essential in breaking down fatty acids to provide energy for embryo expansion and radicle protrusion for the germination process. Mutant of CTS show much ability to elongate roots on medium with 0.5xMS plus 0.5% sucrose plus varying concentrations of 2,4-DB as compared to their relative wild types.

The purpose of this study was to try to define an allelic series of *cts* to help structure function studies and to find out more about the specific role of CTS. This was done using TILLING alleles of CTS. These have point mutations caused by ethy methanesulphate on the exons of the *CTS* locus. The TILLING mutants were determined with the aid of PCR analysis. To achieve the objectives phenotypic analysis experiments were conducted. Root elongation phenotypic analysis results after 8 days on medium with 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB after 8 days showed that the TILLING mutants had a higher mean root elongation length than the wild type, Columbia big mama. The TILLING mutants C22 and C60 exhibited significant root elongation (students, *t*- test, $P< 0.05$). The other known mutant of CTS, *cts-1* had a much higher significant mean root elongation length when compared to its wild type Ler (students, *t*- test, $P< 0.01$).

The results obtained indicated that the TILLING mutants have a slightly defective β-oxidation pathway when compared to the other known mutants of CTS.

# CHAPTER 1 INTRODUCTION

## 1.1 WHAT IS ARABIDOPSIS THALIANA?

*Arabidopsis thaliana* is a small dicotyledonous plant belonging to the brassicaceae (mustard) family and has become the model system of choice for research in plant biology (Meinke *et al.*, 1998). Although it has achieved this status of being a model system of choice for research and is related to crops such as cabbage cauliflower and oil seed rape, it is regarded as a weed of no economic value.

*Arabidopsis* is assumed to have originated in the central Asia highlands of the Western Himalayas (Redei, 1992) and it has now spread through out Asia, Europe and North Africa (Price *et al.*, 1994). Many different ecotypes of *Arabidopsis* have been collected from natural populations and are available for experimental analysis at stock centres (Meinke *et al.*, 1998). Among the known wild type flowering ecotypes collected include Colombia -O and Wassilewskija.

### 1.1.1 Advantages of using *Arabidopsis thaliana*

*Arabidopsis thaliana* offers many advantages to the researcher. It has a rapid life cycle (approximately 8 weeks from seed to seed), small stature and prolific seed production. This makes it ideally suited for genetic and mutational analysis (Slater *et al.*, 2003). In addition its genome size, chromosome number and the technical importance of available stocks and bioinformatics make it a more preferable model species.

#### *1.1.1.1. Genome size and chromosome number*

*Arabidopsis* has a small genome of about 125Mb (Rensink and Buell, 2004) and this makes it easier for genetic and mutational analysis as it is characterised by a small

level of repetitive sequences. Compared to the genome of maize and wheat which are 2500Mb and 16000Mb (Dennis and Surridge, 2000) respectively, its genome is more than 20 times smaller.

Its genome contains an estimated 25000 genes organised into five chromosomes (Clarke, *et al.*, 2003). The low chromosome number (five) allows a more efficient linkage analysis and the high degree of polymorphism at the DNA level between ecotypes (accessions) allows the use of molecular markers in almost any populations derived from ecotype crosses (Koornneef, 1994). It is a diploid plant (Perazza., et al., 1999) and this facilitates the identification of recessive traits and allows research to be done without complications of varying gene dosages encountered in other plant species.

In December 2000, *Arabidopsis* became the first plant to be sequenced (The *Arabidopsis* Genome Initiative, 2000) and this has broadened and enhanced research (Dennis and Surridge, 2000). The sequenced regions of the *Arabidopsis thaliana* genome cover 115.4 Mb of the 125 Mb genome and extend into centromeric regions (The *Arabidopsis* Genome Initiative, 2000).

### *1.1.1.2 Technical importance of Arabidopsis thaliana*

The availability of *Arabidopsis* seed stocks have made the technicalities involved in securing research materials easier and increased the power of *Arabidopsis* as model species. Before 1990, extensive collections of *Arabidopsis* mutants were maintained by individual scientists (Drs. A. R. Kranz, G. P. Redei and M. Koornneef) (Scholl *et al.*, 2000). Three major facilities (stock centres) were then established to greatly expedite the dissemination of *Arabidopsis* seeds to the international research community (Scholl *et al.*, 2000). These are the National Science Foundation-supported *Arabidopsis* Biological Resource Centre (ABRC) in the United States, the Biotechnology and Biological Sciences Research Council-supported Nottingham Arabidopsis Stock Centre (NASC) in the UK, and Sendai *Arabidopsis* Seed Stock Centre (SASSC) in Japan. The function of seed stock centres is to collect, propagate,

preserve and distribute seed stock centres which are of use to researchers (Scholl *et al.*, 2000)

The computer information technology (bioinformatics) used to record stored seeds, and retrieval of genomic data has made it easier for scientist to locate stocks, obtain detailed stock information and communicate with centre staff. The computational tool known as Basic Local Alignment Search Tool (BLAST) can now be used to search for *Arabidopsis* gene sequences (Altschul *et al.*, 1990).

## 1.2 TRANSFER OF INFORMATION TO CROP PLANTS

The essence of generating molecular genetic information from model species such as *Arabidopsis* is to transfer it to crop plants. This can help to solve important agricultural problems. Information transfer can be done using synteny and genetic modification.

### 1.2.1. Using synteny

Synteny is a Greek word; syn meaning together with and taenia which refer to loci contained within the same chromosome (Eckardt, 2001). In comparative genomics, it is often used as a synonynm for colinearity and refers to loci contained within the same chromosome. After a thorough understanding of a particular gene in the model species, a homologue gene can be found in the crop using synteny and then cloned.

The wheat vernalization gene VRN 1 was cloned using synteny between rice and sorghum (Yan *et al.*, 2003). The VRN 1 gene has been mapped in collinear regions of the long arm of chromosomes 5A, 5B and 5D and this region of wheat chromosome 5 is colinear with a region from rice chromosome 3, that includes Hd-6 (Kato *et al.*, 2002)

Some degree of synteny has been shown in comparing *Arabidopsis* with more distantly related species. Significant synteny was reported between soybean (Leguminosae) and *Arabidopsis* along the entire length of *Arabidopsis* chromosome

1 and soybean linkage group A2 (Eckardt, 2001), and blocks of synteny among other chromosomes were as well found (Grant, 2000).

The construction of genetic and physical maps is possible by using synteny. A high resolution map of the ovate-containing region in tomato chromosome 2 was constructed using synteny between *Arabidopsis* and tomato. This was achieved by screening of sequences from the Bacteria Artificial Chromosomes (BAC) clone between the segment of tomato chromosome 2 and the chromosome 4 region of *Arabidopsis* containing the BAC clone ATAP 22 (Ku *et al.*, 2001)

### 1.2.2 Genetic modification

Transfer through genetic modification involves the use of *Agrobacterium tumefaciens* bacteria or rapidly propelled tungsten microprojectiles that have been coated with DNA (Bent, 2000). Electroporation, microinjection or delivery by virus have also been exploited (Bent, 2000). Thus, genes of interest can be isolated from model species to be introduced in crop plants to form transgenic plants. They are however negative concerns about producing genetically modified plants (Slater *et al.*, 2003). Table 1.1. displays some examples of crop plants that have been genetically modified from the *Arabidopsis* genes.

**Table 1.1. Engineered crop species from *Arabidopsis* genes**

| Gene Name | Transgenic Species | Traits |
|---|---|---|
| AtNHX1 | *Brassica Napus* | Salt tolerance |
| AtNHX1 | Tomato | Salt tolerance |
| CBF1, CBF2, CBF3 | *Brassica Napus* | Freezing, Salt, and drought tolerance |
| CBF1 | Strawberry | Freezing tolerance |
| CBF3 | Wheat | Drought tolerance |

(Source: Zhang *et al.*, 2004)

## 1.3 GERMINATION IN *ARABIDOPSIS THALIANA*

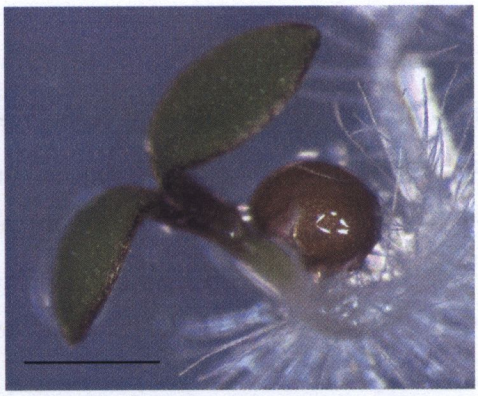
Seeds have developed different survival strategy characteristics to ensure continuity of life. They are resistant to desiccation, posses food reserves, and they exhibit seed dormancy (Koorneef and Karssen, 1994). Seed dormancy is defined as failure of a viable seed to germinate even when given favourable environmental conditions. The seed will only germinate under favourable environmental conditions once dormancy is lost through the period of afterripening (Footitt *et al.*, 2002).The transition from embryo dormancy to germination involves the mobilisation of seed storage reserves which result in radicle emergence [fig. 1.1 (a)], seedling establishment [fig. 1.1 (b)] and finally photoautotrophic growth (Berwley, 1997).

In *Arabidopsis,* germination is defined as radicle emergence from the seed coat while establishment as cotyledon expansion and greening in light grown plants (Zolman *et al.*, 2001). For dark grown plants establishment is defined as hypocotyls elongation (Zolman *et al.*, 2001).

Fig. 1.1(a) Germinated seed

*Bar represents 0.5mm*

Fig. 1.1 (b). Established seedling



*Bar represents 0.5mm*

### 1.3.1 Genetics of germination

Genetic studies in *Arabidopsis thaliana* have reviewed many loci that regulate the transition from embryo to seedling (Koorneef and Karssen, 1994). The majority of which influence many stages of plant growth and development and a small number which specifically affect the phenotype of an embryo (Holdsworth, *et al.,* 1999).

The large group of loci encodes proteins that regulate the production or sensitivity of hormone that are related to the synthesis or perception of absicissic acid (ABA) and gibberillin (GA) and to a less extent ethylene ($C_2H_4$) (Koorneef and Karssen, 1994). ABA is known to inhibit germination while GA promotes it (Holdsworth *et al.*, 1999). $C_2H_4$ was found out by Beaudoin *et al.* (2000) to suppress seed dormancy by inhibiting ABA action. The *aba* mutants of *Arabidopsis* contain low levels of ABA which disrupt embryo dormancy (Holdsworth *et al.,* 1999) while mutation of GA loci results in decrease in germination potential. This has been confirmed by the GA response mutants including, *gai* (Peng *et al.,* 1987*), rga* (Silverstone *et al.,* 1998) and *sly1* (Steber *et al.,* 1998).

Mutations in the second group of loci (that which specifically affect the phenotype of an embryo), do not appear to be directly related to hormone synthesis or signalling (Holdsworth *et al.,* 1999). Most of them function as repressors of germination and enhances embryo dormancy. The loci which are transcription factors are FUSCA3

(FUS3) (Baumlein *et al.*, 1994; Keith *et al.*, 1994), *ABSCISIC ACID INSENSITIVE3 (ABI3)* (Giraudat *et al.*, 1992), and *LEAFY COTYLEDON1 (LEC1)* (Lotan *et al.*, 1998).

The COMATOSE (*CTS*) locus was identified by Russel *et al.* (2000), as having a very strong effect on the enhancement of germination. It was mapped using CAPS/SSLP molecular markers to the bottom of chromosome IV close to the marker DHS1 (Konieczny and Ausbel, 1993). Mutants of *CTS* requires prior action of *ABA1*, *ABI3*, *LEC1* and *FUS3* to induce embryo dormancy (Russel *et al.*, 2000), implying that there is an interaction between these loci and *CTS*.

*1.3.1.1 Role of CTS in Germination*

CTS plays a role in germination by repressing embryo dormancy and enhancing germination potential. It functions as a transporter of Very Long Chain Fat Acids (VLCFA) and as a primary target for regulation of the initiation of germination (Holdsworth *et al.*, 2002). Thus, CTS promotes the β- oxidation pathway which involves the abolishing of fatty acids to gain energy. The catabolism of fatty acids supports germination until the seedling becomes photoautotrophic (Lange and Graham, 2000). A large proportion of VLCFA produced is converted via the glyoxylate cycle to produce succinate that is metabolised further to sucrose (Fulda *et al.*, 2004).

*Arabidopsis* seeds require either stratification (chilling) or after-ripening for prompt germination (Colucci *et al.*, 2002). Work done by Holdsworth *et al.* (2001), showed that CTS regulates germination potential by enhancing after ripening, sensitivity to gibberellins and pre-chilling, and by repressing the activities of loci that activate embryo maturation. Even though the morphology of CTS mutants embryos is not altered, physiological analysis reveals that mature CTS mutants seeds do not respond to gibberellins and prolonged chilling of imbibed seeds only partially restores germination potential (Russell, *et al.*, 2000).

There is a marked reduction in germination potential associated with mutation of the *CTS* locus and CTS mutant seeds do not after ripen (Russell, *et al.*, 2000). This is due to a disruption in the transition from embryo development to germination, but other aspects of development are not affected (Russell, *et al.*, 2000). CTS has a significant identity to the human X-linked adrenoleukodystrophy protein (ALDP). Like X-ALDP patients, CTS mutant embryos and seedlings exhibit pleiotropic phenotypes associated with perturbation in fatty acid metabolism (Footitt et al, 2002)

The genome of *Arabidopsis* contains 129 ATP binding cassettes (ABC) genes of which 51 are full size. Of the 51 full size genes only CTS has significant similarity to ALDP (Footitt *et al.*, 2002). Therefore mutations in CTS are unlikely to be restored by other genes.

### 1.3.2 Phenotypes associated with mutations in CTS gene

Mutations in *CTS* gene have led to several associated phenotypes. Table 1.2 summarises some known phenotypes associated with these mutations.

#### *1.3.2.1 Lateral Root Initiation*

The CTS mutant *pxa1* has defects in lateral root initiation (Zolman, *et al.*, 2001). The root initiation is examined by growing plants on sucrose supplemented medium for 4 days and then transferring seedlings to media supplemented by IAA and IBA (Zolman, *et al.*, 2001; Zolman, *et al.*, 2000).

#### *1.3.2.2. Hypocotyl elongation*

Mutants with a defect or reduced rates of β-oxidation are unable to elongate hypocotyls in the medium without sucrose (Zolman, *et al.*, 2001; Zolman, *et al.*, 2000). The CTS mutant *pxa-1* is unable to elongate hypocotyls in the medium without sucrose (Zolman, *et al.*, 2001). *Arabidopsis* seeds are grown on MS medium

and MS plus sucrose medium for 24 hrs before transferring them to the dark and then hypocotyls measured after 5 days.

*1.3.2.3 Root elongation*

The herbicide 2,4-D is known to inhibit root elongation of *Arabidopsis* at an early stage of seedling growth. Wain and Wightman (1954) as cited by Hayashi *et al.* (1998), demonstrated that 2,4-DB is metabolised to produce 2,4-D by the action of peroxisomal fatty acid in higher plants. CTS mutants that have a defect in peroximal fatty acid β- oxidation are unable to convert 2,4-DB to 2,4-D and hence they are resistant to 2,4 DB (Hayashi *et al.*, 1998). It is assumed that resistance to 2,4-DB could be caused by disruption of transport into peroxisome or by disruption of the β-oxidation cycle itself (Lange and Graham, 2000). The peroximal defective mutants ( *ped 1* to *3*) exhibit a resistance to 2,4-DB and these germinate on MS growth medium but fail to establish in the absence of exogenous sucrose (Hayashi *et al.*, 1998). Work done by Footitt *et al.* (2002), showed that the mutants *cts-1* and *cts-2* were resistant in the medium with 2,4 DB as compared to their respective wildtypes, Ler and Ws, where root elongation was inhibited.

*1.3.2.4 Plant size in relation to wild type*

Zoolman *et al.*, (2001) demonstrated that the CTS mutant *pxa1* does not only have defects in germination but that the resulting plant is smaller than the wild type observed over time (Table 1.2). This is because the mutant catabolise lipids very slowly. Though it flowers with similar number of leaves as the wild type, it has a delayed time to flowering. The mutant plants are morphologically similar to wild type despite the small size.

Table 1.2 Physiological tests for germination

| *Phenotype* | *Test* | *Basis for expected phenotype* | *Citation* |
|---|---|---|---|
| Lateral root intiation | Seeds germinated and grown on PNS for 4 days, then transferred to medium containing IBA or IAA. Counting lateral roots after an additional 4 days | Mutants with defects in Lateral root initiation. Unresponsive to IBA but respond to the stimulatory effects of IAA. | Zolman *et al,* 2000, Zolman *et al,* 2001 |
| Hypocotyl elongation | Seeds plated on PN or PNS and incubated for 24 hr under white light before being transferred to the dark. Length measured for 5 or 8 additional days. | Mutants with reduced rates of fatty acid β-oxidation. They show a reduced ability to elongate hypocotyls in the dark. | Zolman *et al,* 2000, Zolman *et al,* 2001 |
| Size in relation to wild type | Examine wild type plants and mutants over time. Compare size of rosette, number of leaves and size of primary inflorescence. In addition time taken to flowering | Mutants catabolise seed storage fatty acids slowly. | Zolman *et al,* 2001 |
| Root elongation | Grow 8-day old seedlings on a range of IBA (0-30µl) and IAA (0-100nm) varying medium concentrations. Also on 2,4-DB, 2,4-D and NAA | Mutants resistance to inhibition by IBA and 2,4-DB but sensitive to IAA, 2,4,-D and NAA | Zolman *et al,* 2001 |
| Stunted phenotype | Grow seedlings on PN medium. Grow in light and dark to check phenotypic differences. Also in different day lengths- 24 h light, long days-16 h light and short days- 8 h light. | Mutants unable to convert acetate from fatty acid β-oxidation into sugars. Restored when seedlings are grown on PNS or in high light conditions | Cornah, *et al.*, 2004, |
| Establishment | Plate seeds on medium with PNS and PN | Mutants with compromised β-oxidation capacity. Seedlings establish on medium with PNS | Hayashi *et al*, 2002, Footitt et al., 2002 |
| Germination | Nick and Plate mutant seeds on medium with PNS and PN | Mutants with a striking inability to break down lipids. Germinate when nicked and plated on PNS. | Footitt et al., 2002 |

### 1.3.3 Alleles of CTS

There are six different alleles of CTS were much studies have been done so far (Table 1.3)

**Table 1.3 *CTS* alleles**

| ***CTS* allele** | **Background Wild type** | **Nature of mutation** | **Citation** |
|---|---|---|---|
| *cts-1* | Ler | chromosomal translocation | Footitt *et al.*, 2002 |
| *cts-2* | WS-2 | T-DNA insertion | Footitt *et al.*, 2002 |
| *pxa-1* | Col-0 | point mutation | Zolman *et al.*, 2001 |
| *ped3-1* | Ler | point mutation | Hayashi *et al.*, 2002 |
| *ped3-2* | Ler | point mutation | Hayashi *et al.*, 2002 |
| *ped3-3* | Ler | point mutation | Hayashi *et al.*, 2002 |

In this thesis work was done on Targeting Induced Local Lesions IN Genomes (TILLING) alleles of the *CTS* genome to determine the germination potential. All the TILLING lines used have Columbia big mama background. TILLING is a general reverse-genetic strategy that provides allelic point mutations in genes of interest (McCallum *et al.*, 2000; Till et al, 2003). These lines had point mutations caused by ethyl methanesulfonate on the exons of the CTS locus forming different alleles.

The TILLING alleles used were N93440 (C22), N91660 (C55), N93169 (C60) and N91953 (C73). There nucleotide changes on the entire CTS gene were at G3609A (G at position 3609 changed to A) C3357T, G3877A and G3756A respectively (Fig. 1.2). The first T shown being on position 3098. The amino acid change in the CTS protein were at D568N for C22, L514F for C55, S657N for C55 and E617K for C73 (Fig. 1.4).

**Fig 1.2. Nucleotide changes for TILLING alleles**

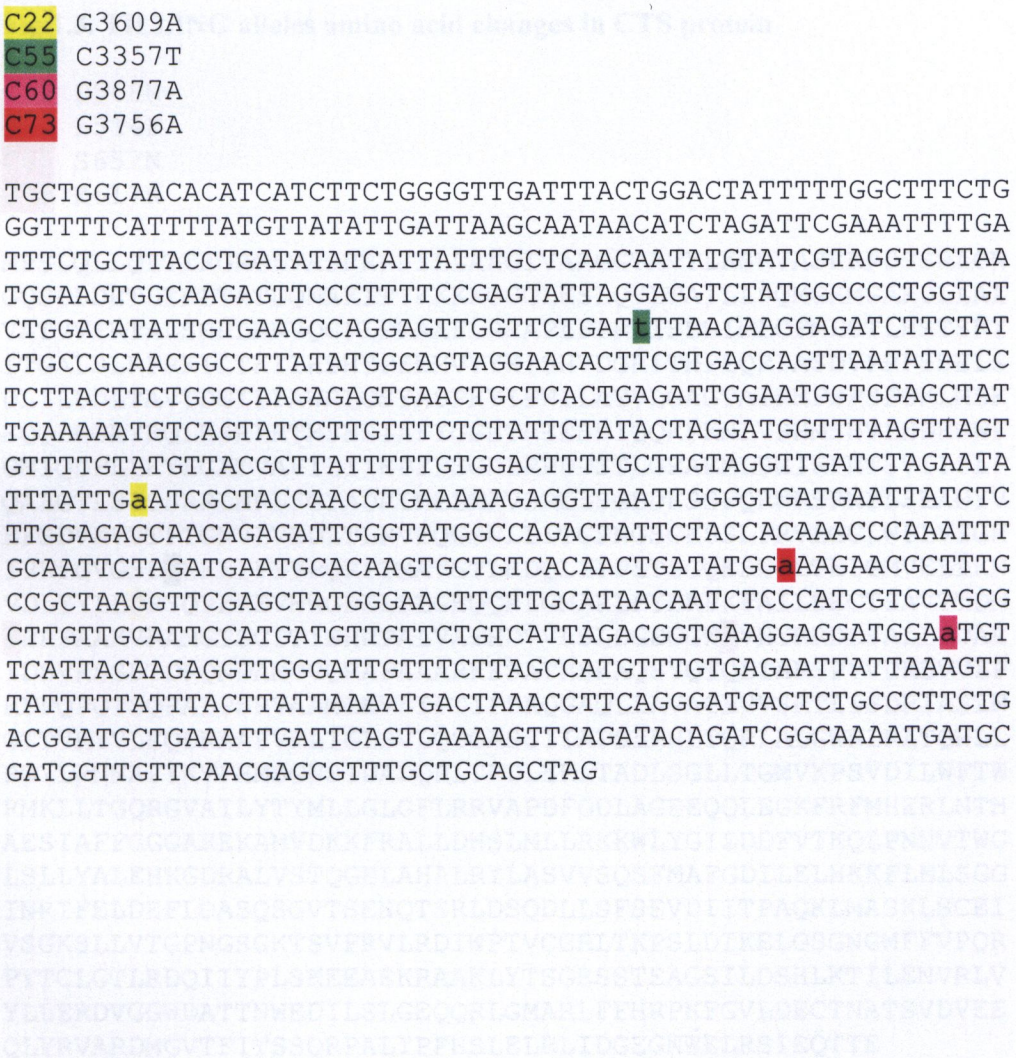


**Fig 1.3. TILLING alleles amino acid changes in CTS protein**

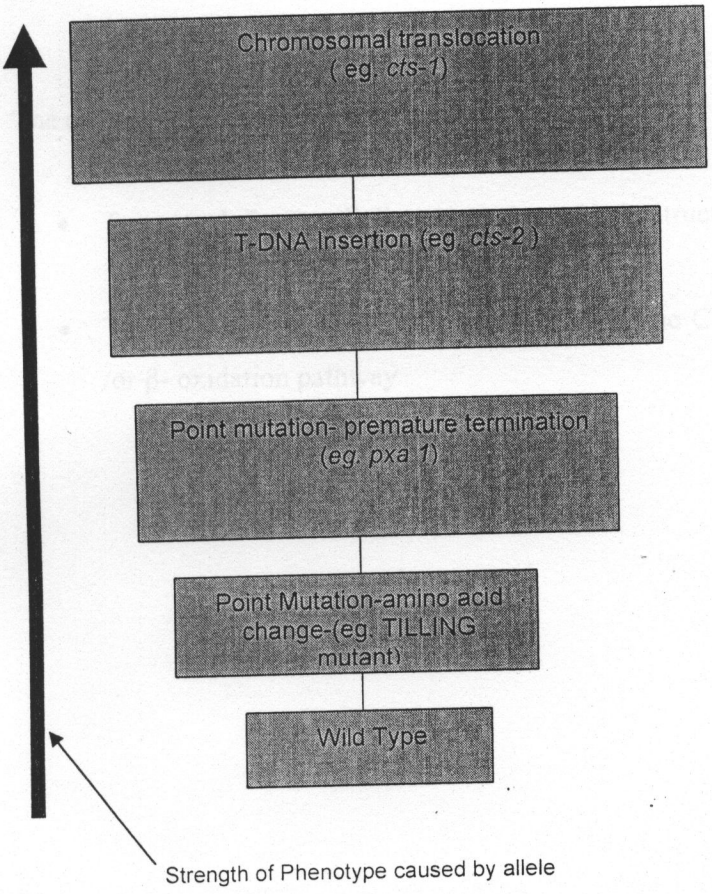
C22 D568N
C55 L514F
C60 S657N
C73 E617K

MPSLQLLQLTERGRGLVASRRKSILLAAGIVAAGGTAVYLKSRVASRRPDSSRLCN
GQSDDDETLEKLTATDQNAKITTKKKKGGGLKSLQVLTAILLSQMGKMGARDLLAL
VATVVFRTALSNRLAKVQGFLFRAAFLRRAPLFLRLISENIMLCFMLSTLHSTSKY
ITGALSLRFRKILTKIIHSHYFENMVYYKISHVDGRITHPEQRIASDVPRFSSELS
DLILDDLTAVTDGILYAWRLCSYASPKYIFWILAYVLGAGTAIRNFSPSFGKLMSK
EQQLEGEYRQLHSRLRTHSESIAFYGGETREESHIQQKFKNLVSHMSHVLHDHWWF
GMIQDFLLKYLGATVAVILIIEPFFSGHLRPDDSTLGRAEMLSNIRYHTSVIISLF
QALGTLSISSRRLNRLSGYADRIHELMAVSRELSGDDKSSFQRNRSRNYLSEANYV
EFSDVKVVTPTGNVLVEDLTLRVEQGSNLLITGPNGSGKSSLFRVLGGLWPLVSGH
IVKPGVGSDLNKEIFYVPQRPYMAVGTLRDQLIYPLTSGQESELLTEIGMVELLKN
VDLEYLLDRYQPEKEVNWGDELSLGEQQRLGMARLFYHKPKFAILDECTSAVTTDM
EERFAAKVRAMGTSCITISHRPALVAFHDVVLSLDGEGGWSVHYKRDDSALLTDAE
IDSVKSSDTDRQNDAMVVQRAFAAARKESATNSKAQSYQTQLIARSPVVDKSVVLP
RFPQPQTSQRALPSRVAAMLNVLIPTIFDKQGAQLLAVACLVVSRTLISDRIASLN
GTTVKYVLEQDKAAFVRLIGLSVLQSGASSIIAPSLRHLTQRLALGWRIRLTQHLL
RNYLRNNAFYKVFHMSGNSIDADQRLTRDLEKLTADLSGLLTGMVKPSVDILWFTW
RMKLLTGQRGVAILYTYMLLGLGFLRRVAPDFGDLAGEEQQLEGKFRFMHERLNTH
AESIAFFGGGAREKAMVDKKFRALLDHSLMLLRKKWLYGILDDFVTKQLPNNVTWG
LSLLYALEHKGDRALVSTQGELAHALRYLASVVSQSFMAFGDILELHKKFLELSGG
INRIFELDEFLDASQSGVTSENQTSRLDSQDLLSFSEVDIITPAQKLMASKLSCEI
VSGKSLLVTGPNGSGKTSVFRVLRDIWPTVCGRLTKPSLDIKELGSGNGMFFVPQR
PYTCLGTLRDQIIYPLSKEEAEKRAAKLYTSGESSTEAGSILDSHLKTILENVRLV
YLLERDVGGWDATTNWEDILSLGEQQRLGMARLFFHRPKFGVLDECTNATSVDVEE
QLYRVARDMGVTFITSSQRPALIPFHSLELRLIDGEGNWELRSIEQTTE

*1.3.3.1 Allelic series of CTS*

The relative strength of CTS mutants in the allelic series (Fig 2) shows that the TILLING mutants have the weakest strength through to *cts-1* which has the most severe alteration

**Fig 1.3. CTS allelic Series**



## 1.4 OBJECTIVE OF THE STUDY

Work has been done so far which has shown that CTS plays a vital role in enhancing germination potential (Footitt et al, 2002; Russell, *et al.*, 2000; Hayashi *et al.*, 2002; Zolman *et al.*, 2001). It promotes the β- oxidation pathway which involves the abolishing of fatty acids to gain energy for the transition from embryo development to germination.

The objectives of the study were:

- To try to define an allelic series of *cts* to help structure function studies
- To find out more about the specific role of the CTS in the germination and /or β- oxidation pathway

# CHAPTER 2 MATERIALS AND METHODS

## 2.1 PLANT MATERIALS AND GROWTH CONDITIONS

### 2.1.1 Seed Materials

The TILLING lines ( Table 2.1) used in this research project have point mutations in the entire genome caused by ethyl methanesulfonate. These were obtained from the Nottingham Arabidopsis Stock Centre (NASC). All the TILLING lines have Columbia (Big mama) background.

**Table 2.1 CTS TILLING lines**

| NASC number | DNA plant number | Nucleotide change | Effect |
|---|---|---|---|
| N93440 | C22 | G3609A | D568N |
| N91660 | C55 | C3357T | L514F |
| N93169 | C60 | G3877A | S657N |
| N91953 | C73 | G3756A | E617K |

*Arabidopsis* wild type ecotypes, Columbia Big Mama, Lansberg *erecta* (*Ler*) Wassilewskija (Ws) were used as control in the phenotypic analysis of germination. In addition known mutants of CTS, *cts-1, cts-2* and *pxa-1* were also used as controls. Details of the phenotypic analysis experiment are given on section 2.4.

### 2.1.2 Medium Preparation

#### *2.1.2.1 Water-agarose, 0.5xMS and 0.5xMS plus 0.5% sucrose medium*

The preparation of water agarose (Table 2.2), 0.5xMS (Table 2.3) and 0.5xMS plus 0.5%sucrose (Table 2.4) all followed similar steps. All the three medium used

distilled water as the solvent. The difference was that with water agarose, only agarose was added; 0.5xMS, MS and agarose were added and with 0.5xMS plus 0.5% sucrose, MS and sucrose were added. The mixing was done using a magnetic stirring rod on the hot plate magnetic stirrer. Agarose was the last to be added in all medium.

Prior to the addition of the solution (water in terms of water agarose medium) to agarose the pH was adjusted to 6.2 using 1M potassium hydroxide (KOH). This was done by carefully putting drops of 1M KOH in the sucrose plus MS solution using a 1000 µl pipette and at the same time reading from the pH meter. The medium was finally autoclaved in the bench omega medium autoclave manufactured by prestige medical. After autoclaving the medium was left to cool to about 50 °C and then poured on plates in the laminar flow hoods where it was left to solidify. It was then stored in the fridge at 4 °C

**Table 2.2 Water agarose medium constitutes for 500ml water**

| Item | Manufacturer | Conc. required | Amount needed |
|---|---|---|---|
| Agarose | FSL | 0.7%(w/v) | 3.5g |

**Table 2.3 0.5xMS medium constitutes for 500ml water**

| Item | Manufacturer | Conc. required | Amount needed |
|---|---|---|---|
| MS Salt mixture | Duchefa | 0.245% (w/v) | 1.225g |
| Agarose | FSL | 0.7%(w/v) | 3.5g |

**Table 2.4 0.5xMS plus 0.5% sucrose medium constitutes for 500ml water**

| Item | Manufacturer | Conc. required | Amount needed |
|---|---|---|---|
| Sucrose | FSL | 0.5% (w/v) | 2.5g |
| MS Salt mixture | Duchefa | 0.245% (w/v) | 1.225g |
| Agarose | FSL | 0.7%(w/v) | 3.5g |

*2.1.2.2 Medium with 2,4-DB*

Three kinds of medium with 2,4-DB were made. These were of 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB; 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB and 0.5xMS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB. This involved the initial preparation of 0.5xMS plus 0.5% sucrose (Table 2.4).

2,4-DB was measured to obtain 0.005g which was later on dissolved in 1 ml sterile distilled water to obtain 5mg/ml 2,4-DB. Since 2,4-DB do not dissolve so easily, 1 drop of 1M Sodium Hydroxide (NaOH) was put in 1 ml sterile distilled water to help in dissolving. From the 1 litre, 5mg/ml 2,4-DB, 33.33µl, 8.33µl and 3.33µl were removed and each put in separate 2ml eppendorf tubes. The eppendorf tubes were then filled up to 2 ml mark with sterile distilled water to make concentrations of 82.5µg/ml, 20.63µg/ml and 8.25µg/ml 2,4-DB respectively. From each concentration (82.5µg/ml, 20.63µg/ml and 8.25µg/ml) 2,4-DB, 1.2ml of each was removed and each added to 500ml medium with 0.5xMSplus 0.5% sucrose to make final medium concentrations of 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB; 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB and 0.5xMS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB respectively. The 2,4-DB was added to the 0.5xMS plus 0.5% sucrose (cooled to about 50 °C) via the sterile filter using a 2ml syringe. This

was done in the laminar flow hood. The medium was then poured on plates and left to solidify. It was then sealed and stored in a fridge at 4 °C.

*2.1.2.3 Medium with 0.5xMS plus 0.5% sucrose plus 0.05 µg/ml 2,4-D*

2,4-D was measured to obtain 0.002g. This was dissolved, with the aid of 1M NaOH (1 drop) in sterile 1ml distilled water to make 2mg/ml 2,4-D concentration. From 2mg/ml 2,4-D concentration, 20.7µl was removed and placed in 2ml eppendorf tube. The tube was then filled to a 2ml mark to make a concentration of 20.7 µg/ml 2,4-D. From the 2ml, 20.7 µg/ml 2,4-D 1.2ml was removed and added to the medium containing 0.5xMS plus 0.5% sucrose (cooled to about 50 °C)(Table 2.4) to make 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D. Just as with 2,4-DB medium, 2,4-D was added from the laminar flow via the sterile filter using a 2ml syringe. The medium was then poured on plates and left to solidify before been taken to the fridge (at 4 °C) for storage.

### 2.1.3 Seed sterilisation and plating

Sterilisation was done under aseptic conditions in the Laminar flow hood .Seeds were first placed in 1.5ml eppendorf tube and 0.5ml, 5% parazone (v/v) bleach added. The parazone bleach was removed after leaving it in the 1.5ml eppendorf tubes containing the seeds for 5minutes with occasional shaking. The seeds were then washed twice with 1 ml sterile distilled water and finally left in 1ml sterile distilled water. Thereafter they were plated on the media using a micropipette. It was then waited till water was soaked in the media before the plates were sealed with a micropore surgical tape.

### 2.1.4 Growth of plants

*Arabidopsis thaliana* seeds were first stratified for 2 days at 4 °C in the fridge (dark) before being taken to a growth room with constant light provided by fluorescent tubes at 22°C for 7 days. Thereafter seedlings were then transferred from the media

to compost containing 4 parts of compost original potting, 2 parts of vermiculite and 1 part of silver chloride in another growth room. The growth room conditions were set at a photo period of 16 hours with day and night temperatures of 23 °C and 18 °C respectively. The lights were provided by fluorescent tubes and bulbs (150 W/ 240 V). Seedlings were transplanted into trays containing pot of size 5 x 5 x 5 cm and were clearly labelled. Each tray had 24 pots and two trays were put in a basin (44 x 25 cm). Watering was done twice a week by a can. Seedlings were left to grow and were harvested when ripened.

To avoid wastage, seeds were harvested when the sliques turned brown or yellow but before the sliques dehisced. The whole plant was cut from the bottom with scissors (to get seeds from the main stem) and then placed in glassine (32 x 26 cm) paper carriers. The seeds were then placed in a cool dry place (24 °C ) to allow adequate drying. After 3 days the seeds were transferred from the large glassine bag to smaller glassine bags (10 x 7 cm). This was done by gently tapping the plants in glassine paper carriers to break the sliques and release the seeds. A small section was then cut in the corner of the glassine paper and the seeds released into smaller glassine carriers (10 x 7 cm) for storage

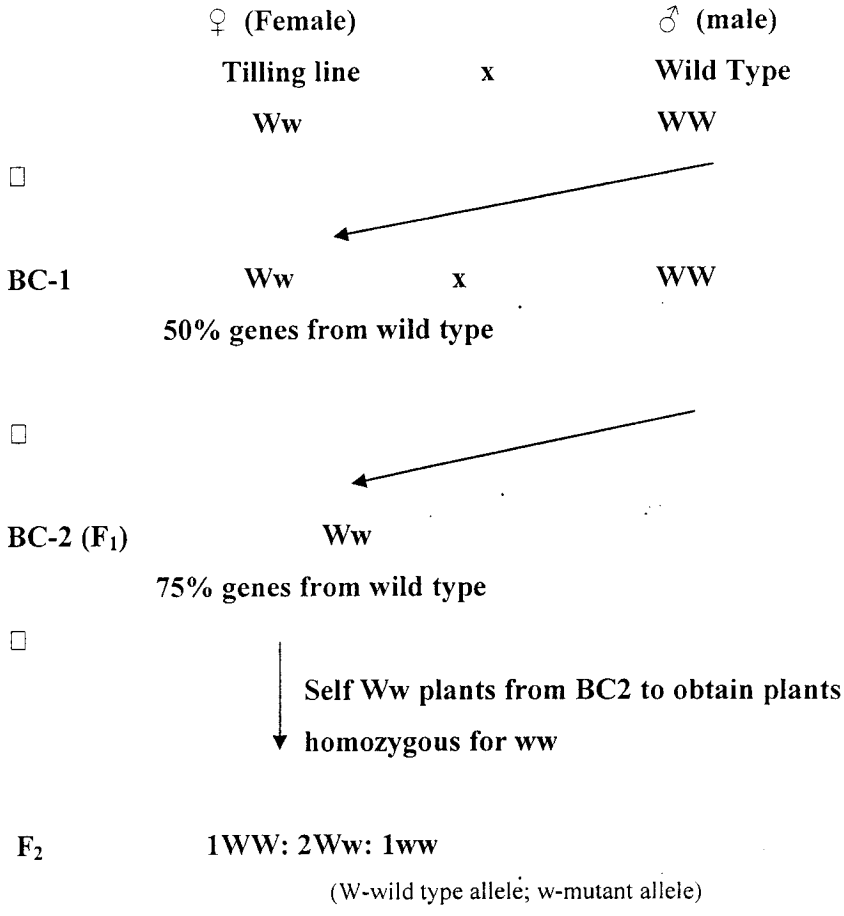
## 2.2 BACKCROSSING

Backcrossing (Fig 2.1) was done on the TILLING lines by Dr Heike Schmuths. This involved the cross between the TILLING line and the parent, wild type Columbia Big Mama. This was done to incorporate as many genes as possible from the wild type in the TILLING line except for the allele of interest. The wild type was used as male (pollen donor) and the TILLING line as the female (pollen receiver). The pollen transfer was done under a microscope. The sepals, petals and anthers were removed from the flowers of the plant receiving pollen using a pair of forceps leaving the stigma. To ensure that selfing did not occur emasculation was done on flowers which were mature but not yet opened. Very small flowers were not chosen as they were too young to develop siliques. From the pollen donor plant, a mature fully opened flower was used. The anthers with pollen were removed using forceps and then the pollen rubbed to the recipient (TILLING line) stigma. The crossed

stigmas were then taken note of by clearly labelling and carefully stamping a piece of paper. The Plants were then taken to the growth room with a photo period of 16 hours with day and night temperatures of 23 °C and 18 °C respectively.

To ensure that mutant allele we want was carried on, PCR analysis was used. Details on PCR analysis will be section 2.3. Plants ($F_1$), which were heterozygous as determined by molecular markers, were taken on from backcross one to use in backcross two. The heterozygous TILLING lines as identified by molecular markers from backcross 2 were followed grown and then selfed to obtain $F_2$ plants.

**Fig 2.1 Backcrossing of TILLING lines to the Wild type (Columbia big mama)**



### 2.3.1 Genomic DNA Extraction

A rapid genomic DNA plant extraction method which does not require any phenol or chloroform extraction as described by Edwards *et al.*, 1991, was used in this research work.

Leaf tissues from *Arabidopsis* were collected in 1.5 ml eppendorf tubes and then placed in liquid nitrogen. A small leaf tissue of a plant was used per each eppendorf tube. The eppendorf tubes were clearly labelled to indicate the plant identity the leaf tissue has been taken from. The eppendorf tubes were removed one at a time from the liquid nitrogen and the tissue ground into powder with sterile grinding pistil. Thereafter 400µl of extraction buffer (Table 2.5) was added into each tube and then the tube was shaken thoroughly to mix the ground tissue and the buffer. The solution was then centrifuged for 5 min at 12000 rotations per minute. Centrifuging enabled the solid materials (sediments) to be brought at the base of the tube, letting the supernatant at the top. From the supernatant, 300µl was removed and then transferred into a clearly labelled fresh tube, which was then taken to the fume hold where 300µl of isopropanol was added making a total solution per tube of 600µl. The solutions were gently mixed and then incubated for 45minutes. They were then centrifuged for 10 minutes at 12000 rotations per minute to bring the genomic DNA at the base. The tubes were then dried at room temperature and then resuspended in 50µl of sterile distilled water.

**Table.2.5 Composition of DNA extraction buffer**

| Item | Amount | Final concentration |
|---|---|---|
| 1 M Tris (pH 7.5) | 20.0 ml | 200mM |
| 0.5 M EDTA (pH 8.0) | 5.0 ml | 25mM |
| 2 M NaCl | 12.5ml | 250mM |
| 20% SDS | 2.5ml | 0.5% |
| Distilled water | Fill up to 100ml | |

To test if the extraction was successful, a genomic DNA check was done. From the 50 µl, of a plant genomic DNA sample, 4 µl was removed and mixed with 2 µl of loading buffer (0.25% [w/v] bromophenol blue; 0.25% [w/v] xylene cyanol FF; 30% [w/v] glycerol in water). This was then loaded in the slots on the agarose gel plate (1.5% agarose) (w/v) placed in the gel tank. The solvent used in preparing the agarose gel plate was 0.5xTBE buffer made from a diluting 10xTBE buffer with distilled water (1 part 10xTBE buffer to 19 parts distilled water). The gel was then run at 80 V for an hour. Thereafter the gel plate was removed and then checked in the transluminator to confirm positive genomic DNA extraction. The positive DNA extraction was confirmed by a clearly separated band. The DNA extraction was repeated for plants whose genomic plant DNA samples did not show a positive result.

### 2.3.2 PCR Analysis

PCR analysis were used for screening the plants. During backcrossing, screening was done to ensure that the plant used in backcrossing was heterozygous for the mutant allele. When a cross was first made between TILLING line (Ww) and the wild type (WW), the expected offspring were assumed to be in the ratio of WW : Ww. Being that the phenotypic appearance was the same for these two genotypes, molecular markers and PCR analysis were used to screen for the heterozygous plant and then using the heterozygous plant for the other backcross (B2). The selfed plants from backcross 2 ($F_2$) were screened for homozygosity so as to obtain mutant plants

(ww) plants to use in phenotypic analysis experiment. The TILLING lines were screened by Cleaved Amplified Polymorphic Sequences (CAPS) markers and selective primers. The TILLING lines N93440, N91660 and N91953 were screened using CAPS markers and N93169 was screened using selective primers.

*2.3.2.1 Screening using CAPS markers*

The initial stage in screening using CAPS marker involved the amplification of the genomic DNA to obtain PCR products.

A forward CTS primer with the sequence, 5'tgctggcaacacatcatcttctgg3' and a reverse primer with the sequence, 5'ctagctgcagcaaacgctcgttga3' was used as part of the master mix (Table 2.6) in the production of PCR products for the N93440, N91660 and N91953 TILLING lines. From the master mix 21µl was added to the PCR tubes to which 4 µl of the genomic DNA of a particular plant from any of the three TILLING lines (N93440, N91660 and N91953) was added. This made a reaction volume for the PCR machine of 25 µl. The tubes were clearly labelled to indicate the plant identity source of the DNA sample.

**Table 2.6 Composition of master mix for 10 DNA samples for N93440, N91660 and N91953 TILLING lines**

| Item | Single sample | Master mix for 10 samples |
|---|---|---|
| *Sterile distilled water* | *15.95µl* | *159.5µl* |
| Buffer ( 10 x PCR-$MgCl_2$) | 2.5µl | 25µl |
| $MgCl_2$ (50nM) | 1.25µl | 12.5µl |
| dNTPs (5nM) | 1.0µl | 10µl |
| l-primer (50pM) | 0.1µl | 1µl |
| r-primer (50pM) | 0.1µl | 1µl |
| Taq polymerase enzyme (5 Units/ µl) | 0.1µl | 1µl |
| **Total volume** | **21µl** | **210µl** |

The thermal cycler was set for the first stage at 94 °C for 3 min. It was set at three holds for the second stage, the denaturing temperature of 94 °C for 30 sec; annealing temperature of 66 °C for 45 sec and the extension temperature of 72 °C for 2 min. The third stage had two holds and it was set at 72 °C for 10min and 10 °C at infinite (∞). The number of cycles was set at 32.

The amplified PCR product was 984bp

The restriction enzymes (Table 2.7) Hinf I, Mbo I and Mbo II were used for the TILLING lines N93440, N91660 and N91953 respectively

**Table 2.7 Restriction Products for N93440, N91660 and N91953 TILLING lines**

| NASC Number | Restriction Enzyme | Restriction Product (bp) |
|---|---|---|
| N93440 | Hinf I | Wild type: 98+783+30+73<br>Mutant: 98+413+370+30+73 |
| N91660 | Mbo I | Wild type: 257+14+223+18+423+49<br>Mutant: 270+223+18+423+49 |
| N91953 | Mbo II | Wild type: 20+256+383+325<br>Mutant: 20+256+708 |

The restriction enzymes were prepared as master mix. From the master mix 4 µl was put into 0.5 ml tubes, to which 6 µl of appropriate PCR product was added. Thus a PCR product from a particular plant of a TILLING line (Appendix 1) was added into a tube containing a master mix portion with an appropriate restriction enzyme. The tubes were carefully labelled indicating the plant identity of the PCR product. The restriction mix was then incubated over night in an oven at 37 °C. Thereafter 2µl loading buffer was added directly into each 0.5ml tubes containing the restriction enzyme product and then the resulting solution was centrifuged up to 2 rotations per minute to thoroughly mix the loading buffer. The loading buffer-restriction enzyme product was then loaded on the gel in the gel tank and then run at 80 V until separation of bands was clear to check for the restriction products. The **gel plate** was prepared at **1.5%** agarose (w/v [0.5xTBE buffer]). The 100bp ladder was loaded as well in a separate slot for deducing or reading the sizes at which the bands appeared. From the restricted fragments bands of a TILLING line it was then determined weather a particular plant was homozygous for the mutant allele or not (Table 2.7).

*2.3.2.2 Screening using Selective Primers*

Screening for the mutants of the N93169 TILLING line was done using selective primers. Selective primers used were the CTS forward primer (5'tgctggcaacacatcatcttctgg3') with either CTS-Mutant primer

(5'gaaacaatcccaacctcttgtaatgaacac3') or CTS-Wild type primer (5'gaaacaatcccaacctcttgtaatgaacat3') as a reverse primers. Thus two master mixes (Table 2.8) were prepared.

**Table 2.8 Composition of master mix for 10 DNA samples for N93169 TILLING line**

| Item | Single sample | Master mix |
|---|---|---|
| ***Strile distilled water*** | ***19.05µl*** | ***190.5µl*** |
| Buffer ( 10 x PCR-$MgCl_2$) | 2.5µl | 25µl |
| $MgCl_2$ (50nM) | 1.25µl | 12.5µl |
| dNTPs (5nM) | 1.0µl | 10µl |
| l (forward)-CTS primer (50pM) | 0.05µl | 0.5µl |
| r-Wild type or mutant primer (50pM) | 0.05µl | 0.5µl |
| Taq polymerase enzyme (5 Units/ µl) | 0.1µl | 1µl |
| **Total volume** | **24 µl** | **240 µl** |

The steps of obtaining the PCR products was done in the same way as in section 2.3 2.1 except that 24 µl was obtained from the master mix and 1 µl of genomic DNA added. The PCR product from the master mix potion with the CTS forward primer and the CTS mutant primer (reverse primer) was compared to the PCR product sample but with the CTS wild type primer as reverse primer derived from the same genomic DNA sample. The wild type DNA sample was used to act as a control. The PCR product for either the CTS mutant primer or CTS wild type primer was 780bp. The presence of the band from the checked PCR product denoted the presence of an allele. The presence of band from the PCR product which had CTS mutant as reverse primer in a master mix denoted the presence of the mutant allele while that with wild type reverse primer denoted the presence of wild type allele. The homozygous mutant was expected to show a band with the PCR product from the master mix with CTS mutant primer but no band with PCR product from the master mix with CTS

wild type primer as reverse primer. The opposite was expected from PCR product from the wild type DNA, a band from the CTS wild type reverse primer and no band from the mutant type primer. Thus a PCR from the DNA sample of a heterozygous plant for a mutant allele was expected to show a band for both reverse primers.

The Thermal Cycler was set for the first stage at 94 °C for 3 min. It was set at three holds for the second stage, the denaturing temperature of 94 °C for 30 sec; annealing temperature of 69.5 °C for 45 sec and the extension temperature of 72 °C for 2 min. The third stage had two holds and it was set at 72 °C for 10min and 10 °C at infinite (∞). The machine was set at 30 cycles.

## 2.4 PHENOTYPIC ANALYSIS EXPERIMENTS

Four plants of each TILLING line (Appendix 1) which were found to be homozygous for the mutant allele were used for the phenotypic analysis experiment together with wild type Columbia big mama. Other known mutants of CTS and wild types were as well used. Details are highlighted in the sub sections of section 2.4.

### 2.4.1 Hypocotyl elongation

The experiment was done on two types of media, with 0.5xMS and 0.5xMS plus 0.5% sucrose. Four TILLING mutants (Appendix 1) per each TILLING line were used for this experiment. Wild type accessions Columbia Big Mama, Landsberg *erecta* and CTS mutant *cts-1,* were used as controls in this experiment. Four plants per each accession were used. This made a total of 28 plants (7 accessions x 4 plants).

Accessions were plated on two medium, 0.5xMS and 0.5xMS plus 0.5% sucrose. Fifty seeds were plated for each plant per medium except *cts-1. cts-1* seeds were nicked before plating and only 10 seeds per medium per plant were plated.

*2.4.1.1 Transfer to growth room and hypocotyl length measurement*

The seeds were transferred to growth room with temperature of 22 °C at constant light after stratifying them for 2 days at 4 °C. After spending 24 hrs in light, the seeds were placed in the dark. This was done by wrapping the plates with aluminium foil. After five days, the aluminium foil was removed and the length of the hypocotyls measured for all the 28 accessions. At least six randomly grown hypocotyls were chosen from each plant to calculate the hypocotyls length and then the mean length for each plant determined. This was done by laying the hypocotyls on black A3 paper and taking photographs using a digital camera.

The hypocotyls lengths were measured using the image J computer soft ware. This was done by importing the photography to the software, setting the scale and them measuring the hypocotyls length using a free hand line selection.

**2.4.2 Root elongation**

Three types of growth media were initially used with the root elongation experiment. Medium with 0.5xMS plus 0.5% sucrose, 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB and 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.

The same plants used in hypocotyl experiment (section 2.4.1) were used for the experiment. Ten seeds per plant were plated in each medium. The *cts-1* seeds were nicked nicked before plating. The seeds were plated in a line about 2.5 cm from one edge of the plate.

A further experiment was done in the same way on media with 0.5MS plus 0.5% sucrose 0.02µg/ml 2,4-DB, 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB and 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.

*2.4.2.1 Transfer to growth room and root length measurement*

The seeds were transferred to growth room with temperature of 22 °C at constant light after stratifying them for 2 days at 4 °C. The plates were placed in a vertical position so as to facilitate the downward growth of roots. The plates were removed from the growth room after 8 days and photographs of the plate taken with the digital camera.

The length of the roots were measured just as with the hypocotyl elongation by using the Image J computer soft ware.

**2.4.3 Germination and establishment**

Germination and establishment experiment made use of 40 plants (10 accessions x 4 plants). In addition to the accessions used for the hypocotyls and root elongation experiment, *cts-2, pxa-1 and* Ws were used. Three types of media was used, water agarose medium 0.5xMS and 0.5xMS plus 0.5% sucrose. Fifty seeds were plated for each plant in each medium. Of the 40 plants only the plants which were harvested almost at the same time (7 and 10 June 2005)(Appendix 6) were compared The experiment was arranged into two categories. The first experimental category was taken for stratification for 2 days before transferring to growth room with constant light at 22 °C and the other category was not stratified. It was taken straight to growth room with constant light at 22 °C. .

To investigate the effect of afterripening all the 40 plants harvested at different days (Appendix 6) were plated on water-agarose medium and were not stratified.

*2.4.3.1 Transfer to growth room and germination measurements.*

Measurement of germination and establishment was done over 10 days for each accession and each experimental category. Germination as earlier on defined was taken as appearance of a radicle and establishment as cotyledon expansion and

greening. The number of seeds which germinated and established for each accession per day was calculated as a mean percentage of the total number of seeds per each accession plated.

For the investigation of afterripening the measurements were done on the seventh day after transfer to the growth room with constant light at 22 °C.

# CHAPTER 3 RESULTS

## 3.1 GENETIC ANALYSIS

### 3.1.1 Homozygosity screening using CAPS markers

The obtained PCR products for $F_2$ plants from N93440 (C22), N91660 (C55) and N91953 (C73) were screened using CAPS markers to identify homozygous individuals to use in phenotypic analysis. The digested products were separated on the agarose gels placed in the gel tank and then finally viewed on the transiluminator. Fig. 3.1, 3.2 and 3.3 displays PCR analysis results for some plants for C22, C55 and C73 respectively.

The number of plants analysed and the results for C22, C55, and C73 are presented on Appendices 2, 3 and 4 respectively.

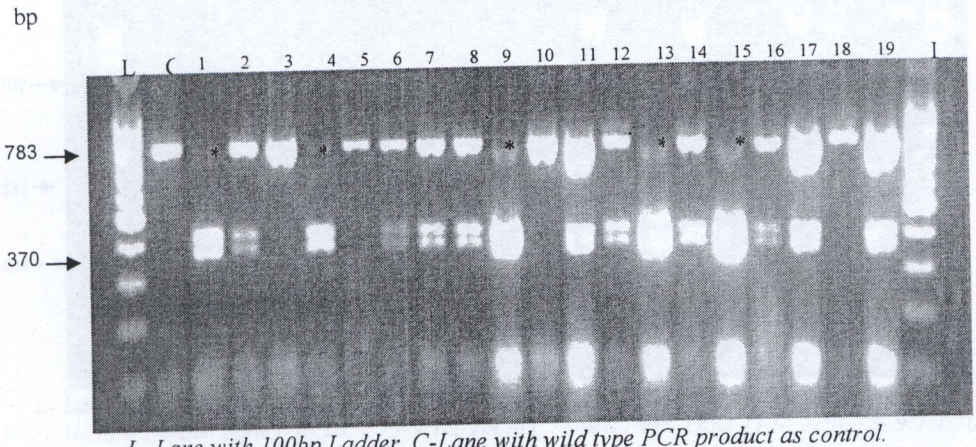
From the 22 plants analysed for C22 (Appendix 2) 5 were found to be homozygous for the mutant (recessive) allele, 13 were found to be heterozygous for the mutant allele and 4 were found to be homozygous for the wild type (dominant) allele. There was no significant difference of C22 outcome genotypic ratios to the expected ratio of 1:2:1 ($\chi^2 = 0.81818$, *d.f.* = *2*, $p > 0.05$).

The results for C55 (Appendix 3) showed that, from the 22 plants analysed 5 were found to be homozygous for mutant allele, 12 were found to heterozygous for the mutant allele and 5 were found to be homozygous for the wild type allele. These out come showed no significant difference with the expected ratio of 1:2:1($\chi^2 = 0.13044$, *d.f.* = *2*, $p > 0.05$).

The results obtained from the analysed plants for C73 (Appendix 4) showed that there was no significant difference between the observed outcome ratio and the expected ratio of 1:2:1 ($\chi^2 = 4.90909$, *d.f.* = *2*, $p > 0.05$). The analysis showed that of the 22 analysed plants, 4 plants were homozygous for the mutant allele, 12 were

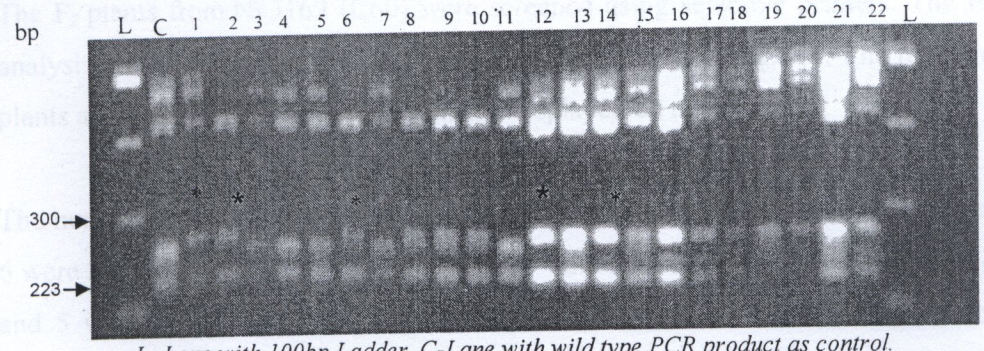
heterozygous for the mutant allele and 2 were homozygous for the wild type allele (Appendix 4).

**Fig. 3.1: Genetic results for homozygosity screening for C 22 individuals**



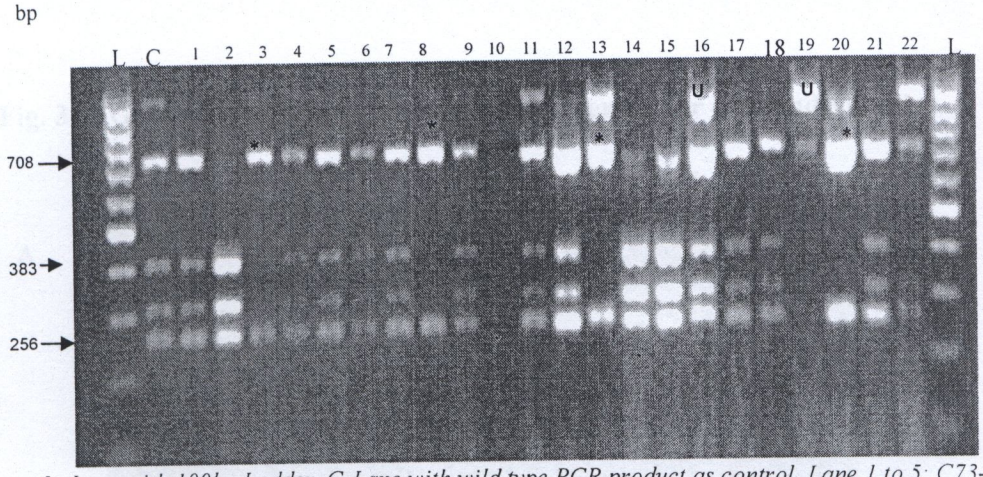

*L- Lane with 100bp Ladder. C-Lane with wild type PCR product as control.*
*. Lane 1 to 4: C22-6/4/2/1/1w to 6/4/2/1/4w. Lane 5: C22-6/4/2/1/6w. Lane 6 to 8: C22-6/4/2/1/4m to 6/4/2/1/6m. Lane 9 to 15: C22-6/4/2/1/10 to 6/4/2/16. Lane 16 to 19: C22-6/4/2/1/18 to 6/4/2/1/21.*
** - Lane with C 22 homozygous for mutant allele products.*

**Fig.3.2: Genetic results for homozygosity screening for C 55 individuals**




*L- Lane with 100bp Ladder. C-Lane with wild type PCR product as control.*
*Lane 1 to 6: C55-1/2/5/1w to 1/2/5/6w. Lane 7: C55-1/2/5/2m. Lane 8 to 9: C55-1/2/5/5m to 1/2/5/6m. Lane 10: C55-1/2/5/10m. Lane 11 to 22: C55- 1/2/5/11 to C55-1/2/5/22.*
** - Lane with C 55 homozygous for mutant allele products.*

**Fig. 3.3: Genetic results for homozygosity screening for C 73 individuals**




*L- Lane with 100bp Ladder. C-Lane with wild type PCR product as control. Lane 1 to 5: C73-9/1/1/1w to 9/1/1/5w. Lane 6 to 10: C73-9/1/1/2m to C73-9/1/1/6m. Lane 11 to 22: C73- 9/1/1/10 to C73-9/1/1/21.*

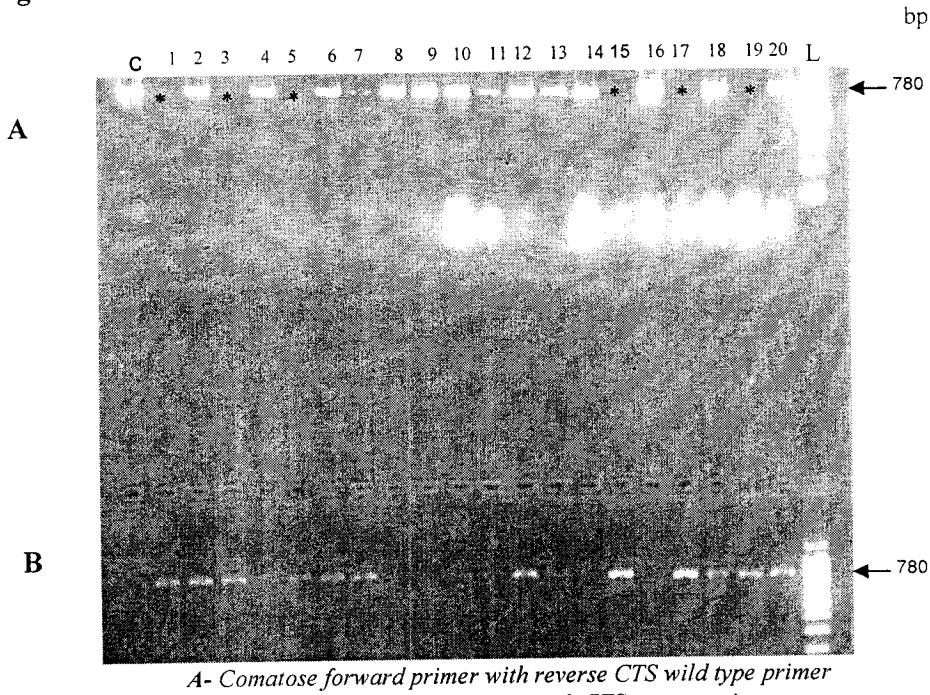
** - Lane with C 73 homozygous for mutant allele products. U- undigested product.*

### 3.1.2 Homozygosity screening using selective primers

The $F_2$ plants from N93169 (C60) were screened using selective primers. The PCR analysis results for some of the C60 plants are presented on Fig. 3.4. The number of plants analysed for C60 and the results are displayed on appendix 5.

The number of plants analysed (Appendix 5), showed that of the 23 plants analysed, 6 were homozygous for the mutant allele, 12 were heterozygous for the mutant allele and 5 were homozygous for the wild type allele. This out come does not differ significant from the expected ratio of 1:2:1($\chi^2 = 0.13044$, *d.f.* = 2, $p > 0.05$).

**Fig. 3.4: Genetic results for homozygosity screening for C 60 individuals**




*A- Comatose forward primer with reverse CTS wild type primer*
*B-Comatose forward primer with CTS mutant primer*
*L- Lane with 100bp Ladder. C-Lane with wild type PCR product as control. Lane 1 to 4: C60-3/1/1/5/1w to 3/1/1/5/4w. Lane 5 to 6: C60- 3/1/1/5/2m to 3/1/1/5/3m. Lane 7: C60-3/1/1/5m. Lane 8: C60- 3/1/1/5/8m. Lane 9 to 20: C60-3/1/1/5/10 to 3/1/1/5/21*
** - Lane with C 60 homozygous individuals for mutant allele products.*

## 3.1 PHENOTYPIC ANALYSIS

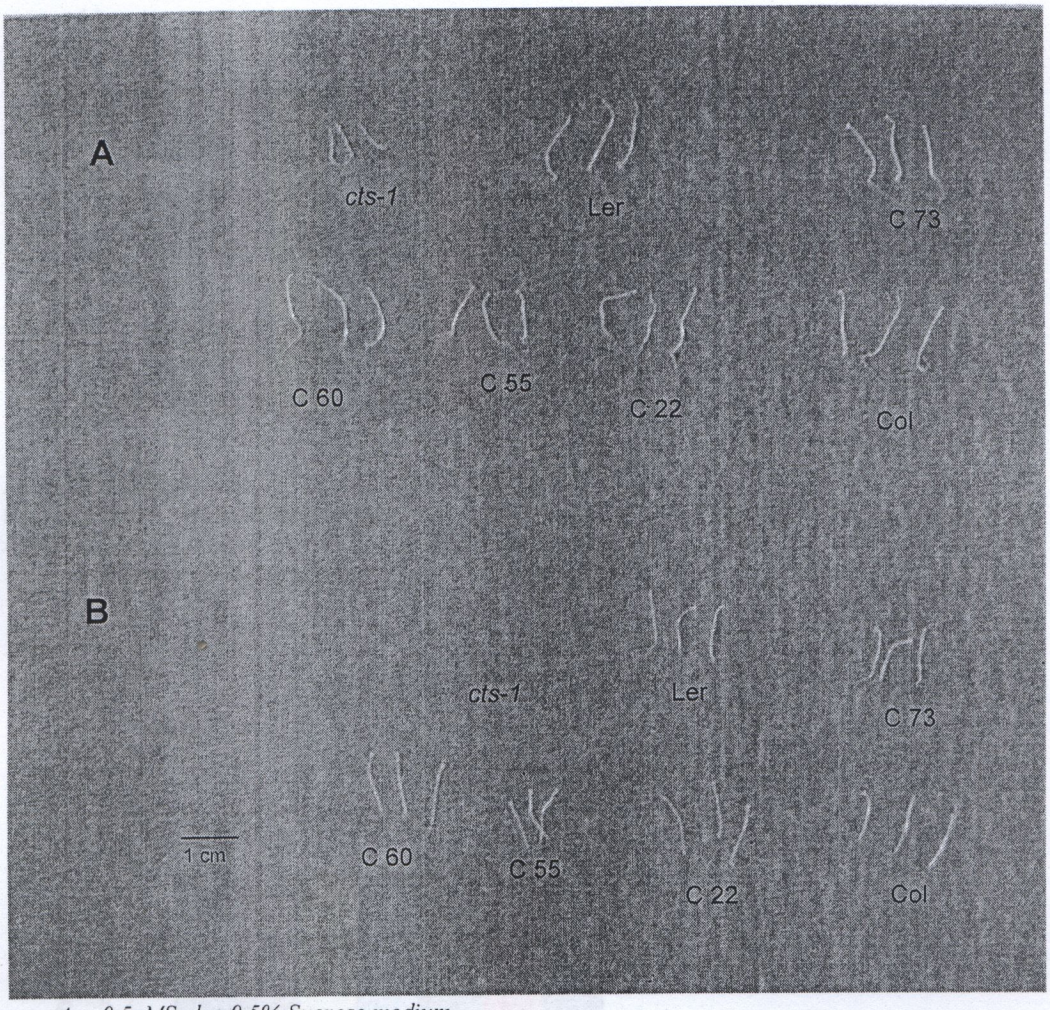
### 3.1.1 Hypocotyl elongation analysis

The hypocotyl elongation experiment was done to investigate TILLING mutants ability to metabolise stored lipids to sucrose.

The results obtained from measuring the hypocotyl lengths after five days from the dark showed no significant differences in the mean values between the TILLING mutants (student's *t*-test, P> 0.05)and the wild type accessions on both 0.5xMS plus 0.5% sucrose and 0.5xMS medium (Fig. 3.5, 3.6 and 3.7). Differences between the

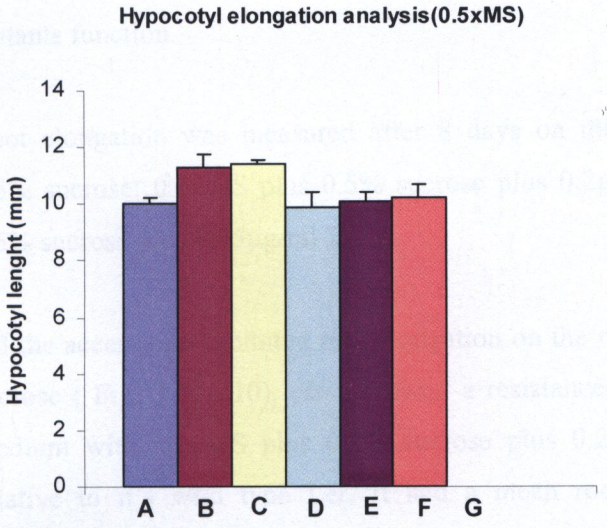
mutant *cts-1* and other accessions was clearly visible. It failed to germinate on the medium with 0.5MS. but germinated on 0.5xMS plus 0.5% sucrose. Though *cts-1* germinated on medium with 0.5xMS plus 0.5% sucrose, the mean length was significantly shorter than its wild type Ler (student's *t*-test, $P < 0.05$)

**Fig. 3.5 Hypocotyl elongation on medium with 0.5xMS plus 0.5% Sucrose and 0.5xMS**



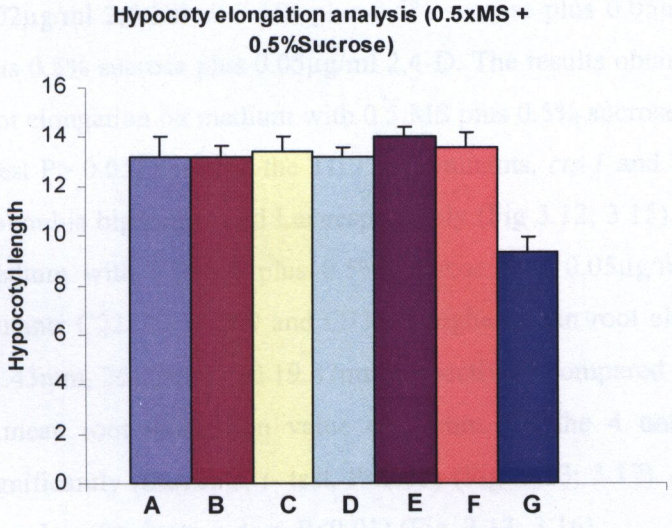

*A- 0.5xMS plus 0.5% Sucrose medium*
*B- 0.5xMS medium*
The bar shown is 1 cm

**Fig. 3.6**




***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

**Fig 3.7**




***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

### 3.1.2 Root elongation analysis

This experiment was done investigate how the β-oxidation pathway of the TILLING mutants function.

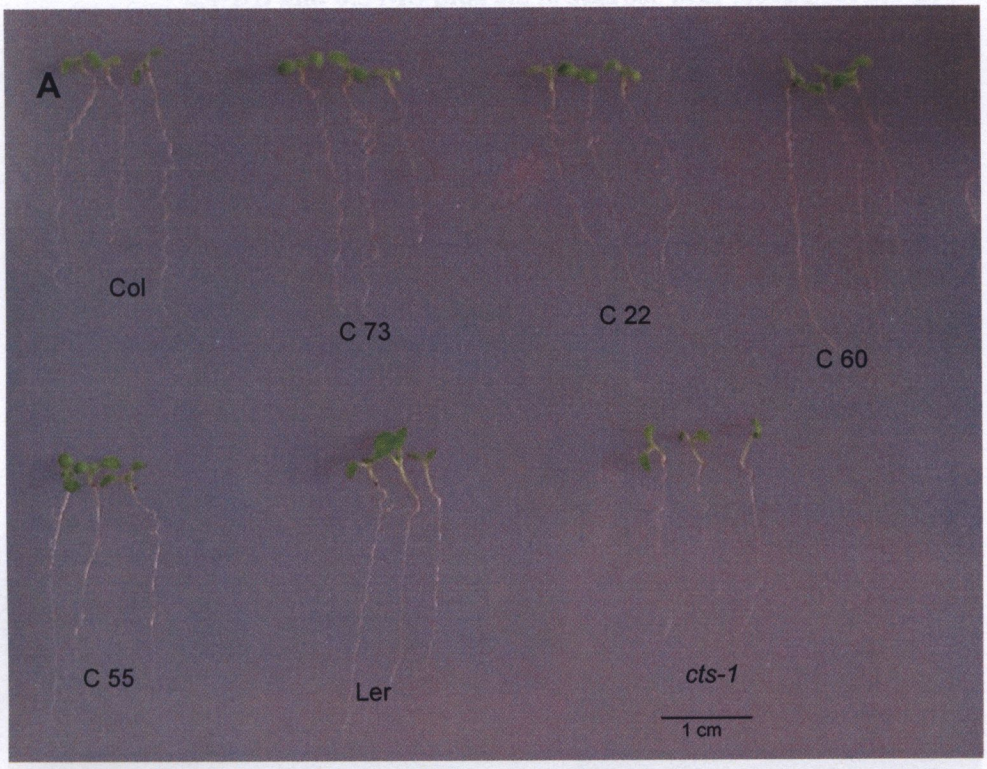
Root elongation was measured after 8 days on medium containing 0.5xMS plus 0.5% sucrose; 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB and 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.

All the accessions exhibited root elongation on the medium with 0.5xMS plus 0.5% sucrose ( Fig. 3.8; 3.10). *cts-1* showed a resistance (able to elongate roots) on the medium with 0.5 MS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB (Fig 3.9; 3.11). relative to it's wild type Ler. It had a mean root elongation value of 15 mm compared to Ler which had a mean value of almost 0 mm. All the accessions exhibited a hindered root elongation on medium with 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D (fig 3.14).

A further experiment was done on medium with 0.5 MS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB; 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB and 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D. The results obtained showed no significant root elongation on medium with 0.5 MS plus 0.5% sucrose plus 0.02µg/ml (students, *t*-test $P > 0.05$) between the TILLING mutants, *cts-1* and their respective wild types Columbia big mama and Ler respectively (Fig 3.12; 3.15). They were differences on medium with 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB. The TILLING mutants C22, C55, C60 and C73had higher mean root elongation values of 25mm, 22.43mm, 26.33mm and 19.87mm respectively compared to the wild type which had a mean root elongation value of 13mm. Of the 4 only C22 and C60 differed significantly (Students, *t*- test, $P<0.05$) (Fig. 3.13; 3.17). *cts-1* differed significantly from Ler (Students, *t*- test, $P<0.01$) (Fig. 3.13; 3.16).

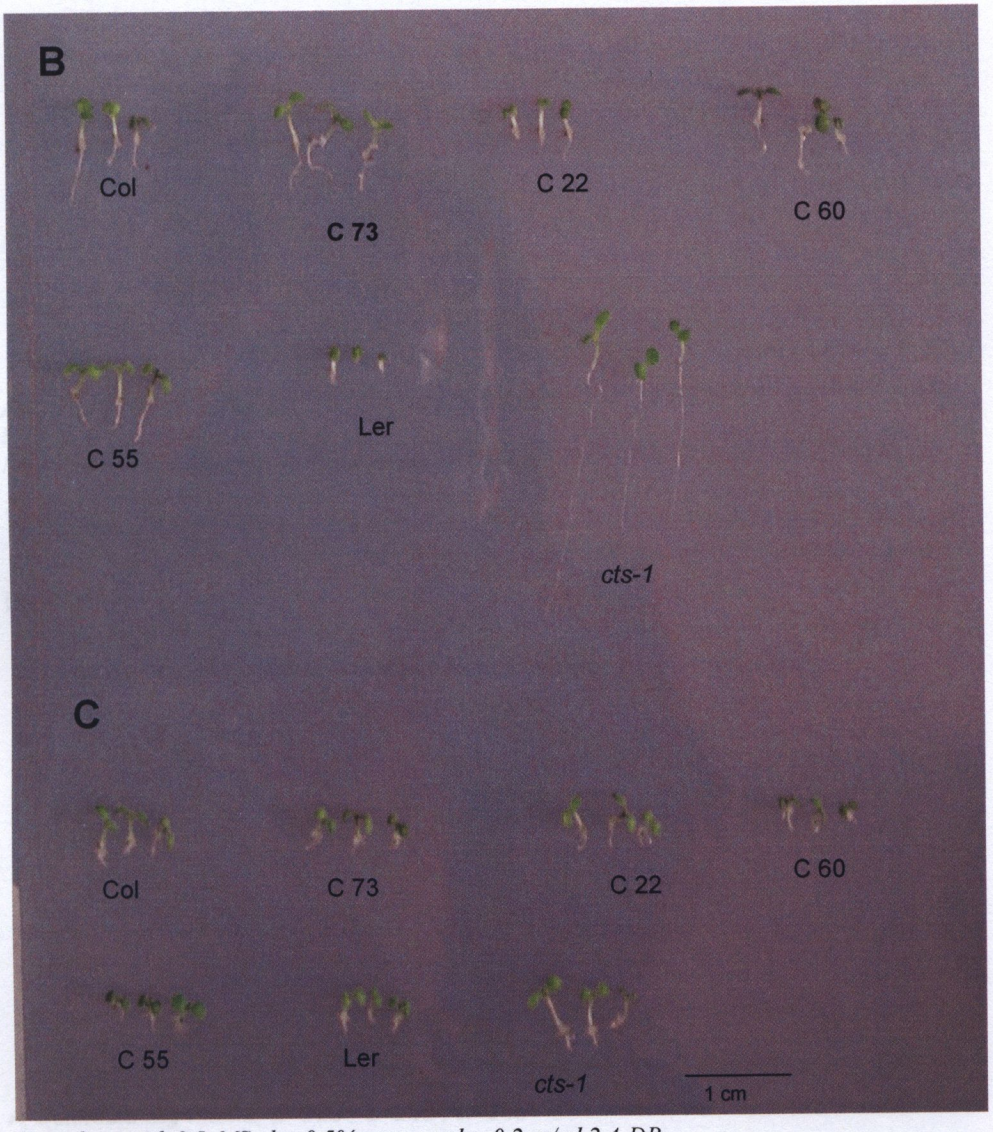
Just as with the earlier experiment there was a hindered root elongation on all accessions on medium with 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D (Fig. 3.14)

**Fig. 3.8. Root elongation on medium with 0.5xMS plus 0.5% sucrose**



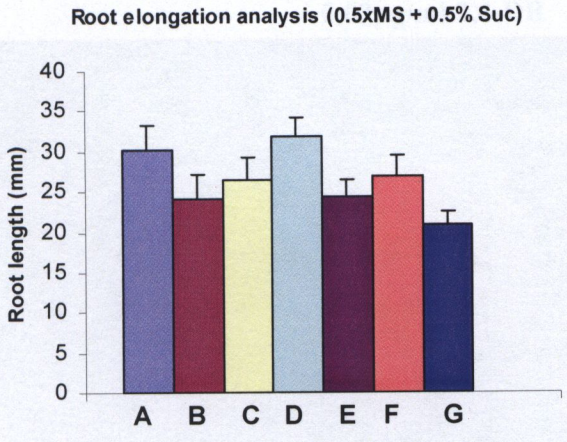

*A-medium with 0.5xMS plus 0.5% sucrose*
*Bar represents 1 cm*

**Fig 3.9. Root elongation medium with 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB and 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.**



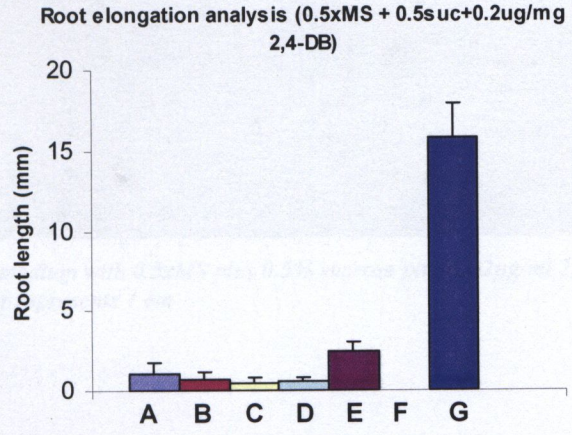

***B**-medium with 0.5xMS plus 0.5% sucrose plus 0.2µg/ml 2,4-DB*
***C**- medium with 0.5xMS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.*
*Bar represents 1 cm*

**Fig. 3.10**



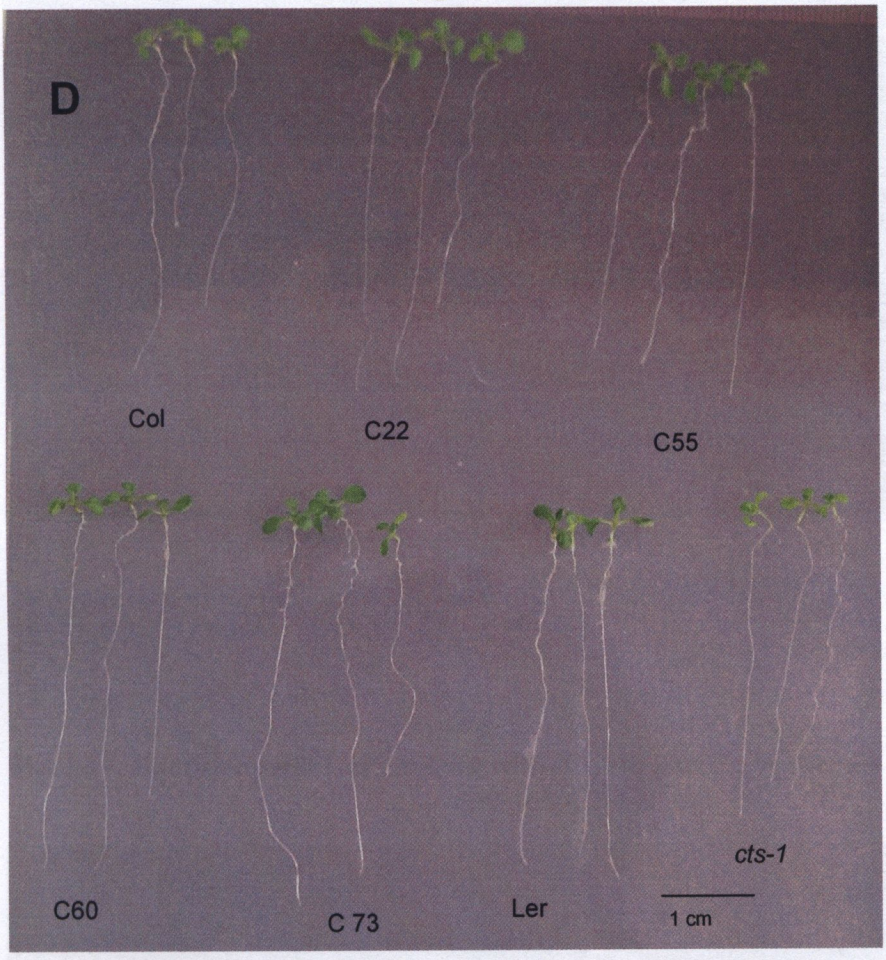

***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

**Fig. 3.11**



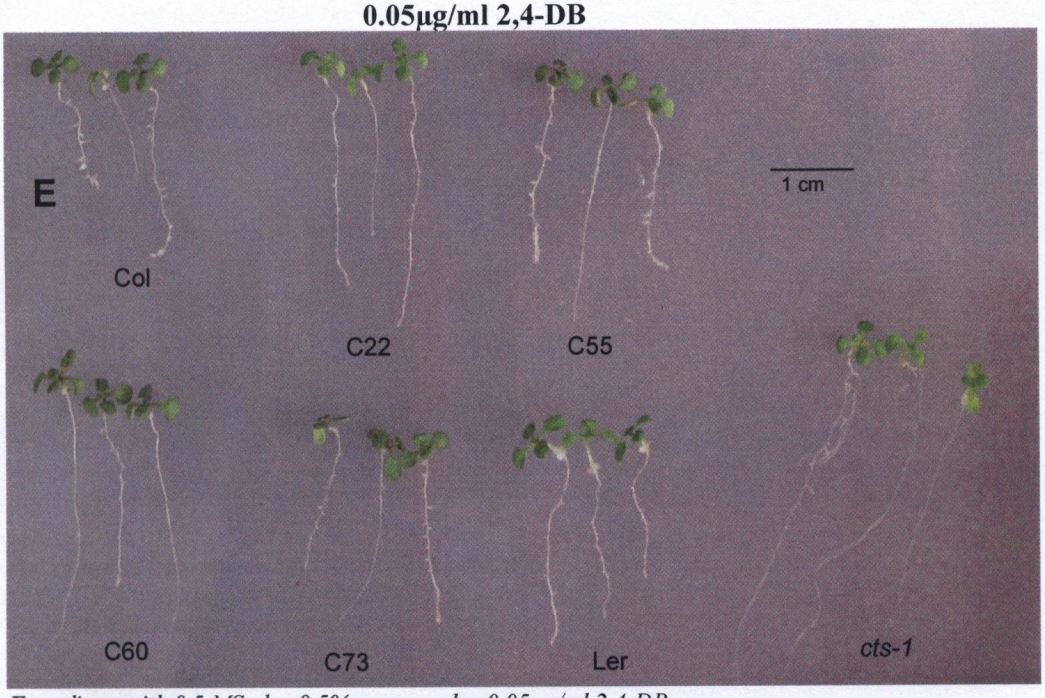

***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

**Fig. 3.12. Root elongation on medium with 0.5 MS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB**




***D**-medium with 0.5xMS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB*
*Bar represents 1 cm*

**Fig 3.13. Root elongation on medium with 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB**




*E-medium with 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB*
*Bar represents 1 cm*

**Fig. 3.14. Root elongation on medium with 0.5 MS plus 0.5% sucrose plus 0.05µg/ml 2,4-D.**

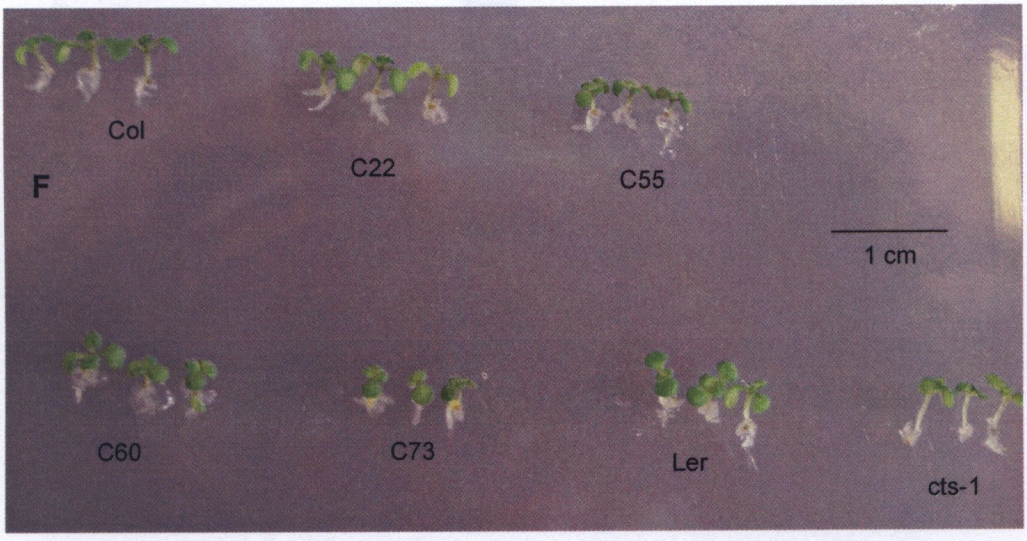
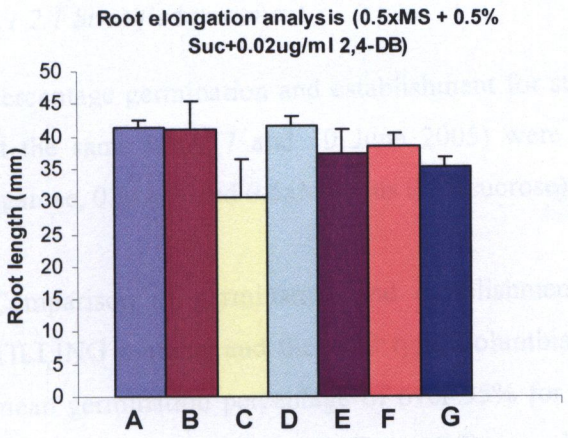
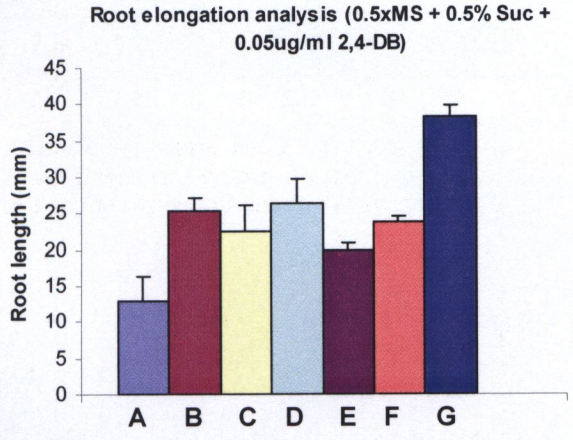


Fig. 3.15




***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

Fig. 3.16




***A**-Columbia Big mama, **B**-C22 , **C**-C55 , **D**-C60*
***E**-C73, **F**-Landsberg erecta, **G**-cts-1*
*Error bars shown presents standard errors*

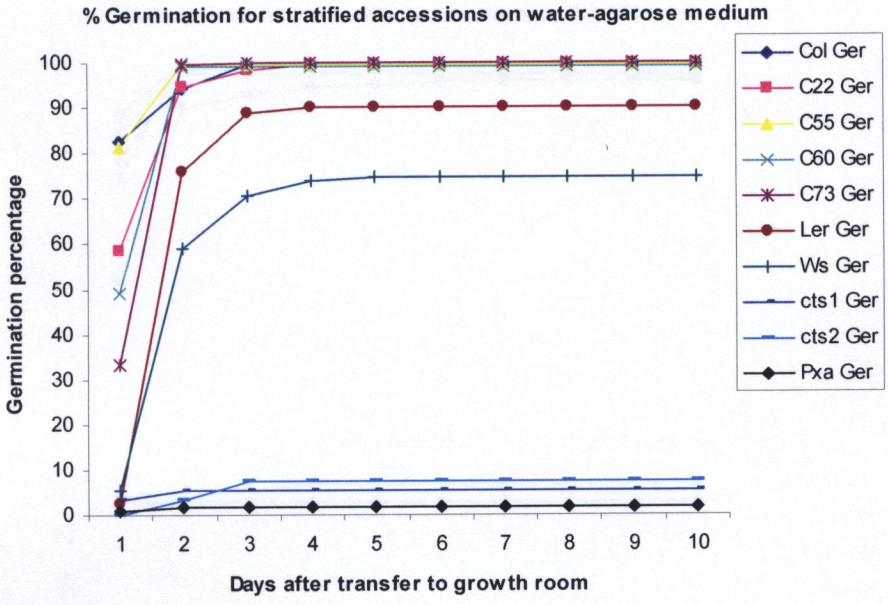
### 3.1.2 Germination and establishmet

*3.1.2.1 Stratified treatment*

Percentage germination and establishment for stratified accessions harvested almost at the same time (7 and 10 June 2005) were measured across all media (Water agarose, 0.5xMS and 0.5xMS plus 0.5%sucrose) over 10 days (Fig.3.17; 3.18; 3.19).

Comparison of germination and establishment percentage at day 7 for all the TILLING mutants and the wild type Columbia big mama across all media gave a mean germination percentage of over 95% for both the TILLING mutants and the wild type. There was a significant difference between the mutants *cts-1* and *cts-2* with there respective wild types Ler and Ws (student's *t*- test, $P< 0.001$) across all the growing medium. Though, all the three mutants, *cts-1, cts-2* and *pxa-1* were able to germinate, (mean germination value of less than 10%) on all the medium, only *pxa-1* got established on all media. *cts-1* and *cts-2* only got established on medium with 0.5xMS plus 0.5% Sucrose

Fig 3.17 (a)


% Germination for stratified accessions on water-agarose medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

(b)

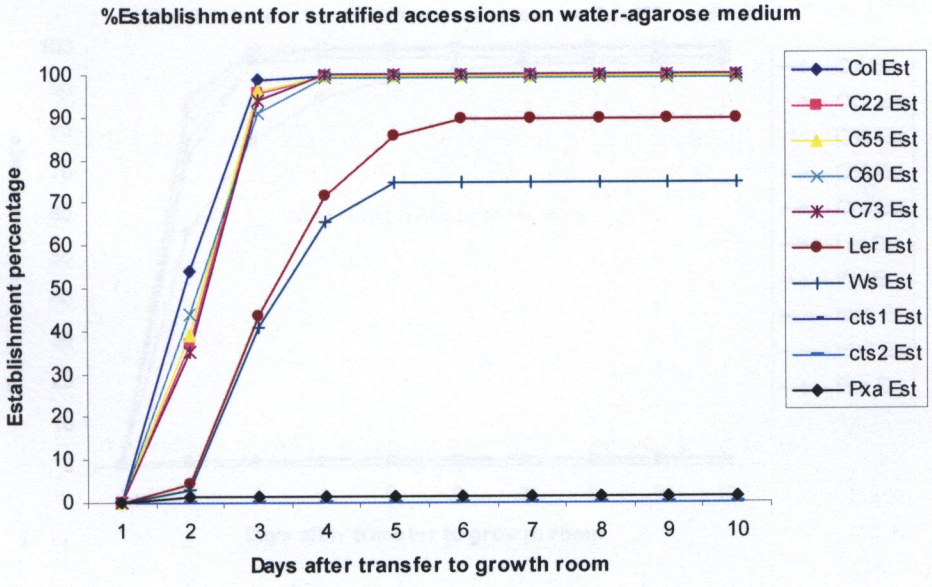
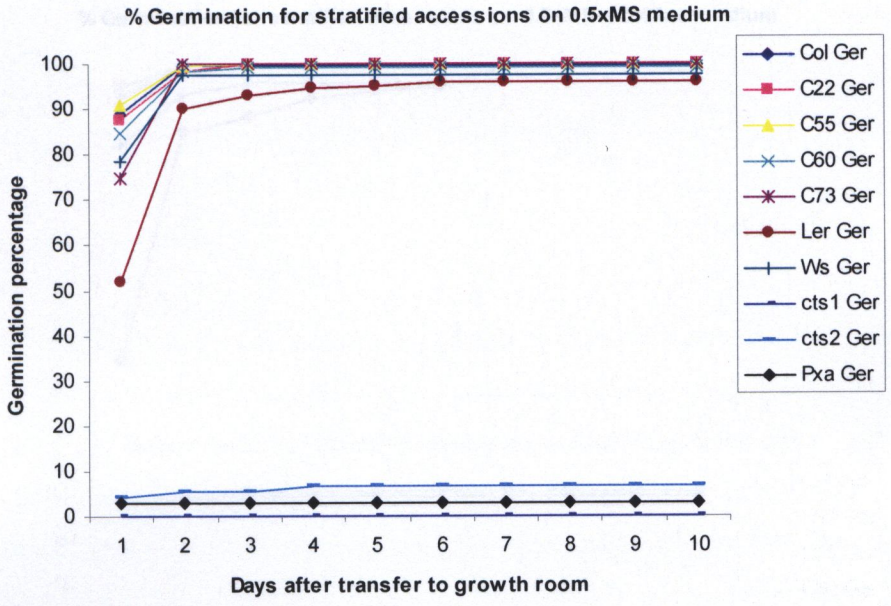

%Establishment for stratified accessions on water-agarose medium
Establishment percentage
Days after transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

Fig 3.18 (a)


% Germination for stratified accessions on 0.5xMS medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

(b)

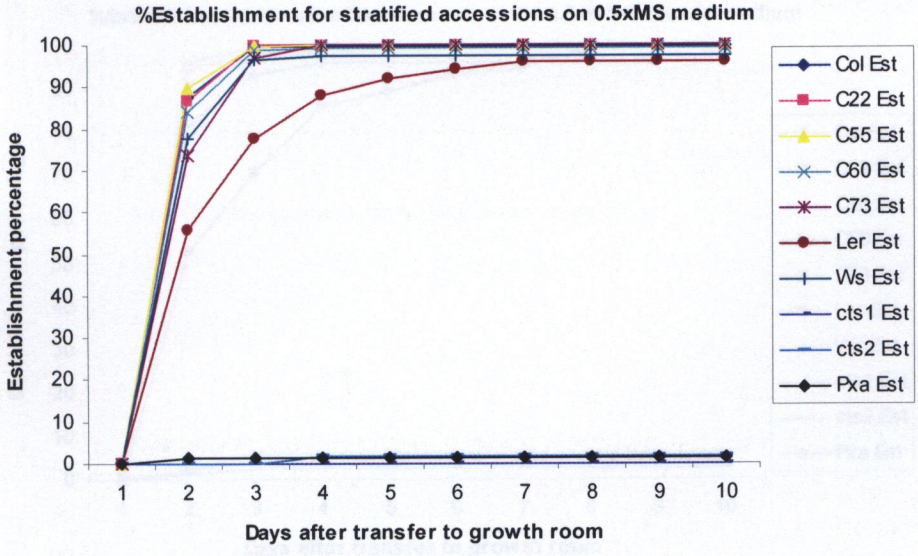
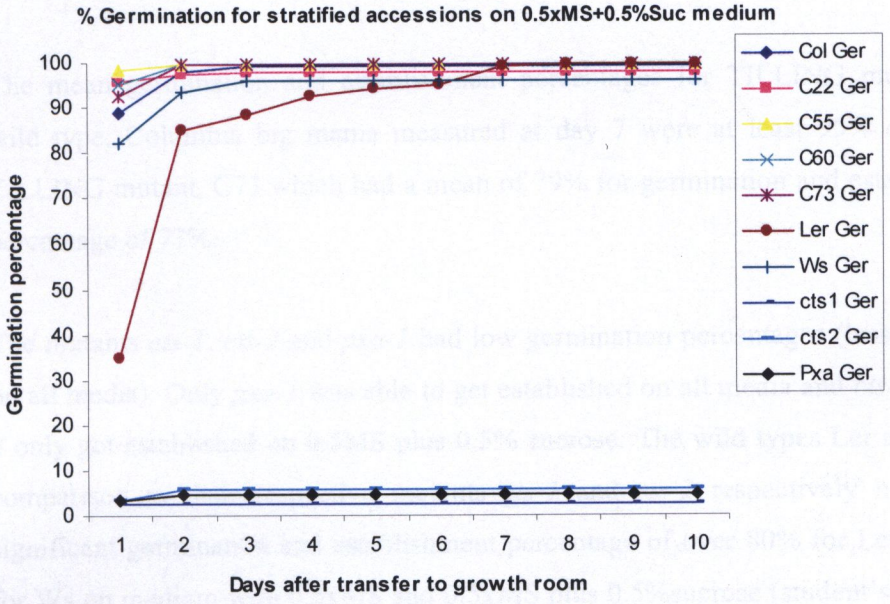
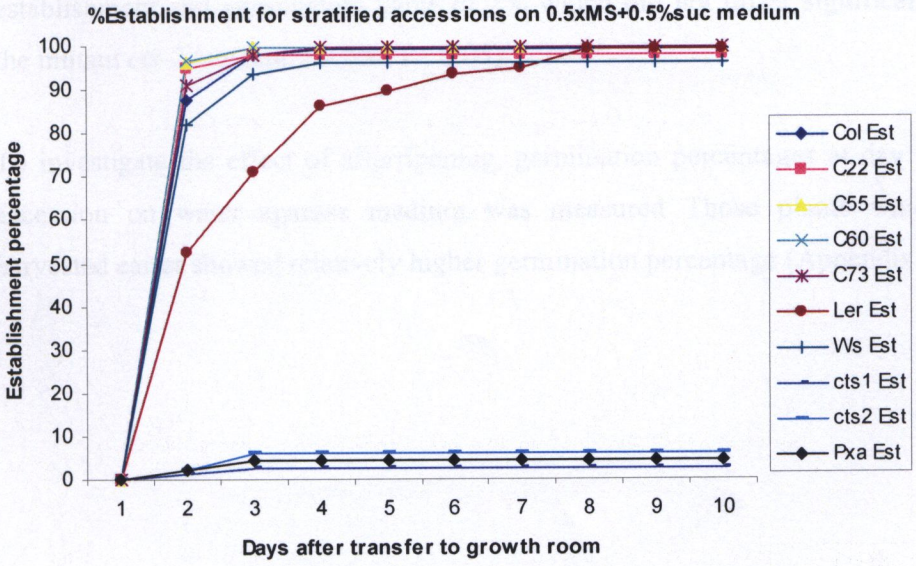

%Establishment for stratified accessions on 0.5xMS medium
Establishment percentage
Days after transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

**Fig 3.19 (a)**


% Germination for stratified accessions on 0.5xMS+0.5%Suc medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

**(b)**


%Establishment for stratified accessions on 0.5xMS+0.5%suc medium
Establishment percentage
Days after transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

*3.1.2.2 Unstratified treatment*

Germination and establishment percentages for unstratified accessions harvested almost at the same time (7and 10 June 2005) were measured across all media over 10 days (Fig. 3.20; 3.21; 3.22).

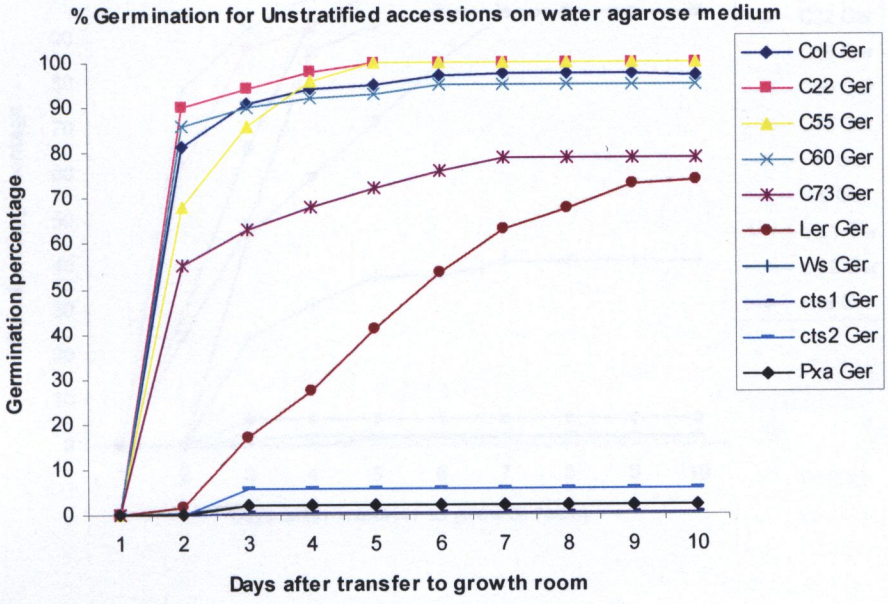
The mean germination and establishment percentages for TILLING mutants and wild type, Columbia big mama measured at day 7 were at least 95% except the TILLING mutant, C73 which had a mean of 79% for germination and establishment percentage of 77%.

The mutants *cts-1*, *cts-2* and *pxa-1* had low germination percentages (less than 10% on all media). Only *pxa-1* was able to get established on all media and *cts-1* and *cts-2* only got established on 0.5MS plus 0.5% sucrose. The wild types Ler and Ws, in comparison to their respective mutants *cts-1* and *cts-2* respectively had a high significant germination and establishment percentage of over 80% for Ler and 35% for Ws on medium with 0.5xMS and 0.5xMS plus 0.5%sucrose (student's *t*- test, $P< 0.01$) . On the medium with water agarose Ler had a significant mean germination and establishment percentage in comparison to *cts-1* of over 66% (student's *t*- test, $P< 0.01$). Ws performed poorly on medium with Water-agarose exhibiting a mean establishment and germination value of 2% which did not differ significantly with the mutant *cts-2* (student's *t*- test, $P> 0.05$).

To investigate the effect of afterripening, germination percentages at day 7 for all accession on water agarose medium was measured Those plants which were harvested earier showed relatively higher germination percentage (Appendix 6).

Fig 3.20

(a)


% Germination for Unstratified accessions on water agarose medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

(b)

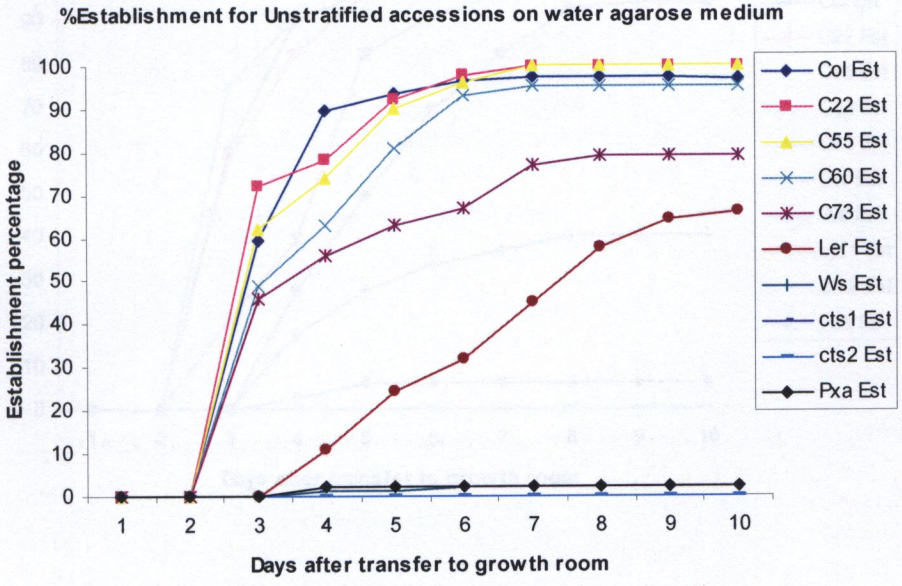
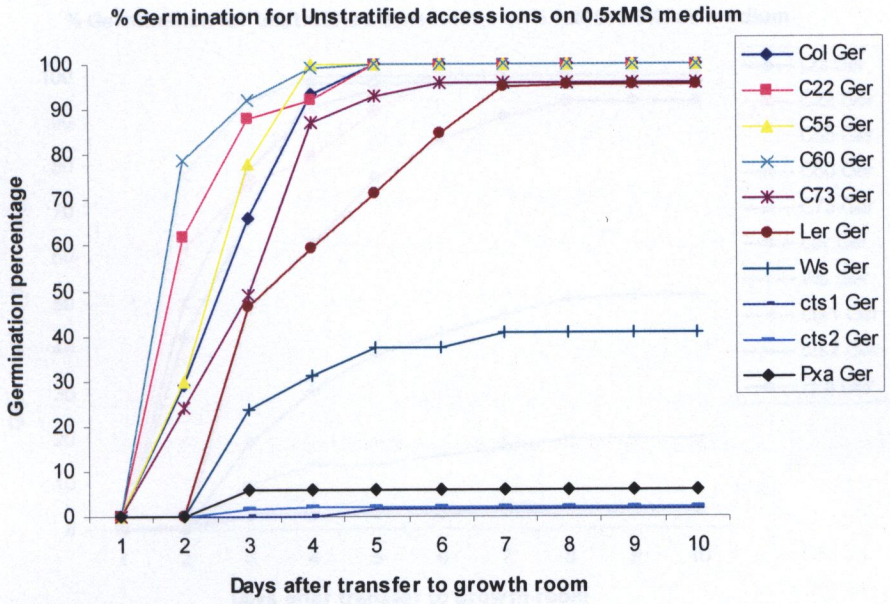

%Establishment for Unstratified accessions on water agarose medium
Establishment percentage
Days after transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

**Fig 3.21**
**(a)**


% Germination for Unstratified accessions on 0.5xMS medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

**(b)**

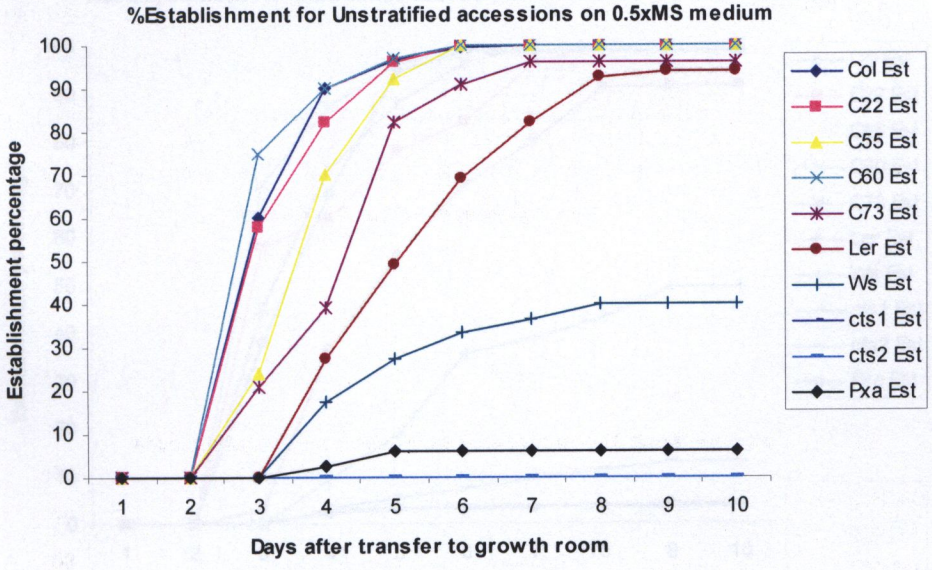
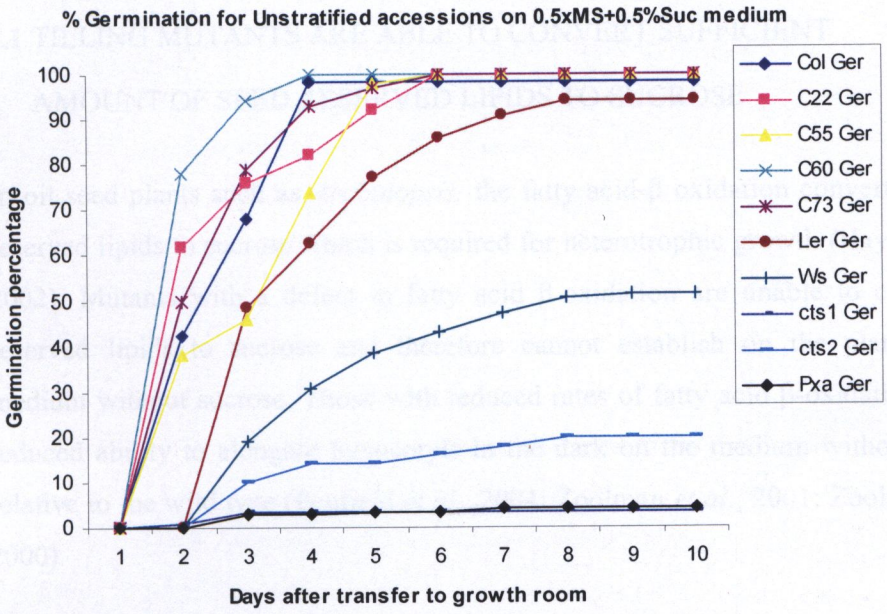
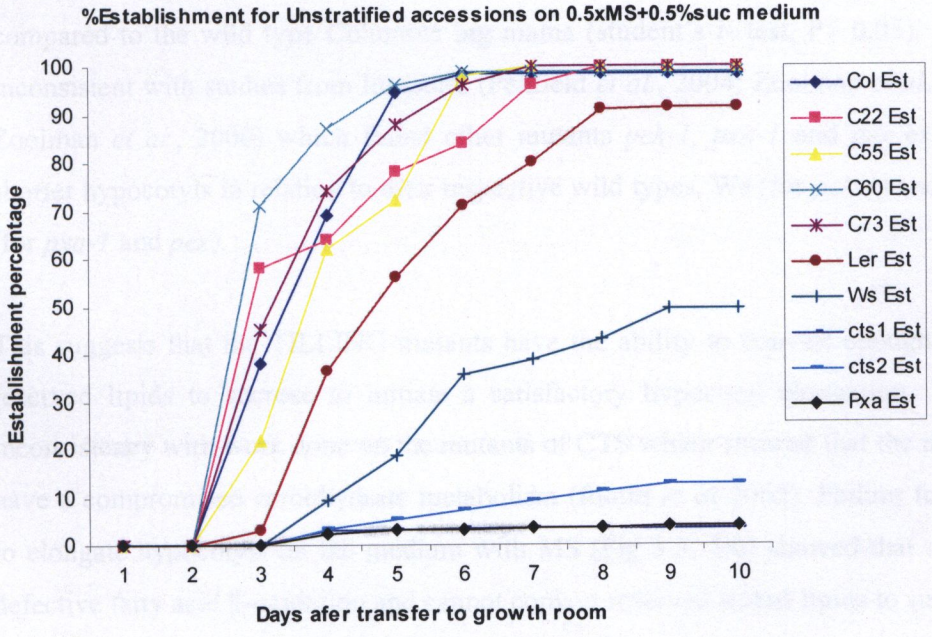

%Establishment for Unstratified accessions on 0.5xMS medium
Establishment percentage
Days after transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

**Fig 3.22**
**(a)**


% Germination for Unstratified accessions on 0.5xMS+0.5%Suc medium
Germination percentage
Days after transfer to growth room
Col Ger
C22 Ger
C55 Ger
C60 Ger
C73 Ger
Ler Ger
Ws Ger
cts1 Ger
cts2 Ger
Pxa Ger

**(b)**


%Establishment for Unstratified accessions on 0.5xMS+0.5%suc medium
Establishment percentage
Days afer transfer to growth room
Col Est
C22 Est
C55 Est
C60 Est
C73 Est
Ler Est
Ws Est
cts1 Est
cts2 Est
Pxa Est

## CHAPTER 4 DISCUSSION

### 4.1 TILLING MUTANTS ARE ABLE TO CONVERT SUFFICIENT AMOUNT OF SEED RESERVED LIPIDS TO SUCROSE

In oil seed plants such as *Arabidopsis,* the fatty acid-β oxidation converts the seed reserved lipids to sucrose which is required for heterotrophic growth (Hayashi *et al.,* 2002). Mutants with a defect in fatty acid β-oxidation are unable to convert the reserved lipids to sucrose and therefore cannot establish on the plant nutrient medium without sucrose. Those with reduced rates of fatty acid β-oxidation show a reduced ability to elongate hypocotyls in the dark on the medium without sucrose relative to the wild type (Penfield *et al.*, 2004; Zoolman *et al.*, 2001; Zoolman *et al.*, 2000).

Results obtained analysing hypocotyl elongation (Fig 3.5, 3.6) on 0.5MS medium showed that the TILLING mutants were able to elongate hypocotyls under these conditions. There was no significant difference between each TILLING mutant when compared to the wild type Columbia big mama (student's *t*- test, P> 0.05). This is inconsistent with studies from literature (Penfield *et al.*, 2004; Zoolman *et al.*, 2001; Zoolman *et al.*, 2000) which found other mutants *pck-1, pxa-1* and *pex* exhibited shorter hypocotyls in relation to their respective wild types, Ws (for *pck-1*) and Col-0 (for *pxa-1* and *pex*).

This suggests that the TILLING mutants have the ability to convert enough stored reserved lipids to sucrose to initiate a satisfactory hypocotyl elongation. This is inconsistency with work done on the mutants of CTS which showed that the mutants have a compromised carbohydrate metabolism (footitt *et al* 2002). Failure for *cts-1* to elongate hypocotyls on the medium with MS (Fig 3.5, 3.6) showed that it has a defective fatty acid β-oxidation and cannot convert reserved stored lipids to sucrose.

Hypocotyl elongation results on medium with 0.5% MS plus 0.5% sucrose (Fig 3.5, 3.7) showed no significant difference between the TILLING mutants and the wild

type (student's *t*- test, P> 0.05). The *cts-1* was however able to elongate hypocotyls length in the dark on the medium with 0.5 x MS and 0.5% sucrose. There was, however, a significant difference in hypocotyl elongation (student's *t*- test, P< 0.01) between the mutant *cts-1* and its wild type Landsberg *erecta*. This is consistent with work done by footitt *et al.*, 2002. The *cts-1* is unable to metabolise stored lipids to sucrose to provide energy for the process of germination and therefore unable to elongate hypocotyls on medium without sucrose.

## 4.2 AFTER RIPENING ENHANCES GERMINATION POTENTIAL OF TILLING MUTANTS

Afterripening has been described to enhance germination potential (Collucci *et al.*, 2002; Footitt *et al.*, 2002). This is consistent with the results obtained on the TILLING mutants (Appendix 6). All the seeds were plated and transferred to growth room on 26 July 2005 and no stratification was done. The seeds were not stratified and the nutrient free medium was used to ensure that the seeds were not given a dormancy breaking treatment.

The TILLING mutants accessions which were harvested earlier (7$^{th}$ June 2005) had the germination percentage measured at 7 days of over 90% except one accession for C73 TILLING mutant which had a germination percentage of 58%. From those which were harvested later (27 June and 12 July, 2005) 7 out of 10 had germination percentages of less than 50% while 3 out of 10 had germination percentages of greater than 50% but less than 70%. Comparison with the wild type accessions, Columbia big mama which was harvested almost at the same time (10$^{th}$ June, 2005) showed that there was no significant differences in germination percentages with the earlier harvested TILLING mutants ( Student's *t* test, P> 0.05). The TILLING mutants which were harvested later showed a highly significant difference when compared to the wild type (Student's *t* test, P< 0.001). These results confirm that after ripeninig has an impact on germination of the TILLING mutants. This is inconsistent with what was found out by Russell *et al.*, 2000 who demonstrated that CTS mutants do not afterripen.

## 4.3 TILLING MUTANTS HAVE A SLIGHT DEFECTED B-OXIDATION PATHWAY

2,4-DB is assumed to be converted to 2,4-D via the peroxisomal β-oxidation pathway (Lange and Graham, 2000) similar to convention of the seed reserved lipids to sucrose. Mutants with a defect in β-oxidation pathway are unable to covert 2,4-DB to 2,4-D. Since 2,4-D hinders root elongation, mutants with a defect or slight defect in β-oxidation pathway are expected to be more resistance to 2,4-DB (elongate longer roots) on the medium with 0.5 MS plus 0.5% sucrose and varying concentration of 2,4DB in relation to their respective wild types.

Results obtained on the medium with 0.2 µg/ml 2,4-DB (Fig. 3.9, 3.11) showed that the TILLING mutants did not show any significant root elongation in relation to the wild type (Student's *t*-test, $P > 0.05$). The mutant *cts-1* showed a highly significant result when compared to its wild type Landsberg *erecta* (Student's *t*-test, $P < 0.01$), consistent with what Footitt *et al.* (2002) found.

On the medium with 0.5xMS plus 0.5% sucrose both the wild types and there respective mutants exhibited root elongation and there differences were not significant (Student's *t*-test, $P > 0.05$). However all the accessions showed inability to elongate roots on the medium with 2,4-D. These results coincide with results obtained in previous studies (Footitt *et al.*, 2002; Hayashi *et al.*, 2002). It can therefore be deduced that the ability for *cts-1* to elongate roots was due to the β-oxidation pathway which was inhibited suggesting it has an impaired lipid storage mobilisation. Failure for TILLING mutants to exhibit significant root elongation on the medium with 0.2µg/ml 2,4 DB suggests that the TILLING mutants might have at least a functional β-oxidation pathway.

Further experiments on lower levels of 2,4-DB (0.5 x MS plus 0.5% sucrose plus 0.02µg/ml 2,4-DB (Fig. 3.12, 3.15) and 0.5 x MS plus 0.5% sucrose plus 0.05µg/ml 2,4-DB (Fig. 3.13, 3.16) proved that the TILLING mutants have a β-oxidation pathway which is slightly defective in comparison to wild type, Columbia big mama. While the TILLING mutants and *cts-1* did not show any significant difference to

their respective wild types on medium with 0.02μg/ml 2,4-DB (student's *t*- test, P> 0.05), differences were shown on medium with 0.5 x MS plus 0.5% sucrose plus 0.05μg/ml 2,4-DB.

The TILLING mutants C22, C55 C60 and C73 had mean root elongation values of 25.4mm, 22.43 mm, 26.33mm and 19.87mm respectively compared to the wild type which had a mean root elongation value of 13 mm. Of the four TILLING mutants C22 and C60 showed a significant root elongation compared to the wild type (students, *t*-test, P< 0.05). The mutant *cts-1* had a significant root elongation (Student's *t*-test, P< 0.01) with a mean root elongation of 38.4mm compared to the wild type landsberg *erecta* which had 23.91mm.

It should be pointed out, however, that a much larger sample size (n ≥10) per plant would have increased the chances of the mutants C55 and C73 being found significant in relation to the wild type and probably reduce further the significant probability levels of the other mutants. Time constraints limited the number of plants per TILLING mutant to four.

Further studies should focus on subjecting the TILLING mutants to several concentrations less than 0.2μg/ml 2,4-DB of the plant nutrient medium but greater than 0.02μg/ml 2,4-DB so that to check the trend of root elongation in different concentrations.

## 4.4 CONCLUSION

Previous studies have shown that the *CTS* locus promotes germination potential (Holdsworth *et al.*, 2001; Russell *et al.*, 2000). This entails that mutations on the *CTS* locus are suppose to reduce or inhibit germination potential. This has been confirmed by work done on CTS mutants, *cts-1* (Footitt *et al.*, 2002), *cts-2* (Footitt *et al.*, 2002), *pxa-1* (Zoolman *et al.*, 2001) and *ped3* (Hayashi et al., 2002).

Results of the CTS TILLING mutants have reviewed that these mutants as compared to the previous ones studied (Footitt *et al.*, 2002; Hayashi *et al.*, 2002 Zolman *et al.*,

2001) have a very small effect on germination potential. That is they have a slight defect on the Fatty-acid β-oxidation pathway. This has been proved by the TILLING mutants response on the medium with 0.5 x MS plus 0.5% sucrose plus 0.05μg/ml 2,4-DB.

It can be deduced that TILLING mutants causes the least severe alteration on germination potential when compared to the CTS mutants which have been studied so far (Footitt *et al.*, 2002; Hayashi *et al.*, 2002 Zolman *et al.*, 2001). Therefore the TILLING mutants occupy the least position on the CTS allelic series among the known CTS mutants (Fig 1.4).

## APPENDICES

**Appendix 1. Plants used in Phenotypic Analysis**

| Plant | Plant Identity No. |
|---|---|
| TILLING mutant N93440 | C22-6/4/2/1/1w |
| TILLING mutant N93440 | C22-6/4/2/1/10 |
| TILLING mutant N93440 | C22-6/4/2/1/14 |
| TILLING mutant N93440 | C22-6/4/2/1/16 |
| TILLING mutant N91660 | C55-1/2/5/1w |
| TILLING mutant N91660 | C55-1/2/5/1/2w |
| TILLING mutant N91660 | C55-1/2/5/12 |
| TILLING mutant N91660 | C55-1/2/5/14 |
| TILLING mutant N93169 | C60-3/1/1/5/1w |
| TILLING mutant N93169 | C60-3/1/1/5/3w |
| TILLING mutant N93169 | C60-3/1/1/5/16 |
| TILLING mutant N93169 | C60-3/1/1/5/18 |
| TILLING mutant N91953 | C73-9/1/1/3w |
| TILLING mutant N91953 | C73-9/1/1/4m |
| TILLING mutant N91953 | C73-9/1/1/12 |
| TILLING mutant N91953 | C73-9/1/1/19 |
| Columbia big Mama | Col/1 |
| Columbia big Mama | Col/2 |
| Columbia big Mama | Col/5 |
| Columbia big Mama | Col/6 |
| Landsberg *erecta* | Ler/1 |
| Landsberg *erecta* | Ler/2 |
| Landsberg *erecta* | Ler/4 |
| Landsberg *erecta* | Ler/6 |
| Wassilewskija | Ws/2 |
| Wassilewskija | Ws/3 |
| Wassilewskija | Ws/5 |
| Wassilewskija | Ws/8 |
| *pxa-1* | *pxa*-1/1 |
| *pxa-1* | *pxa*-1/3 |
| *pxa-1* | *pxa*-1/4 |
| *pxa-1* | *pxa*-1/6 |
| *cts-1* | *cts* 1/2 |
| *cts-1* | *cts*-1/4 |
| *cts-1* | *cts*-1/5 |
| *cts-1* | *cts*-1/6 |
| *cts-2* | *cts*- 2/1 |
| *cts-2* | *cts*-2/2 |
| *cts-2* | *cts*-2/3 |
| *cts-2* | *cts*-2/4 |

Appendix 2. $F_2$ results for C22 plants

| No. | TILLING mutant | Identity plant No. | Result |
|---|---|---|---|
| **1** | **N93440** | **C22-6/4/2/1/1wt** | **Homozygous for mutant allele** |
| 2 | N93440 | C22-6/4/2/1/2wt | Heterozygous for mutant allele |
| 3 | N93440 | C22-6/4/2/1/3wt | Homozygous for wild type allele |
| **4** | **N93440** | **C22-6/4/2/1/4wt** | **Homozygous for mutant allele** |
| 5 | N93440 | C22-6/4/2/1/5wt | Heterozygous for mutant allele |
| 6 | N93440 | C22-6/4/2/1/6wt | Homozygous for wild type allele |
| 7 | N93440 | C22-6/4/2/1/1m | Heterozygous for mutant allele |
| 8 | N93440 | C22-6/4/2/1/2m | Heterozygous for mutant allele |
| 9 | N93440 | C22-6/4/2/1/4m | Heterozygous for mutant allele |
| 10 | N93440 | C22-6/4/2/1/5m | Heterozygous for mutant allele |
| 11 | N93440 | C22-6/4/2/1/6m | Heterozygous for mutant allele |
| **12** | **N93440** | **C22-6/4/2/1/10** | **Homozygous for mutant allele** |
| 13 | N93440 | C22-6/4/2/1/11 | Homozygous for wild type allele |
| 14 | N93440 | C22-6/4/2/1/12 | Heterozygous for mutant allele |
| 15 | N93440 | C22-6/4/2/1/13 | Heterozygous for mutant allele |
| **16** | **N93440** | **C22-6/4/2/1/14** | **Homozygous for mutant allele** |
| 17 | N93440 | C22-6/4/2/1/15 | Heterozygous for mutant allele |
| **18** | **N93440** | **C22-6/4/2/1/16** | **Homozygous for mutant allele** |
| 19 | N93440 | C22-6/4/2/1/18 | Heterozygous for mutant allele |
| 20 | N93440 | C22-6/4/2/1/19 | Heterozygous for mutant allele |
| 21 | N93440 | C22-6/4/2/1/20 | Homozygous for wild type allele |
| 22 | N93440 | C22-6/4/2/1/21 | Heterozygous for mutant allele |

Appendix 3. $F_2$ results for C55 plants

| No. | TILLING Mutant | DNA Identity plant No. | Result |
|---|---|---|---|
| 1 | N91660 | C55-1/2/5/1w | **Homozygous for mutant allele** |
| 2 | N91660 | C55-1/2/5/2w | **Homozygous for mutant allele** |
| **3** | N91660 | C55-1/2/5/3w | Homozygous for wild type allele |
| 4 | N91660 | C55-1/2/5/4w | Homozygous for wild type allele |
| 5 | N91660 | C55-1/2/5/5w | Homozygous for wild type allele |
| 6 | N91660 | C55-1/2/5/6w | **Homozygous for mutant allele** |
| 7 | N91660 | C55-1/2/5/2m | Homozygous for wild type allele |
| 8 | N91660 | C55-1/2/5/5m | Heterozygous for mutant allele |
| 9 | N91660 | C55-1/2/5/6m | Heterozygous for mutant allele |
| **10** | N91660 | C55-1/2/5/10w | Heterozygous for mutant allele |
| 11 | N91660 | C55/1/2/5/11 | Homozygous for wild type allele |
| 12 | N91660 | C55-1/2/5/12 | **Homozygous for mutant allele** |
| 13 | N91660 | C55-1/2/5/13 | Heterozygous for mutant allele |
| 14 | N91660 | C55-1/2/5/14 | **Homozygous for mutant allele** |
| **15** | N91660 | C55-1/2/5/15 | Heterozygous for mutant allele |
| 16 | N91660 | C55-1/2/5/16 | Heterozygous for mutant allele |
| 17 | N91660 | C55-1/2/5/17 | Heterozygous for mutant allele |
| 18 | N91660 | C55-1/2/5/18 | Heterozygous for mutant allele |
| 19 | N91660 | C55-1/2/5/19 | Heterozygous for mutant allele |
| 20 | N91660 | C55-1/2/5/20 | Heterozygous for mutant allele |
| 21 | N91660 | C55-1/2/5/21 | Heterozygous for mutant allele |
| **22** | N91660 | C55-1/2/5/22 | Heterozygous for mutant allele |

Appendix 4. $F_2$ results for C73 plants

| No. | TILLING mutant | Identity plant No. | Result |
|---|---|---|---|
| 1 | N91953 | C73-9/1/1/1wt | Heterozygous for mutant allele |
| 2 | N91953 | C73-9/1/1/2wt | Homozygous for wild type allele |
| **3** | **N91953** | **C73-9/1/1/3wt** | **Homozygous for mutant allele** |
| 4 | N91953 | C73-9/1/1/4wt | Heterozygous for mutant allele |
| 5 | N91953 | C73-9/1/1/5wt | Heterozygous for mutant allele |
| 6 | N91953 | C73-9/1/1/6wt | Homozygous for wild type allele |
| 7 | N91953 | C73-/9/1/1/1m | Heterozygous for mutant allele |
| 8 | N91953 | C73-9/1/1/2m | Heterozygous for mutant allele |
| 9 | N91953 | C73-9/1/1/3m | Heterozygous for mutant allele |
| **10** | **N91953** | **C73-9/1/1/4m** | **Homozygous for mutant allele** |
| 11 | N91953 | C73-9/1/1/5m | Heterozygous for mutant allele |
| 12 | N91953 | C73-9/1/1/10 | Heterozygous for mutant allele |
| 13 | N91953 | C73-9/1/1/11 | Heterozygous for mutant allele |
| **14** | **N91953** | **C73-9/1/1/12** | **Homozygous for mutant allele** |
| 15 | N91953 | C73-9/1/1/13 | Heterozygous for mutant allele |
| 16 | N91953 | C73-9/1/1/14 | Heterozygous for mutant allele |
| 17 | N91953 | C73-9/1/1/15 | Heterozygous for mutant allele |
| 18 | N91953 | C73-9/1/1/16 | Heterozygous for mutant allele |
| 19 | N91953 | C73-9/1/1/17 | Heterozygous for mutant allele |
| **20** | **N91953** | **C73-9/1/1/19** | **Homozygous for mutant allele** |
| 21 | N91953 | C73-9/1/1/20 | Heterozygous for mutant allele |
| 22 | N91953 | C73-9/1/1/21 | Heterozygous for mutant allele |

Appendix 5. $F_2$ results for C60 plants

| No. | TILLING Mutant | Identity plant No. | Result |
|---|---|---|---|
| **1** | N93169 | C60-3/1/1/5/1w | **Homozygous for mutant allele** |
| 2 | N93169 | C60-3/1/1/5/2w | Heterozygous for mutant allele |
| 3 | N93169 | C60-3/1/1/5/3w | **Homozygous for mutant allele** |
| **4** | N93169 | C60-3/1/1/5/4w | Heterozygous for mutant allele |
| 5 | N93169 | C60-3/1/1/5/5w | Heterozygous for mutant allele |
| 6 | N93169 | C60-3/1/1/5/6w | Heterozygous for mutant allele |
| 7 | N93169 | C60-3/1/1/5/1m | Homozygous for wild type allele |
| 8 | N93169 | C60-3/1/1/5/2m | **Homozygous for mutant allele** |
| 9 | N93169 | C60-3/1/1/5/3m | Heterozygous for mutant allele |
| 10 | N93169 | C60-3/1/1/5/5m | Heterozygous for mutant allele |
| 11 | N93169 | C60-3/1/1/5/8m | Homozygous for wild type allele |
| **12** | N93169 | C60-3/1/1/5/10 | Homozygous for wild type allele |
| 13 | N93169 | C60-3/1/1/5/11 | Heterozygous for mutant allele |
| 14 | N93169 | C60-3/1/1/5/12 | Heterozygous for mutant allele |
| 15 | N93169 | C60-3/1/1/5/13 | Heterozygous for mutant allele |
| **16** | N93169 | C60-3/1/1/5/14 | Heterozygous for mutant allele |
| 17 | N93169 | C60-3/1/1/5/15 | Homozygous for wild type allele |
| **18** | N93169 | C60-3/1/1/5/16 | **Homozygous for mutant allele** |
| 19 | N93169 | C60-3/1/1/5/17 | Homozygous for wild type allele |
| 20 | N93169 | C60-3/1/1/5/18 | **Homozygous for mutant allele** |
| 21 | N93169 | C60-3/1/1/5/19 | Heterozygous for mutant allele |
| 22 | N93169 | C60-3/1/1/5/20 | **Homozygous for mutant allele** |
| 23 | N93169 | C60-3/1/1/5/21 | Heterozygous for mutant allele |

**Appendix 6. Germination percentage for unstratified accessions used in phenotypic analysis on water-agarose medium**

| Plant | Plant Identity | Date harvested | %Germ.- 7 days |
|---|---|---|---|
| TILLING mutant N93440 | C22-6/4/2/1/1w | 7 June 05 | 100 |
| TILLING mutant N93440 | C22-6/4/2/1/10 | 12 July 05 | 64 |
| TILLING mutant N93440 | C22-6/4/2/1/14 | 12 July 05 | 30 |
| TILLING mutant N93440 | C22-6/4/2/1/16 | 12 July 05 | 38 |
| TILLING mutant N91660 | C55-1/2/5/1w | 7 June 05 | 100 |
| TILLING mutant N91660 | C55-1/2/5/2w | 27 June 05 | 68 |
| TILLING mutant N91660 | C55-1/2/5/12 | 12 July 05 | 10 |
| TILLING mutant N91660 | C55-1/2/5/14 | 12 July 05 | 18 |
| TILLING mutant N93169 | C60-3/1/1/5/1w | 7 June 05 | 96 |
| TILLING mutant N93169 | C60-3/1/1/5/3w | 7 June 05 | 94 |
| TILLING mutant N93169 | C60-3/1/1/5/16 | 12 July 05 | 24 |
| TILLING mutant N93169 | C60-3/1/1/5/18 | 12 July 05 | 44 |
| TILLING mutant N91953 | C73-9/1/1/3w | 7 June 05 | 100 |
| TILLING mutant N91953 | C73-9/1/1/4m | 7 June 05 | 58 |
| TILLING mutant N91953 | C73-9/1/1/12 | 12 July 05 | 22 |
| TILLING mutant N91953 | C73-9/1/1/19 | 12 July 05 | 54 |
| Columbia big Mama | Col/1 | 10 June 05 | 96 |
| Columbia big Mama | Col/2 | 10 June 05 | 100 |
| Columbia big Mama | Col/5 | 10 June 05 | 98 |
| Columbia big Mama | Col/6 | 10 June 05 | 96 |
| Landsberg *erecta* | Ler/1 | 10 June 05 | 30 |
| Landsberg *erecta* | Ler/2 | 10 June 05 | 88 |
| Landsberg *erecta* | Ler/4 | 10 June 05 | 70 |
| Landsberg *erecta* | Ler/6 | 10 June 05 | 64 |
| Wassilewskija | Ws/2 | 10 June 05 | 2 |
| Wassilewskija | Ws/3 | 10 June 05 | 0 |
| Wassilewskija | Ws/5 | 10 June 05 | 4 |
| Wassilewskija | Ws/8 | 10 June 05 | 2 |
| *pxa-1* | *pxa*-1/1 | 10 June 05 | 2 |
| *pxa-1* | *pxa*-1/3 | 10 June 05 | 0 |
| *pxa-1* | *pxa*-1/4 | 10 June 05 | 2 |
| *pxa-1* | *pxa*-1/6 | 10 June 05 | 4 |
| *cts-1* | *cts* 1/2 | 10 June 05 | 0 |
| *cts-1* | *cts*-1/4 | 10 June 05 | 0 |
| *cts-1* | *cts*-1/5 | 10 June 05 | 0 |
| *cts-1* | *cts*-1/6 | 10 June 05 | 2 |
| *cts-2* | *cts*- 2/1 | 10 June 05 | 6 |
| *cts-2* | *cts*-2/2 | 10 June 05 | 6 |
| *cts-2* | *cts*-2/3 | 10 June 05 | 8 |
| *cts-2* | *cts*-2/4 | 10 June 05 | 4 |

## REFERENCES


ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, W. E. and LIPMAN, J.D. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, 215: 403- 410.

BAUMLEIN, H., MISERA, S., LUESSEN, H., KOLLE, K., HORSTMANN, C., WOBUS, U. and MULLER, A.J. (1994) The *Fus3* gene of *Arabidopsis thaliana* is a regulator gene expression during late embryogenesis. *The Plant Journal*, 6: 379-387.

BEAUDOIN, N., SERIZET, C., GOSTI, F. and GIRAUDAT, J. (2000) Interactions between abscisic acid and ethylene signaling cascades. *Plant Cell*, 12: 1103-1115.

BENT, F.A. (2000) *Arabidopsis* in planta transformation. Uses, mechanisms, and prospects for transformation of other species. *Plant physiology,* 24: 1540-1547.

BEWLEY, J.D. (1997) Seed germination and dormancy. *Plant Cell,* 9: 1055-1066.

CLARKE, B., LAMBRECHT, M., and RHEE, Y.S. (2003) Arabidopsis genomic information for interpreting wheat EST sequences. *Funct. Integr. Genomics*, 3: 33-38

COLUCCI, G., APONE, F., ALYESHMERNI, N., CHALMERS, D and CHRISPEELS, M.J (2002) GCR1, the putative *Arabidopsis* G protein-coupled receptor gene is cell cycle-regulated, and its overexpression abolishes seed dormancy and shortens time to flowering. *PNAS*, 99: 4736-4741.

CORNAH, E.J., GERMAIN, V., WARD, L.J., BEALE, H.M. and SMITH, M.S. (2004) Lipid utilization , gluconeogenesis, and seedling growth in *Arabidopsis* mutants lacking the glyoxylate cycle enzyme malate synthatase. *Journal of Biological Chemistry* 279: 42916-42923

DENNIS, C and SURRIDGE, C (2000) *A. thaliana* genome. *Nature*, 408: 791.

ECKARDT, A.N. (2001) Everything in its place: Conservation of gene order among distantly related plant species. *The Plant Cell*, 13: 723-725.

EDWARDS, K., JOHNSTONE, C., and THOMPSON, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic acid research*, 19: 1349

FOOTITT, S., SLOCOMBE, P.S., LARNER, V., KURUP, S., WU, Y., LARSON, T., GRAHAM, I., BAKER, A. and HOLDSWORTH, M. (2002) Control of germination and lipid mobilization by Comatose, the Arabidopsis homologue of human ALDP. *The EMBO Journal*, 21, 2912-2922.

FULDA, M., SCHNURR, J., ABBADI, A., HEINZ, E. and BROWSE, J. (2004) Peroxisomal Acyl-CoA synthetase activity is essential for seedling development in *Arabidopsis thaliana. The Plant Cell*, 16: 394-405.

GIRAUDAT, J., HAUGE, B.M., VALON, C., SMALLE, J., PARCY,F. and GOODMAN, H.M. (1992) Isolation of the *Arabidopsis* Abi3 gene by positional cloning. *Plant Cell,* 4: 1251-1261.

GRANT, D., CREGAN, P. and SHOEMAKER, C.R. (2000) Genome organization in dicots: Genome duplication in *Arabidopsis* and synteny between soybean and *Arabidopsis. Proc. Natl. Acad. Sci,* 97: 4168-4173

HAYASHI, M., NITO, K., TAKEI, R., YAGI, M., KONDO, M., SUENAGA, A., YAMAYA, T and NISHIMURA, M. (2002) Ped3p is a peroxisomal ATP-binding cassette transporter that might supply substrates for fatty acid β-oxidation. *Plant Cell Physiol.*, 43: 1-11.

HAYASHI, M., TORIYAMA, K., KONDO, M. and NISHIMURA, M. (1998) 2,4-Dichlorophenoxybutyric Acid-Resistant Mutants of Arabidopsis Have Defects in Glyoxysomal Fatty Acid β-Oxidation. *The Plant Cell*, 10: 183-195.

HOLDSWORTH, M., KURUP, S., and MCKIBBIN, R. (1999) Molecular and genetic mechanisms regulating the transition from embryo development to germination. *Trends in Plant Science,* 4: 275-280.

HOLDSWORTH, M., LENTON, J., FLINTHAM, J., GALE, M., KURUP, S. MCKIBBIN, R., BAILEY, P., LARNER, V. and RUSSELL, L. (2001) Genetic control mechanism regulating the intiation of germination. *Journal of Plant Physiology*, 158, 439-445.

HOLDSWORTH, M., LENTON, J., FOOTITT, S., and WILKINSON, M. (2002) *Annual Report 2001-2002.* Crop Performance. Understanding the genetics of germination. IACR

KATO, K., KIDOU, S., MIURA, H., and SWADA, S. (2002) Molecular cloning of the wheat CK2alpha gene and detection of its linkage with Vrn-A1 on chromosome 5A. *Theor. Appl. Genet*, 104: 1071- 1077.

KEITH, K., KRAML, M., DENGLER, N.G. and McCOURT, P. (1994) *fusca3*: a heterochronic mutation affecting late embryo development in *Arabidopsis*. *Plant Cell,* 6, 589-600.

KONIECZNY, A. and AUSUBEL, F.M. (1993) A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific PCR-based markers. *The Plant Journal,* 4: 403-410.

KOORNNEEF, M. (1994) *Arabidopsis* Genetics. In: *Arabidopsis* (M,E. Meyerowitz and R.C. Somerville, eds), pp. 89-120. Cold Spring Harbor Laboratory Press, New York.

KOORNNEEF, M. and KARSSEN, C.M. (1994) Seed dormancy and germination. In: *Arabidopsis* (M,E. Meyerowitz and R.C. Somerville, eds), pp. 313-334. Cold Spring Harbor Laboratory Press, New York.

KU, H., LIU, J., DOGANLAR, S., and TANKSLEY, D.S. (2001) Exploitation of *Arabidopsis*-Tomato synteny to construct a high resolution map of the ovate-containing region in tomato chromosome 2. *Genome*, 44: 470-475.

LANGE, P.R. AND GRAHAM, I (2000) Arabidopsis thaliana mutants disrupted in lipid mobilisation. *Biochemical Society Transactions*, 28: 762-765.

LOTAN, T., OHTO, M., YEE, K.M., WEST, M.A.L., Lo, R., KWONG, R.W., YAMAGISHI, K., FISCHER, R.L., GOLDBERG, R.B. and HARADA, J.J. (1998) Arabidopsis LEAFY COTYLEDONI is sufficient to induce embryo development in vegetative cells. *Cell*, 93: 1195-1205.

McCALLUM, M.C., COMAI, L., GREENE, A.E. and HENIKOFF, S. (2000) Targeting Induced Local Lesions IN Genomes (TILLING) for plant functional genomics. *Plant Physiology*, 123: 439- 442.

MEINKE, W. D., CHERRY, M. J., DEAN, C., ROUNSELEY, D.S. and KOORNNEEF, M. (1998) Arabidopsis thaliana: A model plant for genome analysis. *Science*, 662: 679-681.

PENFIELD, S., RYLOTT, L.E., GILDAY, D.A., GRAHAM, S.G., LARSON R.T. and GRAHAM, A.I. (2004) Reserve mobilization in the *Arabidopsis* endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires phosphoenolpyruvate carboxykinase1. *The Plant Cell*, 16: 2705-2718.

PENG, J.R., CAROL, P., RICHARDS, D.E., KING, K.E., COWLING, R.J., MURPHY, G.P. and HARBERD, N.P. (1997) The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes and Development*, 11: 3194-3205.

PERAZZA, D., HERZOG, M., HULSKAMP, M., BROWN, S., DORNE, A. M. and BONNEVILLE, J. M. (1999) Trichome cell growth in *Arabidopsis thaliana* can be derepressed by mutations in at least five genes. Genetics, 152: 461-476

PRICE, R.A. PALMER, J.D. and AL-SHEHBAZ, I.A. (1994) Systematic relationships of *Arabidopsis*: A molecular and morphological perspective. In *Arabidopsis* (E.M. Meyerowitz, and C.R. Somerville eds), pp. 7-19. . Cold Spring Harbor Laboratory Press, New York.

REDEI, G.P. (1992) A heuristic glance at the past of *Arabidopsis* genetics. In: *Methods in Arabidopsis research*, (C. Koncz, N.H. Chua and J. Schell, eds), pp. 1-15. World Scientific Singapore.

RENSINK, A.W., and BUELL, R.( 2004) *Arabidopsis* to Rice. Applying Knowledge from a Weed to Enhance Our Understanding of a crop Species. *Plant Physiology*, 135: 622- 629.

RUSSELL, L., LARNER, V., KURUP, S., BOUGOURD, S. and HOLDWORTH, M. (2000) The *Arabidopsis* COMATOSE locus regulates germination potential. *Development*, 127: 3759-3767.

SCHOLL, L.R., MAY, T.S. and WARE H.D. (2000) Seed and molecular resources for *Arabidopsis*. *Plant Physiology*, 124: 1477-1480.

SILVERSTONE, A.L., CIAMPAGLIO, C.N. and SUN, T.P. (1998) The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant cell,* 10: 155-169.

SLATER, A., SCOTT, N. and FOWLER, M (2003) *Plant Biotechnology*. Oxford University Press.

STEBER, C.M., COONEY, S.E. and McCOURT, P (1998) Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1-1* in *Arabidopsis thaliana.* Genetics, 149: 509-521.

THE *ARABIDOPSIS* GENOME INTIATIVE. (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, 408: 796-815.

TILL, J.B., REYNOLDS, H.S., GREENE, A. E., CODOMO, C.E., JOHNSON, E.J., BURTNER, C., ODDEN, R.A., YOUNG, K., TAYLOR, E.N., HENIKOFF, G.J., COMAI, L. and HENIKOFF, S. ( 2003) Large-Scale Discovery of Induced Point Mutations With High- Throughput TILLING. *Genome Research*, 13, 524-530.

YAN, L., LOUKOIANOV, A., TRANQUILLI, G., HELGUERA, M., FAHIMA, T. and DUBCOVSKY, J. (2003) Positional cloning of the wheat vernalisation gene VRN1. *PNAS*, 100: 6263- 6268

WAIN, R.L. and WIGHTMAN, F. (1954) The growth-regulating activitiy of cartain *w*-substituted alkyl carboxylic acids in relation to their β-oxidation within the plant. *Proc. R. Soc. Lond. Biol.Sci.*, 142: 525-536.

ZHANG, Z. J., CREELMAN, A.R. and ZHU, J. (2004) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiology*, 135: 615- 621.

ZOLMAN, K.B., SILVA, D.I. and BARTEL, B. (2001) The *Arabidopsis pxa1* mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid β-oxidation. *Plant Physiology*, 127: 1266-1278.

ZOLMAN, K.B., YODER, A. and BARTEL, B. (2000) Genetic analysis of Indole-3-butric acid responses in *Arabidopsis thaliana* reveals four mutant classes. *Genetics*, 156: 1323-1337