Development of frost tolerance in *Brassica napus* using *Arabidopsis ACYL-COENZYME A-BINDING PROTEIN6*

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Abstract

Canola crops in Australia are greatly affected by frost stress, especially at the grain-filling stage, which results in poor seed fill and poor oil quality. Frost tolerance was developed for rapid-cycling *Brassica napus* and *B. napus* 'Westar', using *Agrobacterium*-mediated transformation with *A. thaliana ACYL-COENZYME A-BINDING PROTEIN6* (*ACBP*6). Using four-day-old cotyledon explants, *in vitro* regeneration of rapid-cycling *B. napus* and *B. juncea* was established with an optimum concentration of 5 µM of BAP and 2 µM of NAA. *B. napus* 'Westar' and rapid-cycling *B. napus* were successfully transformed with *ACBP6* through *Agrobacterium*-mediated transformation; with transformation efficiency of 6.1% for rapid-cycling *B. napus* and 4.9% for Westar. The fast development *in vitro* of rapid-cycling *B. napus* accelerated the recovery of transgenic plants within 6-7 weeks, compared to the long duration *B. napus* 'Westar' of at least 10 weeks. The integration of *ACBP6* transgene and *NPTII* marker gene was confirmed in 10 independent transgenic plants (T_0) of rapid-cycling *B. napus* and 'Westar' through PCR and RT-PCR. Western blot analysis confirmed expression of ACBP6 in the leaves. Non-acclimated ACBP6-overexpressing plants were tested for frost tolerance at -4°C (actual mean temperature was -5.2°C ± 0.79). Freezing tolerance in ACBP6-overexpressing rapid-cycling *B. napus* T_3 and *B. napus* 'Westar' T_2 plants was confirmed based on lower electrolyte leakage (at least 37%), minimal reduction of PSII efficiency [Y(II)] and higher CO_2 assimilation rate (*A*). Boundary layer modelling of Y(II), *A*, stomatal conductance (g_{sw}) and intercellular CO_2 concentration (C_i) after freezing showed ACBP6-overexpressing plants performed similarly with non-stressed plants. The tolerance to freezing injury was also observed in the developing seed embryos, based on image analysis of tissue stained with fluorescein diacetate (FDA) viability stain.

Declaration

I certify that this thesis has been composed solely by myself and that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. This thesis contains less than 80,000 words in length, exclusive of table, figures, bibliographies and appendices.

Eden Jane U. Tongson

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And lastly to my family, thank you for understanding.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A - net photosynthetic rate or CO_2 assimilation rate (μmol CO_2 $m^{-2}S^{-1}$)

ACBP6 - acyl-Coenzyme A-binding protein 6

BNFP – *Brassica napus* fast plants or rapid-cycling *B. napus*

BJFP – *Brassica juncea* fast plants or rapid-cycling *B. juncea*

BNW – *Brassica napus* 'Westar'

Ci - intercellular CO_2 concentration (μmol CO_2 mol^{-1})

CTAB - cetyltrimethylammonium bromide

DNA - deoxyribose nucleic acid

E - transpiration rate (mmol H_2O $m^{-2}s^{-1}$)

EDTA – ethylenediaminetetraacetate

EL - electrolyte leakage

ETR - electron transport rate

g_{sw} - stomatal conductance (mol H_2O $m^{-2}s^{-1}$)

NPTII - neomycin phosphotransferase II

RNA - ribonucleic acid

SDS - sodium dodecyl sulphate

TAE - Tris-acetate EDTA (buffer)

Y(II) - effective photochemical quantum efficiency PSII or PSII operating efficiency

Chapter 1

General Introduction

Canola is an oilseed crop grown for its oil product, which is claimed to be the healthiest edible oil in the market today, being a good source of omega-3 and low in saturated fat (Lin et al., 2013; Canola Council of Canada, 2016). Canola was originally bred from oilseed rape (*Brassica napus* L.) to produce oil suitable for human consumption. As a by-product of oil extraction, the canola meal is also highly valued as a high-protein feed for livestock, poultry and fish farming (Gunstone, 2009). The term canola was coined from "Canadian oil, low acid", first defined in Canada in 1978 and refers to cultivars of oilseed rape (*B. napus*.), mustard (*B. rapa*) and *B. juncea* that are 'canola quality' producing oil with less than 2% erucic acid and less than 30 μmoles glucosinolates in the meal (Nelson and Grombacher, 1992). Oil from non-canola quality and inedible rapeseed remained in production for use in industrial applications. Total world production of rapeseed and canola reached to almost 80 million metric tons as of 2013-2014 (FAO, 2016).

Canola has emerged as one of the most important crops in Australia. Rapid expansion of canola production started in the early 1990s as the world demand for canola oil increased. Australia is ranked 6th in the world as a major producer of canola and rapeseed, valued at 4.1 million metric tons (FAO, 2016). The majority of canola in Australia is produced in Western Australia, with more than 50% share of the total production followed by New South Wales, Victoria and Western Australia (AOF, 2015). Canola has an important contribution in the winter crop rotation in the grain-growing

regions of Australia where it provides a break to diseases and weeds in the field following grain and pulses crops (Burton et al., 2008).

Frost has been identified as a major agronomic risk to canola production in Australia. Frost causes complete abortion of open flowers of canola plants while developing seeds die and shrivel resulting in incomplete filled pods (Walton et al., 1999; Jason-Flanagan et al., 1991). Yield and oil quality can be greatly affected when frost affects the pod-filling stage wherein seeds contain approximately 60% moisture. This results in poor seed fill and poor oil quality (Walton et al., 1999). GRDC (2015) reported substantial loss in crop production in major frost events. Damage to crops in New South Wales including cereals, oilseeds and pulses, was estimated to reach $100 M. In South Australia and Victoria, the cost of crop loss due to frost was estimated at an average of more than $33M a year. In 2007, the National *Brassica* Germplasm Improvement Program (NBGIP) identified frost tolerance in seed development stage as one of the key traits for germplasm development. To date, mitigating measures to reduce frost damage has only been through risk management as there is no commercial frost tolerant variety of canola.

The use of biotechnology has become indispensable as a tool for crop improvement to keep up with the increasing demand for food and agriculture in a changing environment. Genetic engineering is a modern approach that employs direct transfer of one or more genes from one organism to another, allowing the introduction of a trait that is absent to the recipient's germplasm (Davies & Reznikoff, 1992). This technique resolves the limits of conventional breeding methods to improve crops such as in dealing with traits that are very hard to improve or takes too long to develop. In Australia, insect resistant genetically modified (GM) cotton has been commercially

grown since 1996. The first GM cotton, Bt Ingard® cotton, developed to express a gene from *Bacillus thuringensis* Berliner, 1915 enabled plants to produce Bt protein, which is toxic to cotton's major insect pests (Schnepf et al., 1998). Later varieties of GM cotton and two commercial varieties of GM canola grown at present are genetically modified for herbicide tolerance. Roundup Ready® canola (Monsanto Australia Ltd.) was developed to tolerate the herbicide glyphosate while InVigor® canola (Bayer CropScience Pty Ltd.) was genetically modified to contain two new characteristics – a hybrid breeding system and tolerance to the herbicide glufosinate ammonium (Thompson et al., 1987). As of 2014, the Roundup Ready ® canola, InVigor® canola, and InVigor® x Roundup Ready® canola were commercially released in Australia. Other commercial genotypes in development or currently in the pipeline include canola with increased lauric acid, fungus resistance, modified photoperiod, reduced glucosinolates, modified plant habit, reduced pod shattering and combined herbicide and hybrid breeding systems (OGTR, 2016).

In the related genome of *Arabidopsis thaliana* L. (Heynh.), a family of six genes encodes acyl-CoA-binding proteins, designated as ACBP1 to ACBP6 (Xiao and Chye, 2011). These proteins are conserved at an acyl-CoA-binding domain, which facilitates the binding of long-chain acyl-CoA esters. Investigations on the *Arabidopsis* ACBP6 have shown that (i) *ACBP6* mRNA and protein are induced by cold (4°C) treatment, (ii) an ACBP6 gene knock-out mutant was freezing (-8°C) sensitive, and (iii) overexpression of ACBP6 in *Arabidopsis* confers freezing (-8°C) tolerance (Chen et al., 2008). Lipid analysis of ACBP6-overexpressors indicated that ACBP6 mediated freezing tolerance by manipulation of phospholipid metabolism (Chen et al., 2008). Lack of adverse phenotypic changes affecting plant growth, development and reproduction in ACBP6-overexpressors

(Chen et al., 2008) are desirable traits that are especially valuable to crops that are harvested for seeds.

The 'fast plants' or rapid-cycling *Brassica* are populations of special *Brassica* developed through artificial selection for rapid completion of seed-to-seed life cycle (Friend & Helson, 1966; Williams & Hill, 1986). Because of their short life cycle, the rapid-cycling *Brassica* collection was perceived as model material for teaching and research (Williams and Tomkins, 1990; Musgrave, 2000; Price & Harding, 1993; Shimabuku, 1991; Briggs & Goldman, 2006). *In vitro* regeneration studies in rapid-cycling *Brassica* are however, limited and were predominantly based on protoplast culture (Loudon et al. 1989; Kik & Zaal, 1993; Hansen and Earle 1994). Direct shoot regeneration from cotyledonary explants of *in vitro* grown rapid-cycling *Brassica*s proved to be a robust method for producing new plants for rapid-cycling *B. rapa* (Teo et al., 1997) and rapid-cycling *B. oleracea* (Cheng et al., 2001), and deemed an ideal method for *in vitro* based *Agrobacterium*-mediated trasnformation. *Agrobacterium*-mediated transformation of rapid-cycling brassicas has not been widely studied. So far, only the rapid-cycling *B. oleracea* has been transformed through *Agrobacterium*-mediated transformation (Beclin et al., 1993; Berthomieu & Jouanin, 1992). Rapid-cycling *B. napus, B. rapa* and *B. juncea* which are oilseed types, have not been used for genetic transformation.

The objective of this thesis was to develop frost tolerant *B. napus* using the rapid-cycling genotype of *Brassica* and the cultivated variety *B. napus* 'Westar', through *Agrobacterium*-mediated transformation with *Arabidopsis thaliana ACYL-COA-BINDING PROTEIN6* (*ACBP6*). Specifically, this study aimed to: (i) establish *in vitro* tissue culture regeneration protocol for rapid-cycling *Brassica napus and B. juncea* (ii)

transform these genotypes, and *B. napus* 'Westar' with *Arabidopsis ACBP6* to produce frost tolerant transgenic plants, and (iii) evaluate transformed plants for frost tolerance in the vegetative and reproductive stage. The *Agrobacterium*-mediated transformation of *Brassica* with *ACBP6* and the screening of ACBP6-overexpressing plants for freezing tolerance were discussed in three experimental chapters (chapters 3-5). Chapter 3 described the development of the *in vitro* regeneration system for rapid-cycling *Brassica*. In chapter 4, rapid-cycling *B. napus* and *B. juncea*, and *B. napus* 'Westar' were used for *Agrobacterium*-mediated transformation with *ACBP6,* and incorporation and inheritance of *ACBP6* were determined. Chapter 5 described the response of non-acclimated ACBP6-overexpressing plants to freezing temperature (-4°C) based on electrolyte leakage, chlorophyll fluorescence and gas exchange. The tissue vitality of seed cotyledons after freezing was also analyzed.

Chapter 2

Review of Related Literature

2.1 The *Brassica* family

The *Brassicaceae* is a diverse family of flowering plants made up of 338 genera and 3709 species, many of which are economically important (Warwick & Al-Shehbaz, 2006). The *Brassicaceae* family is often informally referred to as the mustard family, crucifers, or the cabbage family, and includes many of the important crop species, which have contributed to a large proportion of human nutrition worldwide. Species within the family are valued for their edible roots, buds, stems, flowers and seeds. Some well-known *Brassicacea*e members include *Brassica napus* L. (oilseed rape, rapeseed), *Brassica oleracea* L. (cabbage, broccoli, and cauliflower), *Brassica rapa* L. (turnip), *Raphanus sativus* L., the common radish (Vaughan, 1977) and the model plant *Arabidopsis thaliana* (L.) Heynh. (Rakow, 2004). An estimated 215 brassica species under 57 genera are present in Australia. More than half of these species are native, and some are naturalized species, which were introduced in Australia for their oil and meal. Many species of *Brassicaceae* are important weeds throughout the southern cropping zones of Australia (Cheam et al., 2008).

The botanical and cytogenetic relationships between the *Brassica* species are best described by the famous triangle of U (Figure 2.1; Nagaharu, 1935). The *Brassica* species that have a high number of chromosomes (*B. juncea*, *B. napus* and *B. carinata*) were proposed to be amphidiploids that have undergone chromosome doubling from the diploid species *B. nigra, B. rapa* and *B. oleracea*. *Brassica carinata* (BBCC, n=17) probably arose from hybridisation between *B. oleracea* (CC, n=9) and *B. nigra* (BB, n=8);

B. napus (AACC, n=19) from *B. oleracea* and *B. rapa* (AA, n=10), and *B. juncea* (AABB, n=18) from *B. rapa* and *B. nigra*.

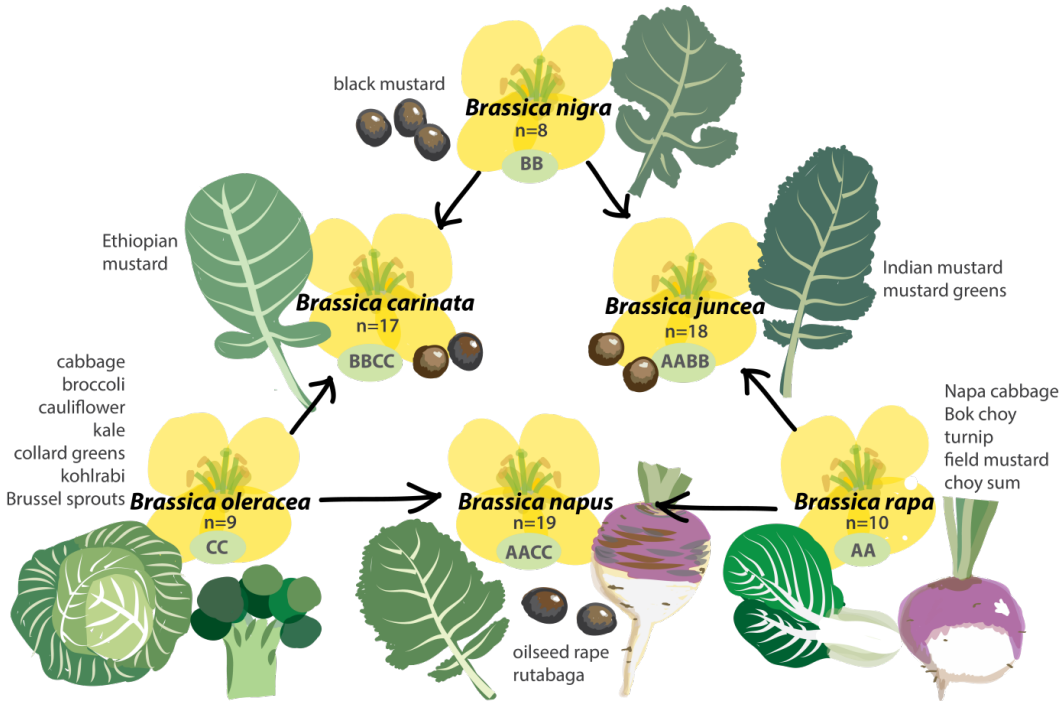


Figure 2.1. Genomic relationships of the different *Brassica* species, shown as the triangle of U (Nagaharu, 1935). The corresponding genome assignments are indicated by letters A - C, with haploid genome number (n). Some examples of cultivated varieties of the species are also illustrated.

Oilseed *Brassica* has been in domestication for thousands of years, utilized as a source of lamp oil and cooking oil (Daun, 2011). Among the many species of the *Brassica* family, oilseed rape species include *B. rapa, B. napus, B. juncea* and *B. carinata. Brassica rapa* (turnip rape) is predominantly grown in western Canada and is adapted to the freezing temperatures in the Northern hemisphere. *Brassica napus* has two seasonal types – spring and winter varieties, which dominate the oilseed rape growing regions in Europe, Canada and China. The winter type requires vernalisation to start the reproductive phase while spring type has no or minimal vernalisation requirement (Raymer et al., 1990).

Brassica juncea is mainly grown in drier areas such as India and China, and has recently been introduced into Australia. Growing of *B. carinata* is limited to Ethiopia and nearby East African countries, wherein the climate is dry (Gunstone, 2009).

2.2 Oilseed rape (*Brassica napus* L.)

How *B. napus* exactly came to rise from the parent species has long been investigated. The majority of the cultivated forms of *B. napus* (AACC) were derived from a cross in which the maternal donor came from a closely related ancestral species of *B. rapa* (AA) and *B. oleracea* (CC). Nagaharu (1935) established that *B. napus* was formed by allopolyploidy between *B. oleracea* and *B. rapa*. Sinskaia (1927) hypothesized that *B. napus* originated in the Mediterranean region where the parents *B. rapa* and *B. oleracea* shared natural and agricultural habitat. Palmer (1988) suggested that some populations of *B. napus* stabilized from repeated backcrossing of the interspecific hybrids to either one of both of the parental population. Analysis of the different accessions of *B. napus* representing diversity in the species, together with associated diploid parental species using nuclear, chloroplast, and mitochondrial RFLPs, supported the hypothesis that *B. napus* arose from different origins (Song & Osborn, 1992).

Chalhoub et al. (2014) sequenced the genome of *B. napus* and determined that approximately 7500 years ago *B. rapa* and *B. oleracea* crossed to form a hybrid that consequently doubled its chromosome number giving rise to the present *B. napus*. In their study, the *B. napus* genome was assembled (849.7 Mb) and contained 101,040 gene models. Of these, 90% matched with *B. rapa* and/or *B. oleracea* expressed protein assembly. The A_n and C_n subgenomes of *B. napus* were found to have high colinearity

with the diploid A_r (*B. rapa*) and C_o (*B. olearacea*) genomes. Figure 2.2 shows the repeated occurrence of genome duplication in *B. napus* (Chalhoub et al., 2014).

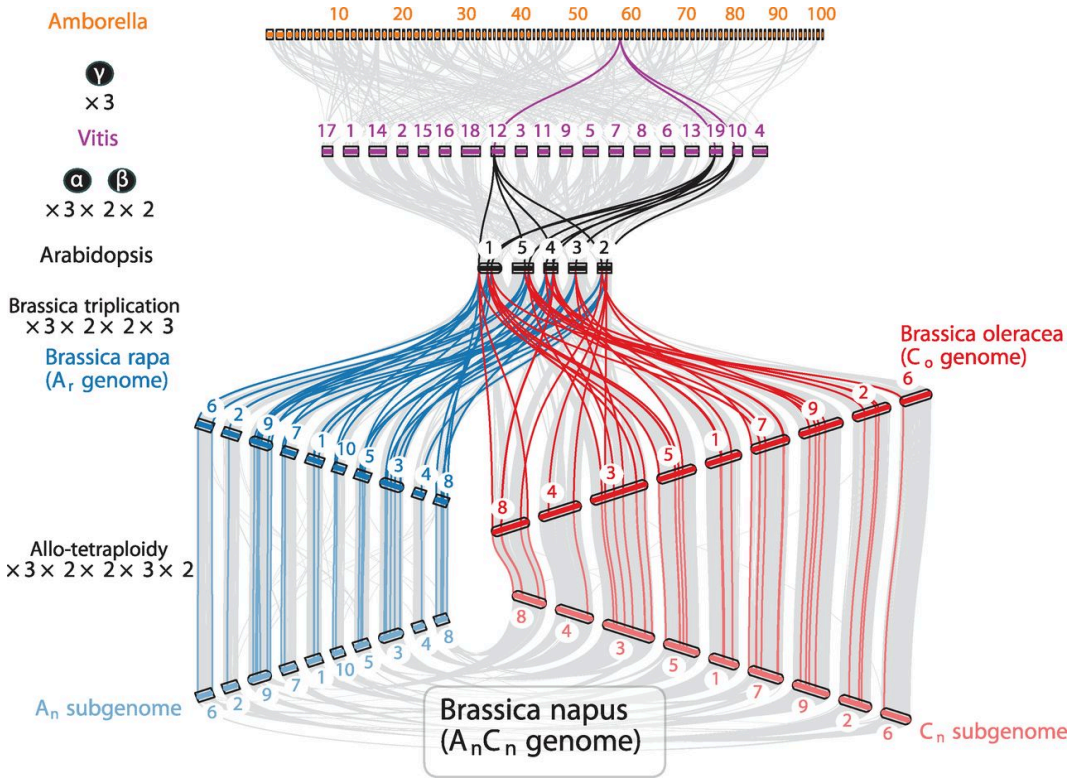


Figure 2.2. The genome of *Brassica napus* originated from a hybrid between *B. rapa* and *B. oleracea* through a series of genome duplications**.** Genomic alignments showing the recurrence of genome duplications in *B. napus*, with reference to basal species of angiosperm (*Amborella trichopoda*), a eudicot (*Vitis vinifera*), model relative *Arabidopsis thaliana*, and parental genomes of *B. oleracea* and *B. rapa*. Gray lines represent conserved synteny blocks containing 10 gene pairs. *From Chalhoub et al. (2014). Early allopolyploid evolution in the post-neolithic Brassica napus oilseed genome. Science, 345(6199), 950-953. Reprinted with permission from AAAS.*

2.2.1 Plant morphology

The botanical illustration of *B. napus* showing the stem, reproductive structures and the silique or pod is shown in Figure 2.3. The compiled descriptors of oilseed rape crops (Callahan et al., 2000; Colton & Sykes, 1992; OECD, 2012), described oilseed rape as an annual growing plant that grows from 70 to 170 cm high. The main structures of the plant are the leaves, stem, roots, flowers, siliques, and seeds. A mature healthy plant produces up to 10 to 15 leaves, although it does not produce a definite number of leaves. The first true leaf grows at the terminal meristem and appears ruffled at the margin when fully developed and expanded. Each leaf is attached to the stem at a node. The oldest leaves at the base of the plant are the largest, and the youngest leaves at the top of the plant are the smallest. The lower leaves have small lobes and are clasped loosely in the stem. The middle and upper leaves are more rounded and thicker and attached to the stem without a petiole. The leaves appear as bluish green. The leaves are attached singly at the stem forming a node, with approximately 15-20 internodes per plant. Oilseed rape has bisexual flowers that develop in the terminal racemes.

Oilseed rape has an indeterminate growth and is able to produce reproductive flowers all throughout its life (Wang et al., 2009). The OECD (2012) described the flowers of *B. napus* to have yellow colour and have 4 sepals and 4 petals, with the petals arranged diagonally opposite to each other at the top of the raceme and narrows at the basal end forming a cross, which accounts for the original family name, Cruciferae (now *Brassica*ceae). The flowers contain 6 stamens (2 of which are shorter and inserted lower than the others); a pistil of 2 carpels and an ovary positioned above the receptacle. The flowers form a bunch at the end of the stem. Newly formed buds sit above the flowers. Seeds develop in a 2-celled, elongated capsule called a silique (or pod) with a prominent

Figure 2.3. Botanical illustration of *Brassica napus* L. showing the stem, reproductive structures and the siliques. Original illustration by Janus Kops (1822), Flora Batava, Vol. 4. *Source:www.biolib.de (Public Domain) via Wikimedia Commons*.

mid-vein. The pods are 5 to 10 cm long, with conical, thin and seedless beaks measuring 6 to 11 mm long. Mature seeds are reddish-brown to bluish-black, round with 1.5 to 3 mm in diameter, and textured.

2.2.2 Cultivation

Brassica napus also known as oilseed rape, rapeseed and canola, is the most economically important species among all brassicas. With human selection as the main driving force for crop domestication and improvement, the present oilseed *Brassica* has rapidly lost its glucosinolate genes while preserving genes for oil biosynthesis (Fehr, 1987). Cultivation of *B. napus* has been documented to be as early as 2000 BC in India and has been used since the 13th century, mainly for fuel in oil lamps (Daun, 2011; Gupta & Pratap, 2007). The early *Brassica* oil contained high amounts of erucic acid while the dry matter contains high glucosinolates. Erucic acid renders the oil unfit for human consumption, while the glucosinolates make the protein-rich meal animal feed almost unpalatable to animals (Bell, 1993). The discovery of *B. napus* variants having low glucosinolate content and zero erucic acid in natural populations lead to intensive breeding programs to utilize these desirable traits in Canada and Europe during 1960-1970. The product of this intensive breeding was the commercial *B. napus*, known as canola (Bell, 1982). The term canola was coined from “Canadian oil, low acid”, first defined in Canada in 1978. Canola quality oil is classified to contain no more than 2% erucic acid and the meal to have less than 30 mol/g of glusinolates (Shahidi, 1990; www.canolacouncil.org). The canola industry quickly expanded since its early development in 1960s.

Edible oil is the main product of oilseed rape, although lower quality oil is also being utilized for industrial purposes. The seeds produce from 35% to over 45% oil (Scarth & McVetty, 1999; Gunstone, 2009). As a by-product of extraction, seed meal is used as a high-protein animal feed (Gunstone, 2009). With the advancement of technology, whereby early and large scale detection of low erucic acid levels can be achieved, breeding for these specific qualities has greatly accelerated. Cultivars containing zero or low erucic acid are produced commercially in Canada, Sweden, France, and Germany (Rai et al., 2007). These characteristics have also been developed in *B. rapa* and *B. juncea* (Gunstone, 2009). Canada, China, India, France, Australia, and Germany are among the highest oilseed rape producers in the world (FAO, 2014). Figure 2.4 shows the volume and the areas of oilseed rape production in the world.

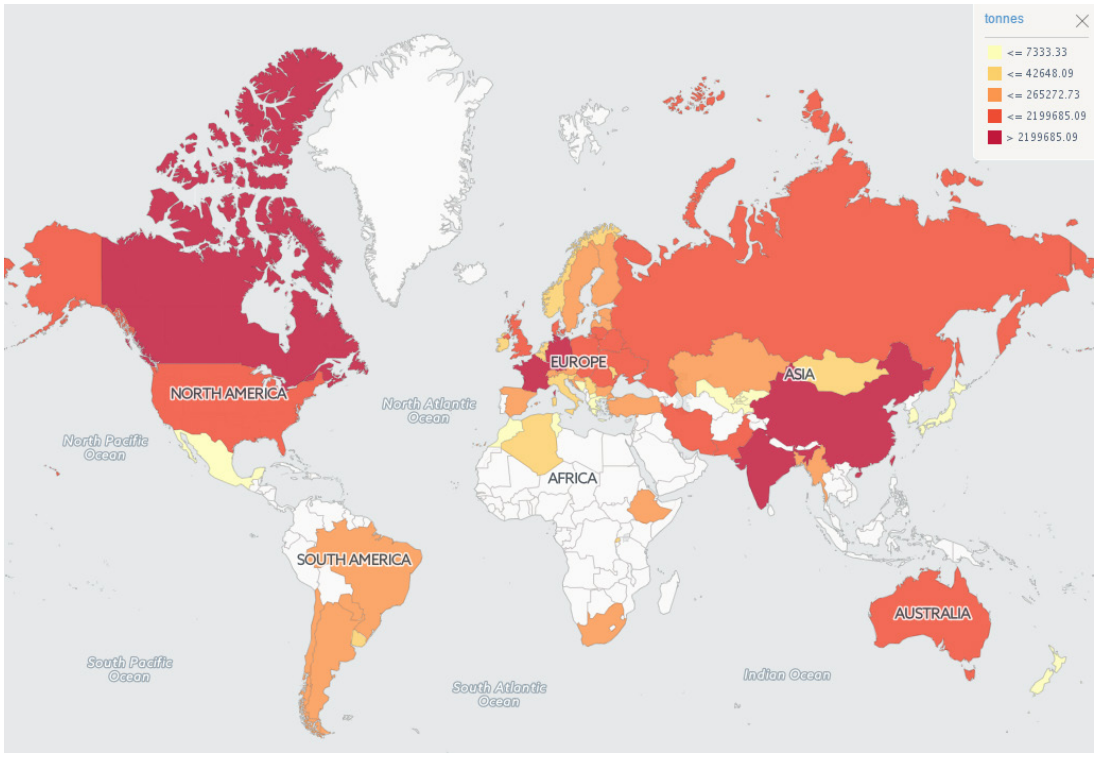


Figure 2.4. Ten-year (2004-2014) average oilseed rape production in the world. *Source: http://faostat3.fao.org*.

2.3 Canola production in Australia

Canola in Australia is a multi-billion dollar industry that emerged from three decades of breeding from rapeseed. A rapid expansion of canola production has been witnessed starting in the early 1990s as the world demand for canola oil increased (Colton & Potter, 1999). At present, Australia ranked 6th in the world as a major producer of oilseed rape, valued at 4.1 million metric tons and the second highest seed exporting country next to Canada (FAO, 2014). Majority of canola in Australia is produced in Western Australia, with almost 50% share of the total production; followed by New South Wales, Victoria and South Australia (AOF, 2015). Figure 2.5 shows the canola growing areas in Australia.

The early varieties of oilseed rape grown in Australia originated from Canada (Salisbury & Wratten, 1999). These were spring varieties that did not require vernalisation to flower. Unlike growing canola in other parts of the world, Australia has different cropping systems. Canola varieties in Australia are bred to fit the short days of the winter-spring season, based on flowering responses. Canola is sown in late autumn or early winter (April to June) during the onset of the rainy season. Australian winter ranges from minimum of 4-5°C to a maximum of 14-18 °C. Harvest is ideally during late spring and early summer in the months of November and December when the seeds contain approximately 30-40% moisture and the plants are ready for swathing (Burton et al., 2003; Potter et al., 1999).

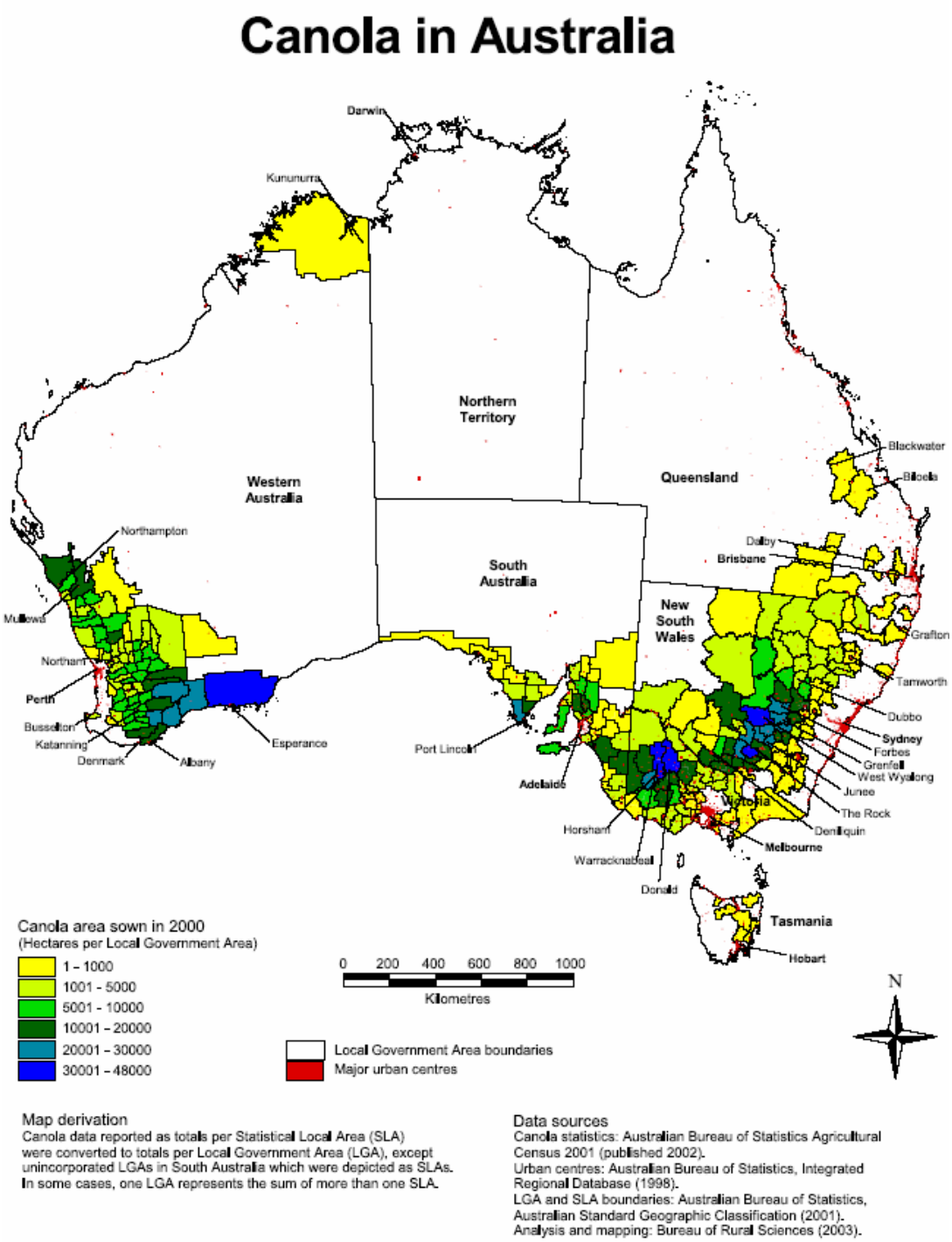


Figure 2.5. Map showing areas in Australia where canola is being sown in the year 2000 (Bureau of Rural Sciences, 2003). The hectarage per local government are indicated by the colours. The bulk of production was concentrated along the southeast regions in New South Wales, Victoria, and South Australia; and the southwestern tip of Western Australia. *Source: OGTR (2008)*.

Canola production can be affected by various abiotic and biotic stresses. In fact, the beginning of the canola industry in Australia in the 1970s was severely affected by blackleg, caused by the fungus *Leptosphaeria maculans* as accounted in Salisbury et al., (1995) and Salisbury & Wratten (1999). Production only turned around in 1990s with the availability of improved varieties with resistance to the disease, good yield and improved oil quality, coupled with increased farming knowledge. Expansion of canola production in Australia is basically limited by water availability (Walton et al., 1999). Majority of Australia receives limited rainfall with canola production extended to areas with as low as 325 mm annual rainfall and is mainly concentrated along the wheat belt regions. Canola breeding programs in Australia started in the 1970s in Victoria, followed by breeding programs New South Wales and Western Australia, which were largely focused on developing open-pollinated varieties that were high-yielding and resistant to blackleg. Private breeding programs began in the 1980s which was started by Pacific Seeds followed by AgSeed Research, Pioneer, etc., which were predominantly focused for hybrid seed production. As a result of extensive breeding, yield had greatly increased with improved oil and meal quality, and the availability of varieties suited to different Australian environments (Salisbury & Wratten, 1999). At present, the average yield of Australian canola ranges from 1.04 t/ha in South Australia to 1.45 t/ha in New South Wales. The average quality of Australian canola has oil content of 44.1%, protein content of 38.8%, and glucosinolates of 6%; the oil composition is composed of 63.7% oleic acid, 17.9% linoleic acid, 9.3% linolenic oil, 7.5 % saturated fatty acid, and less than 0.1% of erucic acid (Seberry et al, 2014).

2.3.1 Frost risk in Australian canola industry

Frost has been identified as a major agronomic risk in growing canola in Australia. Frost causes complete abortion of open flowers of canola plants while developing seeds die and shrivel resulting in incompletely filled pods (Lardon & Triboiblondel, 1995). Yield loss can be greatly affected when frost affects the grain-filling stage wherein seeds contain approximately 60% moisture. This results in poor seed fill and poor oil quality (Johnson-Flanagan et al., 1990; GRDC, 2009).

In a major frost event in October 2001, damage to crops in NSW including cereals, oilseeds and pulses, was estimated to reach $100 M. Each year the entire northern grains region (northern NSW and Queensland) has an estimated loss of production of $100 million in barley and wheat due to frost damage. In South Australia and Victoria, the cost of crop loss due to frost reaches an estimated average of more than $33M a year (AgtransResearch, 2008).

Frost incidence affecting the wheat belt has increased in the last 3 decades, especially in Western Australia (GRDC, 2009). Key areas more prone to frost have been identified based on topography and other related factors. This facilitates better placement of strategies on how to manage frost. The GRDC (2009) defined strategies such as choosing the right variety in a given location, thereby minimizing frost risk without compromising yield potential. Sowing a mixture of long and short season varieties is also a way to minimize risk. To reduce crop susceptibility, proper nutrition must be supplied.

The potential occurrence of frost is predictable based on the preceding atmospheric conditions in the locality. However, temperature recorded in weather stations may not always reflect the real temperature at the crop canopy and records may differ by as much as 5°C or higher. A recorded temperature of 2.2°C was identified to cause frost

damage in susceptible crops (GRDC, 2011). Growers are encouraged to keep track of local weather conditions especially in frost prone areas. The use of temperature data loggers or checking the Bureau of Meteorology Frost Potential website is recommended (Nichols, 2010). Radiative frost events, which is the most common in Australia, cause much of the damage in the majority of the crops (BOM, 2016). Radiant frost occurs when during a sunny day, the soil and plants absorb heat and radiate it back during a clear and still night (Jennings, 2005). The lowest areas of the canopy then become a sink for denser cold air. With frost occurrence, the immediate temperature is low enough to cause ice crystal formation in the plant tissues (Maqbool et al., 2009). The potential frost days in Australia are shown in Figure 2.6.

Managing flowering date and enhancing soil heat retention are two major strategies identified to minimize frost risk (GRDC, 2009; Crimp et al., 2013). Crops at the flowering stage are most vulnerable to frost damage. Sowing is either delayed or long season varieties are used to avoid the period where high incidence of frost is expected. As the extent of frost damage is highly dependent on the temperature in the immediate environment of the plants, management techniques have been practiced to influence the canopy and paddock temperature. This includes clay delving and rolling, which enhance the soil capacity to retain heat and moisture (GRDC, 2009). These practices can increase the temperature by 0.5°C to 1°C at canopy height, reducing frost damage to as much as 80%.

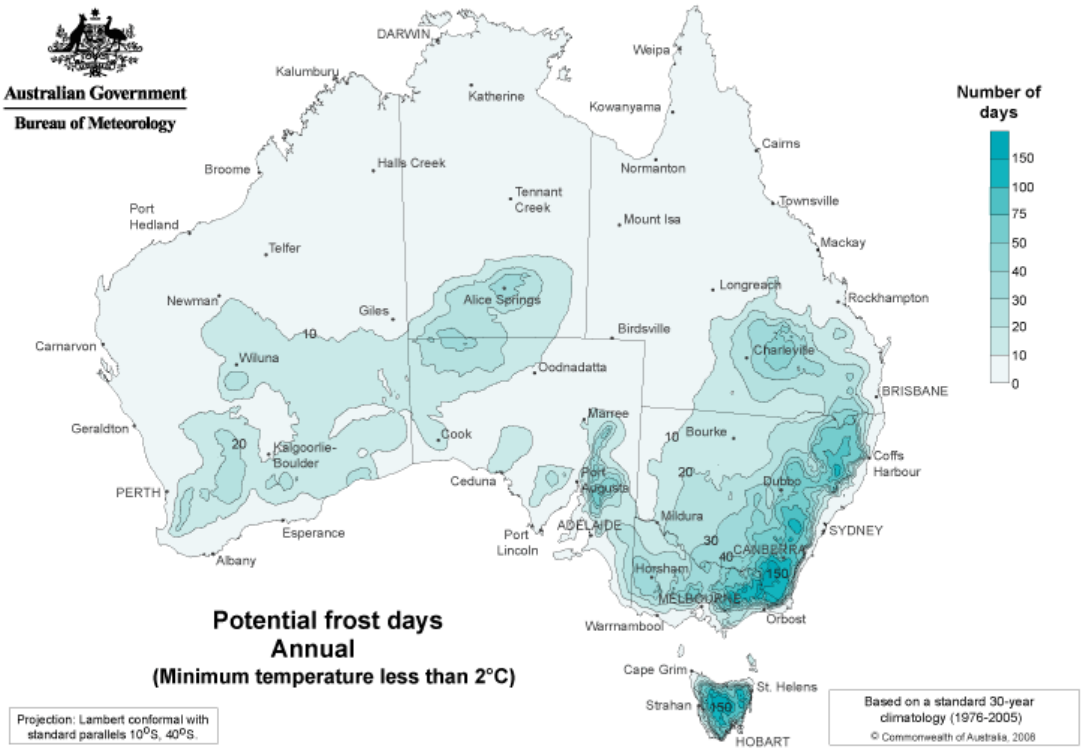


Figure 2.6. Areas in Australia with minimum temperature of 2°C. These areas were identified to have the highest chance of frost occurrence. The colours indicate the number of potential frost days. *Source: www.bom.gov.au.*

As summarized in the broad acre frost trial report of Lynch et al. (2003), sowing canola too early increases its risk to frost while delaying it exposes the crop to hot and dry conditions, affecting the yield potential and oil quality. A week delay in sowing of canola has been shown to contribute up to 5% yield loss in South Australia and Victoria and as much as 10% in central and southern NSW. In general, frost damage during early to middle of flowering can be subsequently compensated for by further flower development since canola flowering occurs from 4-6 weeks. Risk management in terms of sowing time depends on the variety and the determined optimum period for a particular region (Lynch et al., 2003).

The Grains Research and Development Council (GRDC) made a significant investment to fund projects on climate risk management in agriculture and natural resource management as a response to their priority research program in 2002-2003,

which was focused on the reduction of climate variability with emphasis on variable rainfall and frost. The main objective of this program was to equip farmers to properly manage climate-related risks. From this program, options for frost management were tested and techniques and strategies were put in place to minimize frost risk. Information kits for yield and climate risk analysis were generated (AgtransResearch, 2008).

Frost tolerance during seed development was identified as one of the key traits for development by the National *Brassica* Germplasm Improvement Program (NBGIP). The NBGIP is responsible for *Brassica* research and development to identify, develop and enhance germplasm sources for utilization in breeding programs. This effort ensures availability of cultivars especially those with challenging agronomic traits to Australian farmers to enable them to keep up with the world market (Salisbury et al., 2008). There has been no significant progress made in finding frost tolerance in the germplasm surveyed (P. Salisbury, personal communication, 7 November, 2016). In 2014, GRDC established the National Frost Initiative, which aimed to mitigate the damaging effects of frost through integration of genetics, management and environmental prediction (www.grdc.com.au).

2.4 Low temperature stress

One of the major constraints in cultivation of crop plants is temperature, limiting their distribution and productivity. Low temperature stress can be classified as chilling stress and freezing stress, based on the temperature affecting certain plant types (Levitt, 1980; Hale & Orcutt, 1987).

2.4.1 Chilling stress

Chilling stress causes injury (chilling injury) to plants when exposed to low but non-freezing temperatures. Sensitive plants could be damaged when exposed to temperatures below 15°C (Levitt, 1980). Most plant species growing in the tropics and subtropics are susceptible to chilling injury such as common horticultural crops like tomato, potato, cucumber and banana (Wilson, 1985), although temperate plants could be susceptible such as corn and wheat (Levitt, 1980). The symptoms and degree of severity of chilling injury depends on the plant species and variety, the stage of growth, the exposed plant part and the degree and duration of exposure (Wang, 1990).

Symptoms could be directly observed right after exposure (direct injury) or after prolonged exposure (indirect injury). In direct chilling injury, the cell membranes are damaged resulting to leakage of cell contents. With indirect injury, membrane damage can also occur, however, the injury is slower and prolonged brought about by the impairment of some metabolic processes in the plant i.e. photosynthesis and respiration (Levitt, 1980).

Chilling injury symptoms may be observed as changes in the cells such as plasmolysis and altered ultracellular structures such as swelling and deformation of chloroplasts and mitochondria (Levitt, 1980; Kratsch & Wise, 2000). More noticeable symptoms include wilting of leaves and necrosis of tissues, formation of lesions, chlorosis, formation of sunken pits and water-soaked tissues, and early senescence (Wang, 1990). Internal injury can be observed as premature browning of seeds, and discoloration of vascular tissues, while in horticultural products such as fruits, the normal ripening process fails affecting flavour and aroma (Wang, 1994).

2.4.2 Freezing stress

Frost occurs when the surface temperature falls below the freezing point (0°C), while freezing is the change in state of water from liquid into solid ice (Sakai & Larcher, 1987). In horticulture, frost, freezing, or frost-freeze events, are often used interchangeably (Perry, 1998) to describe the occurrence of a localized low temperature (≤ 0°C), which causes ice formation in plant tissues (Guy, 1990; Levitt, 1972; Hale & Orcutt, 1987). Frost events can be radiative or advective type. Radiative frost occurs when heat is lost to the atmosphere causing the ground and ambient air to cool down. This phenomenon is observed when night temperature is below 0°C and day temperature is above 0°C, during calm winds (< 5 mph) and clear skies (Snyder & Melo-Abreu, 2005; Perry, 1998). In advective frosts, the temperature fall below 0°C, and may maintain for an entire day, brought about by cold air blown into the area (Snyder & Melo-Abreu, 2005).

Plants could change temperature to equalize with the ambient temperature in a very short time (Hale & Orcutt, 1987). When losing heat through radiative cooling, a plant could be 1 to 3°C colder than the air temperature, creating a temperature gradient from the ground towards the canopy, and may vary depending on the plant's habit and degree of exposure (Sakai & Larcher, 1987).

The onset of frost or ice formation in plant cells can cause immediate freezing injury to freezing-sensitive cells, resulting to death. Membrane damage and cell rupture ensue once ice starts to form (Levitt, 1980). Extracellular and intracellular fluids in plant tissues freezes at different temperatures, due to the differences in the concentration of solutes. Freezing starts outside the cells, where the fluids are more diluted and ice nucleators are abundant. This creates a vapour pressure difference, which is equilibrated

upon ice formation inside the cell or evaporation of water out of the cell (Hale & Orcutt, 1987). Freezing-tolerant tissues can withstand freezing to a certain degree, such that freezing injury becomes evident at a temperature lower than the freezing temperature. Although freezing already takes place, the cells may retain membrane viability or are able to maintain equilibrium until such point when it becomes dehydrated (Sakai & Larcher, 1987).

Symptoms of freezing injury may be immediately observable after freezing and thawing of plant parts (Wilson, 1985; Wang, 1990). One of the most obvious signs of injury is discoloration, such as darkening of exposed tissues, due to oxidation of cellular contents after breakage of membranes. Chlorosis and bleaching occurs in tissues that withstand cell death, and the photosynthetic machinery may be affected altogether such that newly formed leaves are chlorotic. Necrotic lesion in leaves, flower decay, dieback of shoot tips and blister formation in fruits and fleshy plant parts, and shrinking of leaves of herbaceous plants. Development of new tissues may be affected when meristematic and tissue primordia are injured resulting to malformation of new organs. Maturation and developmental stages may also be delayed (Sakai & Larcher, 1987).

2.4.3 Freezing resistance

Frost or freezing resistance is the ability of the plants to survive temperatures below freezing point of water (Sakai & Larcher, 1987). Levitt (1980) described freezing resistance to be divided into two mechanisms - avoidance and tolerance. Some plants can avoid freezing by supercooling – the ability to lower down temperature without ice formation. However, this state was observed to be temporary and may break down when the temperature lowers further or during longer freezing period. Another avoidance

mechanism described by Levitt (1980) is by freezing point depression in the presence of high concentration of osmolytes in the cell, and/or low water content, such in some buds and desiccating seeds. Levitt (1980) also noted that the avoidance of intracellular freezing is the only mechanism considered as a true form of freezing resistance. However, this avoidance must also correspond with extracellular freezing tolerance to be useful for survival. Freezing resistance of plants is essentially freezing tolerance, specifically tolerance to extracellular freezing.

Freezing resistance also correspond to hardening capacity (cold hardiness). Some plants are capable for acclimation to low temperature, to enhance their freezing avoidance capacity. Sakai and Larcher (1987) categorised freezing resistance based on the inherent resistance to freezing and cold hardiness. Selected freezing-sensitive plants can cold acclimate to avoid frost to a certain degree through supercooling and freezing point depression, while partially freezing-tolerant plants when hardened can acquire resistance in specific tissues.

2.5 Mechanism of low temperature stress tolerance

The mechanism of low temperature stress response is a complex network of processes that alters the metabolism and gene expression of the plant (Cook et al., 2004; Thomashow, 1999). Cold acclimation or prolonged exposure of plants to low temperature induces changes in the plant's development and metabolism that enables it to withstand freezing temperatures (Stefanowska et al., 2002).

Immediate injury upon freezing exposure occurs in the membranes of the plant cells caused by ice formation and consequent dehydration of the cells (Gordon-Kamm & Steponkus, 1984). Other factors contributing to cellular injury include production of

reactive oxygen species and denaturation of proteins (Thomashow, 1999). In response, during cold acclimation, membrane stability is being enhanced, brought about by mechanisms involving changes in lipid composition (Uemura & Steponkus, 1997), accumulation of sugars (Strauss & Hauser, 1986), and involvement of hydrophilic proteins. Numerous cold-regulated of *COR* (*cold-resposnive*) genes were identified to be induced during cold acclimation (cold-inducible), expressing proteins, which were involved in freezing tolerance (Thomashow, 1998).

At the first instance that a plant senses cold stress, a change in the fluidity of the cell membrane occurs – the membrane rigidification effect (Örvar et al., 2000). This is followed by a series of signal transduction that activates cold-responsive genes. Downstream reactions that occur in various signalling pathways involve calcium, reactive oxygen species, protein kinase, protein phosphatase and lipid signalling cascades. The change in calcium levels in the cytosol induces change in the calcium binding proteins, which then triggers a phosphorylation cascade that targets stress-responsive genes and transcription factors (Chinnusamy et al., 2007). In *B. napus*, influx of calcium ions triggered by cold was required in the cold-induction of the *BN115* promoter (White et al., 1994). Changes in gene expression along the cascade of reactions also affected genes involve in the formation of plant hormones. Abscisic acid has been found to play an important role in plant stress response including cold stress (Chinnusamy et al., 2007).

Physiological changes during cold acclimation in plants that enables them to withstand freezing temperatures involve identifiable sequential stages such as hydrolysis of phosphatidycholine molecules, observed in the leaves when exposed to sudden freezing temperature. To study the effect of short and sudden decrease in temperature on inositolphospholipids (PI, PIP and PIP_2) hydrolysis into phosphoinositides and inositol

phosphates (IP_3, IP_2, and IP), cold-acclimated and non-acclimated winter oilseed rape (*B. napus* 'Jantar') were exposed to short and sudden low temperature (Smolenskasym & Kacperska, 1994). An increase in concentration of PI and PIP2 in the leaves was observed in cold-acclimated plants, while exposure of non-acclimated leaves to 0°C and cold–acclimated leaves to -7°C increased concentration of inositol phosphates and decreased concentration of PIP2 and PIP, indicating hydrolysis of PIP2 and PIP into IP3 and IP2. These reactions may have been caused by increased activity of phospholipase C. Cold acclimation caused decrease in responsiveness of the cells to low temperature in terms of induction of the phosphoinositide pathway (Smolenskasym & Kacperska, 1996). Lipid molecules play an important role in the cold stress response signalling complex. Phosphatidic acid which is a component of the membrane lipid is actively induced during cold stress (Testerink & Munnik, 2005). Phospholipase D which produces the phosphatidic acid is also involved with reactive oxygen species. In *Arabidopsis*, the expression of AtPLDd, which is a phosphilipase D, is responsible for cold-inducible freezing tolerance, the enhanced tolerance to freezing when exposed to low temperature (Li et al., 2004).

The response of plants to cold stress, specifically cold and frost tolerance is the result of the interplay of different genetic factors involved in regulation and expression of genes. One of the most studied gene families is the *Arabidopsis COR* (cold-regulated) genes which are regulated by *CBF/DREB1* transcriptional activators; and the wheat *WCS/WCOR* (Thomashow, 1999). These gene families encode for proteins such as the hydrophilic dehydrins, which act by modifying the membrane structure (Trischuk et al., 2014).

The transcription factors and effector genes involved in cold stress response are collectively referred to as cold-regulated (*COR*) genes (Thomashow, 1998). Cold-responsive genes such as the *COR78* and *COR15a*, whose expression is induced during cold stress, have been identified and characterized in *Arabidopsis* (Gilmour et al., 1992). Transcriptions factors such as the dehydration responsive elements or C-repeat binding factors (DREB1/CBF) are also expressed when plants are exposed to cold stress. *Arabidopsis* CBF/DREB1 activates *COR* genes and confers constitutive freezing tolerance in transgenic *Arabidopsis* and oilseed *Brassica* when overexpressed (Cook et al., 2004; Gilmour et al., 1992). Homologous overexpression of *B. napus BNCBF/DREB1*-like genes increased constitutive freezing tolerance and regulated chloroplast development to increase efficiency of photosynthetic machinery (Savitch et al., 2005). However, overexpression of DREB1A from the CaMV *35S* promoter although culminated in tolerance to drought, high-salinity and freezing, resulted to transgenic plants that showed growth retardation under normal growth conditions (Kasuga et al., 1999).

2.5.1 Low temperature stress response in Brassica

Most studies of cold and freezing tolerance in oilseed rape have been done on winter types focusing on winter survival mechanisms necessary to survive winter environments in the temperate zone where the temperature can reach -25°C. Winter survival is a complex trait that involves vernalisation response, cold-acclimation and freezing tolerance (Rife & Zeinali, 2003; Teutonico et al., 1995). Vernalisation is the ability of the plant to hasten flowering through exposure to low temperature (Rife & Zeinali, 2003). Plants with longer vernalisation requirements approach the reproductive

phase longer which is advantageous to survive winter (Rife & Zeinali, 2003). Acclimation ability is the ability of plants to increase freezing tolerance when exposed to low temperature for a period of time (Levitt, 1980). Freezing tolerance enables plants to survive low temperatures in winter. Winter and spring-type oilseed rape are classified based on their vernalisation requirement to be able to produce flowers and not on their ability to survive freezing temperatures (Andersson & Olsson, 1961) although differentiating the two based on freezing tolerance is more perceivable (Rapacz, 1998).

Spring type oilseed and cereals have lower frost tolerance than winter types. This is because the spring type plants have limited ability to be able to undergo proper hardening that involves growth cessation and maintenance of high photosynthetic activity during cold acclimation (Marcin Rapacz, 1998). In other words, temperate winter type oilseed rape plants exhibit freezing tolerance, and this ability is further enhanced through cold-acclimation. Tropical and subtropical plants on the other hand are freezing sensitive and lack the ability to cold-acclimatize (Zhu et al., 2007). Cold acclimation of plants prior to exposure to freezing temperature provides the maximal frost resistance (Rapacz, 1998). In *B. napus* winter cultivars, prehardening of plants to cold temperature (12°C) is necessary for frost hardiness to be differentiated (Rapacz & Janowiak, 1999).

Exposure to freezing temperature affects brassica plants differently based on their growth stages. Based on the study of Crouch & Sussex (1981) during seed maturation, storage lipids and proteins such as napin and curciferin, are being produced at a high rate especially during the predessication, peaking before the moisture content drops to around 50%. At the same moisture level, the seeds exhibit a change in response and sensitivity when exposed to ABA levels and osmotic potential, indicating that at this stage, a major developmental change has occurred (Finkelstein & Crouch, 1986). Exposure of canola

seeds to freezing temperature disrupts the normal development of seeds, especially during seed maturation. Specifically, it affects the degreening process and promotes synthesis of green pigments (Johnson-Flanagan et al., 1990). Seeds with about 55% moisture content have decreased lipid quantity when exposed to freezing. The elongation and unsaturation pattern of fatty acids were also greatly affected (Johnson-Flanagan et al., 1991). Seeds with more than 80% moisture content exposed to freezing temperature are unable to develop and germinate (Johnson-Flanagan et al., 1990) hence, after a freezing, seeds desiccated at high rate (Johnson-Flanagan et al., 1991).

In a study by Lardon & Triboiblondel (1995), they found out that low temperature stress (7°C day/0°C night) in winter can cause loss of flower and pods in *B. napus*, however, the plants are adapted to this kind of environment by changing yield architecture. After exposure, there is an increased number of branching and extended flowering period. Assimilates are concentrated at the lower racemes, producing most of the total seed yield. When winter oilseed rape (*B. napus* 'Samourai') plants in the reproductive stage were exposed to freezing (-3°C for 4 hours), the ovules dissected from pistils were shrivelled and non-viable. Pollens become non-viable only during binucleate stage, while seeds are very sensitive to freezing at 20 DAF and viability remained unaffected at 35 DAF (Lardon & Triboiblondel, 1994).

2.6 Measuring low temperature stress response

2.6.1 Electrolyte leakage assay

The electrolyte leakage method to determine injury to plant tissues is based on an increased concentration of electrolytes or leaked ions as measured by the electrical conductance of a solution, and has been used as early as 1915 (Merrill, 1915). This

method was applied to determine hardiness of plants exposed to various stresses such as freezing, pathogen damage, chemical exposure, and mechanical injury (Dexter et al., 1932). The first studies focusing on freezing injury were done by Dexter et al., (1930) and Dexter et al. (1932) using the electrical conductivity reading as a direct measurement of the plant's response to freezing. To account for the inherent conductivity differences of the plant tissues, Stuart (1940) used specific conductivity as a percentage of electrolyte leakage from total leakage after heat killing the tissues, as defined by Dexter (1956) as relative conductivity, R_t (%). Other proponents include the conductivity of the non-frozen control in the equation, and expressed the freezing damage as an index of injury, I_t (Flint et al., 1967), or by acquiring the temperature to which 50% leakage occurred, TK_{50} (Piotrowska et al., 2000) and LT_{50}, the temperature that resulted in 50% death in the population (Kacperska & Szaniawski, 1993). Whitlow et al., (1992) expressed electrolyte leakage in the tissue as ionic conductance g_{Ti} which accounted for differences between the ion concentration of the solution and the tissue, and the surface area of the tissue.

The different estimations of freezing injury were compared by Manley and Hummel (1996) on freeze-stressed pith, leaf and petiole tissues of cabbage. I_t and g_{Ti} produced similar results in the estimation of freezing injury, although the authors recommended the use of I_t for its simplicity. Prasil and Zamecnik (1998) determined that by just using the R_t (%) = 100 (conductivity of frozen samples / maximal conductivity), freezing injury of samples could be estimated provided that the tissues were identically sampled (same plant parts, size and shape).

Numerous studies on freezing stress measured electrolyte leakage of exposed tissues to estimate freezing injury, in conjunction with other methods such as chlorophyll fluorescence and gas exchange analysis. Electrolyte leakage has been proven an effective

method to measure freezing tolerance in many plant species such as conifers (Mckay, 1992; Peguero-Pina et al., 2008; Wulff et al., 1994), cereals (Clement & vanHasselt, 1996; Rapacz, 2007; Rapacz et al., 2011), and brassicas (Jung et al., 2014; Manley & Hummel, 1996; Prasil & Zamecnik, 1998).

2.6.2 Chlorophyll fluorescence

Chlorophyll fluorescence technique has been commonly used to study plant photosynthetic performance both in field and laboratory conditions. Maxwell and Johnson (2000), and Baker (2008) explained the practical theory behind the technique, and its application to measure photosynthetic performance of plants. When light enters the plant's chloroplast, the energy is absorbed by chlorophyll molecules and used for photosynthesis. Depending on the efficiency of the plant's photochemistry, some of this energy will not be utilized for photosynthesis and instead, re-emitted as light (chlorophyll fluorescence) or released as heat (Figure 2.7). The measurement of the re-emitted light (photon) or chlorophyll fluorescence gives an indication of the state of the plant's photochemistry and ability to dissipate heat (Bose, 1982). In general, maximum fluorescence yield is attained if photochemical efficiency and heat dissipation is at minimum (Bose, 1982; Briantais, et al., Vernotte et al., 1986; Reece et al., 2011).

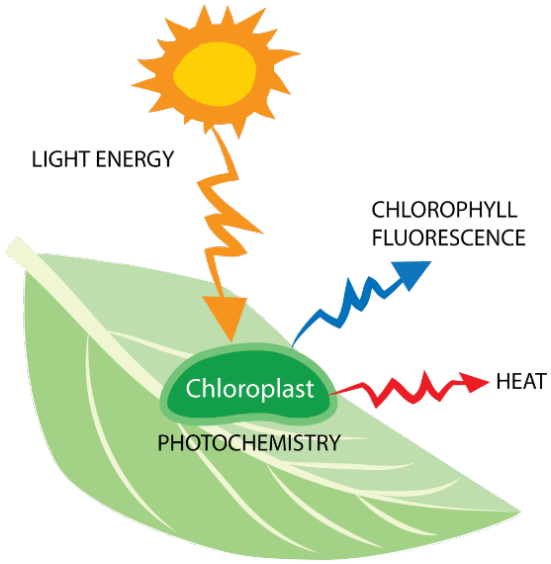


Figure 2.7. Not all light energy is utilised for photochemistry, a portion is emitted back as chlorophyll fluorescence and dissipated as heat (Bose, 1982).

One or 2 % of the total light absorbed by the chloroplast is emitted back as chlorophyll fluorescence (Bose, 1982). Chlorophyll fluorescence is easy to detect because it has a longer wavelength compared to the absorbed light. By exposing a leaf to a specific light wavelength, fluorescence yield can be quantified by measuring the amount of light that is emitted back at a higher wavelength (Reece et al., 2011). To stably capture the re-emitted light, measuring devices use a modulated light source and capture the specific fluorescence excited by the modulated source (Quick & Horton, 1984).

Kautsky (1960) first elucidated the variable response of plants in terms of fluorescence when exposed to light (Maxwell & Johnson, 2000). As described by Maxwell and Johnson (2000), a dark-adapted leaf re-emits higher yield of fluorescence at the first instance of illumination. The moment light is absorbed in the chlorophyll, there is a shortage of electron acceptors in the photosystem II (PSII), specifically the plastoquinone electron acceptor Q_A, creating a state of a PSII reaction centre being 'closed'. This state causes a general reduction of efficiency in photochemistry, and in

consequence, increases the fluorescence yield. However, as Kautsky (1960) found out, this state is very transient and chlorophyll fluorescence will start to decrease in a period of a few minutes, in a process called fluorescence quenching. Fluorescence quenching can either be photochemical or non-photochemical. During photochemical quenching, the decrease in fluorescence yield is caused by an increase of efficiency in the photochemistry as light activates the enzymes that drives carbon metabolism and stomata opening. Subsequently, conversion of energy to heat also increases (non-photochemical quenching, NPQ).

To estimate photosynthesis efficiency using chlorophyll fluorescence yield, one of the two sources of quenching needs to be isolated, or switched off. Bradbury and Baker (1984) and Quick and Horton (1984) developed a method that temporarily closed all PSII reaction centres, resulting in a negligible contribution of photochemical quenching. In this method, a high intensity quick flash of light was emitted to the leaf, causing all reaction centres of PSII to close temporarily. At this moment, maximum fluorescence, *Fm* is attained. The efficiency of PSII can then be estimated by comparing *Fm* to the steady state fluorescence yield in the presence of light (Ft) and yield of fluorescence in the absence of light Fo. To take into account the non-photochemical quenching contribution, the measurement of fluorescence yield needs normalisation from a dark-adapted state, taking *Fm* after dark adaptation, $F°m$.

The most commonly used estimation of photosynthesis on the basis of photochemical quenching is Genty's parameter Φ_{PSII} (Genty et al., 1989), the quantum yield of PSII photochemistry, which measures the efficiency of Photosystem II and is calculated as follows:

$$\Phi_{\mathrm{PSII}} = \frac{Fm' - F_t}{Fm'}$$

where Fm' is the maximum fluorescence when actinic light is applied. From this equation, the proportion of light that entered Photosystem II is being quantified. Φ_{PSII} is also used to estimate the linear electron transport rate (J) as proposed by Genty et. al. (1989) in the following equation;

$$J = \Phi_{PSII} \times PFDa \times (0.5)$$

Where PFDa is the incident light (µmol photon m-2 s-1) and 0.5 is a factor accounting for the energy distribution between the Photosystem II and Photosystem I.

Another fluorescence parameter used to measure PSII efficiency is photochemical quenching, *qP*, which estimates the quantity of open reaction centers in PSII, as given by the equation

$$qP = \frac{Fm' - Ft}{Fm' - Fo'}$$

Genty et. al. (1989) combined the two fluorescence parameters Φ_{PSII} and qP to estimate a third parameter *Fv/Fm* which measures the maximum quantum efficiency of PSII when all PSII reaction centers are open. The following equation describes Fv/Fm where Fv is the variable fluorescence*:*

$$\frac{Fv}{Fm} = \frac{Fm - Fo}{Fm} = \frac{\Phi_{PSII}}{qP}$$

Fv/Fm can be a sensitive indicator of a plant's photosynthesis when measured in a dark-adapted state, as it reflects the potential quantum efficiency of PSII. The change in Fv/Fm is indicative of a change in the non-photochemical quenching efficiency, *NPQ*. In order to accurately estimate *NPQ,* there is a need to measure Fv/Fm value in a dark-adapted state when there is maximal PSII efficiency and minimal heat dissipation. *NPQ* can then be estimated by obtaining the ratio of change in Fm to the final Fm value as described in the following equation by Bilger and Bjorkaman (1990):

$$NPQ = \frac{F°m - Fm'}{Fm'}$$

2.6.2.1 Measuring chlorophyll fluorescence with MINI-PAM

With technology and instrumentation advancement, the methods and analysis for measuring chlorophyll fluorescence has become more practical and straightforward. The invention of fluorometers with a modulated light source – the Pulse-Amplitude-Modulation (PAM), was a major breakthrough in chlorophyll fluorescence research (Schreiber, 2004).

As summarised from the MINI-PAM manual (Walz, 1999), the PAM is able to isolate the two contributors of fluorescence quenching – the photochemical quenching and non-photochemical quenching, by using a 'saturation pulse method' wherein a high intensity pulse of white light is applied which occupies all the electron acceptors in PSII, thereby reducing the rate of electron transport between the two photosystems. The patented measuring principle of the MINI-PAM (Heinz Walz GmbH) is able to tolerate background signal against the fluorescence signal by $1{:}10^6$. The leftover quenching effect is accounted for by the non-photochemical contribution, which can be assumed to be negligible in the period (approx. 1 second) of a saturation pulse.

The MINI-PAM (Walz, 1999) was optimized in such a way that dark-adaptation of samples was not required to estimate the maximal and minimal fluorescence yield. This uses the Genty parameter, Φ_{PSII}, for overall quantum yield of photochemical energy conversion, designated simply as YIELD-parameter in the MINI-PAM measurement:

$$YIELD = \frac{Fm' - F}{Fm'} = \frac{\Delta F}{Fm'}$$

The YIELD measurement is achieved by the MINI-PAM in a very straightforward process (WALZ, 1999). The light source, which is the fibre optics, is pointed into the leaf at approx. 10 mm, followed by pressing the START-key. Within seconds, the fluorescence yield is being acquired, then a saturation pulse is applied, and the final fluorescence, Fm', is measured, followed by the1` calculation of the YIELD parameter. Along with these measurements, environmental parameters are also measured, particularly temperature and light intensity. Photosynthetically active radiation is also being measured, producing a calculation of the apparent rate of electron transport (ETR).

2.5.2.2 Fluorescence yield as a measure of stress response

Chlorophyll fluorescence has been used in various studies to assess stress response including low temperature injury (De Faria et al., 2013; Fracheboud et al., 1999; Griffith et al., 1994; Klosson & Krause, 1981; Melcarek & Brown, 1977; Ottander & Oquist, 1991; Smillie & Hetherington, 1983). Plants that are exposed to stress under ambient light usually have lower Fv/Fm. However, this value could be overestimated because the nonphotochemical quenching contribution increases during stress induction in the presence of light, which decreases the value of Fm. This quenching contribution could not be readily recovered even when the plants are being dark-adapted overnight (Adams III & Demmig-Adams, 2004).

During exposure to stress, nonphotochemcial quenching is also accompanied by deactivation of reaction centers in PSII, and in consequence, favors heat dissipation over photochemistry (Melis, 1999). Looking into changes of either Fo or Fm individually may not be indicative of deactivated reaction centers or increase in nonphotochemical quenching, as these changes can be due to non-quantum related fluorescence yield.

Instead, these changes may be caused by the optical property of the leaf, especially in instances when plant water status is affected. Thus, keeping the fluorescence terms *Fv* and *Fm* as ratios, ensures true estimation of PSII quantum yield (Baker, 2008).

Exposure to low temperature has been observed to cause photoinhibition of photosynthesis, together with reduction in CO_2 assimilation. In field conditions, the occurrence of chilling temperature accompanied with high photon flux greatly reduced the maximum quantum yield of CO_2 uptake (Farage and Long, 1987). Significant reduction (up to 50%) in maximum quantum yield of CO_2 has been observed in *B. napus* planted in the field with temperatures close or below to 0°C, under direct sunlight (Farage & Long, 1991). Immediate damage in the cell membranes caused by freezing has been demonstrated to affect phosphorylation due to the influx of positively charged ions (Santarius, 1973); while more severe damage inhibits electron transport in the photosynthetic pathway (Heber et al., 1973).

In *Zea mays*, the overall quantum yield of PSII (Φ_{PSII}) and Fv/Fm fluorescence parameters were able to distinguish photosynthetic performance of cold tolerant and non-cold tolerant genotypes. Hybrids of selected F3 breeding lines showed 31% increase in photosynthetic capacity, substantiating the potential of Φ_{PSII} as a selection parameter for breeding (Fracheboud et al., 1999). Chlorophyll fluorescence was used to determine the ability of spring-type oilseed rape cultivars to withstand low temperature after cold acclimation (Rapacz, 1999), and effectively screened accessions for frost tolerance in *A. thaliana* (Mishra et al., 2014).

Low temperature exposure close or below 0°C to determine frost hardiness measured by chlorophyll fluorescence strongly correlated with electrolyte leakage assay, and visual assessment in wheat (Clement & vanHasselt, 1996) and white spruce (Gillies

& Binder, 1997). Cold acclimation increased the photosynthetic efficiency (net assimilation rate, μmol (CO_2) $m^{-2}s^{-1}$ from about 2 – 5 in majority of the oilseed rape cultivars, both winter and spring-type. In terms of Fv/Fm, there was no statistically significant difference in cold-acclimated spring type and winter type after exposure to low temperature. Only in winter types where cold acclimation affects Fv'/Fm', with 0.21 in non-cold acclimated and 0.62 in cold acclimated (Rapacz, 1999).

The use of chlorophyll fluorescence as a measure of sensitivity to cold stress has also been used in cereals (Dai et al., 2007; Dolstra et al., 1994; Schapendonk et al., 1989).

2.6.3 Gas exchange

As described by Reece (2011) during photosynthesis, the light reaction utilizes solar energy and converts this to chemical energy, and produces O_2 as a by-product. Through the stomata, CO_2 molecules in the air are absorbed by the plants and incorporated into the chloroplast, initiating the process of the Calvin Cycle. Together with the energy produced from the light reaction, CO_2 is converted into carbohydrates. Oxygen molecules, the by-product of the light reaction, are liberated through the stomata. With sufficient water supply, adequate exchange of CO_2 and O_2 for photosynthesis is maintained. Meanwhile, transpiration – the loss of water vapour by diffusion and evaporation also occurs in the stomata. In response to the changing environmental conditions, the stomata can open and close to conserve water for photosynthetic requirements.

The gas exchange systems quantify photosynthesis and transpiration by measuring CO_2 and H_2O flow with an infrared gas analyser (IRGA). The Li-6400 (LI-COR Inc.) gas exchange system estimates the net photosynthesis (A), transpiration rate

(E), stomatal conductance (g_{sw}) and intercellular CO_2 concentration (C_i) based on Von Caemmerer and Farquhar (1981) (LI-COR Inc., 1998).

Assimilation of CO_2 in the leaves can be affected primarily when plants are exposed to changes in temperature, light, nutrition and other environmental stimuli. The assimilation rate even varies based on the plant's stage of development (Von Caemmerer & Farquhar, 1981).

Figure 2.8 shows the processes in the plant leaf where chlorophyll fluorescence and gas exchange measurements are derived.

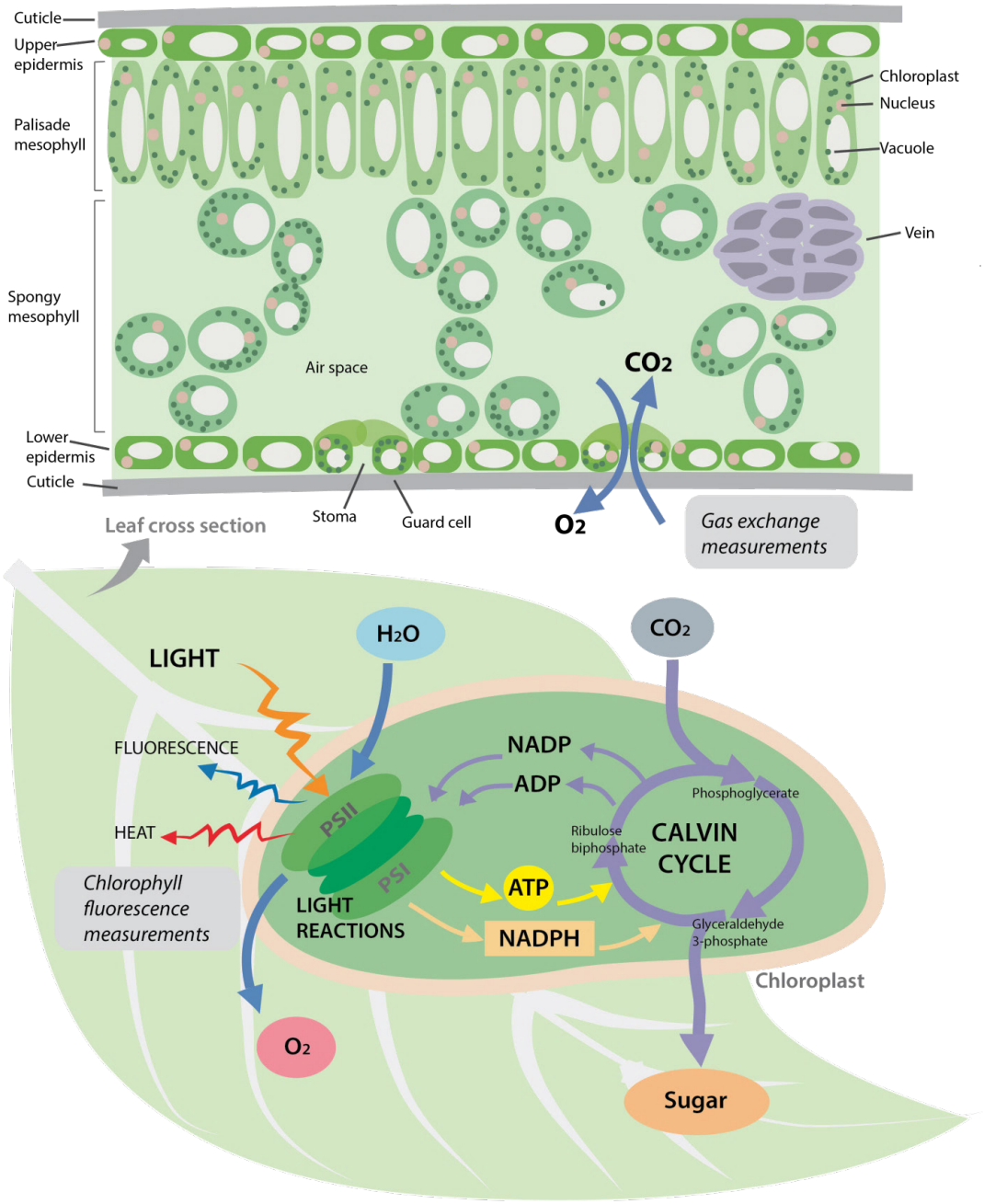


Figure 2.8. Representation of the magnified parts of the leaf where gas exchange and chlorophyll fluorescence measurements are based. Gas exchange measurements are based on the flow of CO_2 coming into the mesophyll layer and O_2 going out, through the stomata (top). Light energy absorbed in photosystem II (PSII) inside the chloroplast is used to drive photosynthesis, while excess energy is dissipated as heat or re-emitted as chlorophyll fluorescence (bottom). The light reaction utilises light energy to split water molecule into hydrogen ions, giving off O_2. The light energy also provides electrons and hydrogen ions, reducing NADP to NADPH, and provides a phosphate group to ADP, producing ATP. ATP and NADH are used in the Calvin cycle. To produce sugar, CO_2 molecules entering through the stomata are incorporated into the Calvin cycle. *Source: Reece et al. (2011). Campbell Biology. Pearson Boston.*

2.7 *Agrobacterium*-mediated transformation of *Brassica napus*

In vitro manipulation of oilseed *Brassica* has been well studied, resulting in the development of protocols for organogenesis of various canola cultivars. Regeneration of fertile plants has been accomplished using different source of explants, such as hypocotyl (Barsby et al., 1986; De Block et al., 1989; Glimelius, 1984; Tang et al., 2011), cotyledons (Bhalla & Singh, 2008; Hachey et al., 1991; Moloney et al., 1989), peduncle (Christey & Earle, 1991), roots (Lazzeri & Dunwell, 1984; Sharma & Thorpe, 1989), leaves (Akasaka-Kennedy et al., 2005; Dunwell, 1981), stem (Fry, et al., 1987; Stringam, 1977) and protoplasts (Barsby et al., 1986; Klimaszewska & Keller, 1987; Kohlenbach et al., 1982; Li & Kohlenbach, 1982; Xu et al., 1982). Factors affecting *in vitro* regeneration have been optimized, such as media composition, type of explants, combination of plant growth regulators, growth conditions and plant genotype. Most importantly, regeneration in *Brassica* is highly dependent on genotype, hence the development of various protocols of specific genotypes for *in vitro* regeneration. Ono et al., (1994) assessed the potential of 100 cultivars for shoot regeneration from cotyledon explants and found regeneration potential ranged from 0% to 97%.

The success of *in vitro* organogenesis of *B. napus* lead to the application of genetic transformation to modify the plant's gene expression for specific traits. Transgenic technology in canola was mostly directed to` oil quality improvement (Katavic et al., 2001; Knutson et al., 1999; Murphy, 1996; Stoutjesdijk et al., 2000), and herbicide tolerance (Gianessi et al., 2002; Kishore et al., 1992; Stringam et al., 2003).

Agrobacterium-mediated transformation is the most widely used method in transgenic technology to commercially produced canola (ISAAA, 2016). Pioneering studies in *Brassica* transformation explored the ability of different strains of

Agrobacterium to infect different cultivars of *Brassica*. *Agrobacterium rhizogenes* and *A. tumefasceins* utilized tumour-inducing (Ti) or root-inducing (Ri) T-DNA, respectively, as a vector for introducing foreign genes. The vector molecules are then reconstructed such that it uses the *trans* acting functions of the *vir* region, in the case of *A. tumefaciencs*, the Ti plasmid, to transfer the segment of DNA bordered by the left and right T-DNA sequences, into the nuclear genome of the host plant. The T-DNA region contains multiple cloning sites such that sequences of interest can easily be inserted (Bevan, 1984; Guerche et al., 1987; Holbrook & Miki, 1985). In *Brassica*, the nopaline strains were found to be more effective to induce teratomas and have higher virulence compared to the octopine strains (Charest et al., 1988; Fry et al., 1987). The use of the binary system of T-DNA, whereby a constructed T-DNA harbouring the gene of interest is used together with a helper plasmid containing the virulence genes from the Ti plasmid of *A. tumefasciens,* proved to be highly effective in producing normal and fertile transformants in *Brassica* (Cardoza & Stewart, 2004; Damgaard et al., 1997; Dutta et al., 2008; Moloney et al., 1998; Radke et al., 1992; Wahlroos et al., 2003).

Selection is an important part of the transformation system for the efficient recovery of transformed cells, which inhibits regeneration of non-transformed cells. Selection is usually done by using selectable marker genes, to which the product can either confer resistance or be less sensitive to the selective agent. The most widely used selectable gene in *Brassica* transformation is neomycin 3'-O-phosphotransferase (NPTII), conferring resistance to aminoglycoside antibiotics such as kanamycin, neomycin and geneticin (Fraley et al., 1983; Poulsen, 1996; Puddephat et al., 1996). Other types of selectable markers include resistance to the herbicides phosphinotricin (De Block et al., 1989), chlorsulfuron, 2,4-dichlorophenoxyacetic acid, bleomycin, bromoxynil and

glyophosate (Bowen, 1993). Positive selectable marker genes have been developed as there were initial health and environmental concerns regarding the use of antibiotics and herbicides. Positive selection uses a physiologically inert substance as the selective agent, which is metabolised by the marker gene in transformed cells, producing a positive signal or expression. Positive markers include gusA (b-glucuronidase), which also serves as a reporter gene, phosphomannose isomerase, and xylose isomerase (Joersbo, 2001; Penna et al., 2002).

2.8 *Arabidopsis* Acyl-Coenzyme A-Binding Proteins (ACBPs)

The first ACBP isolated from plants was cloned from *Brassica napus* (Hills et al., 1994) and was shown to be involved in acyl-CoA transport to glycerol-3-phosphate acyltransferase (Brown et al., 1998). The mRNA was highly expressed in cotyledons, developing embryos and flowers, and at lower levels in leaves and roots, indicating the possible role of ACBP in lipid metabolism in plants. The cloned ACBP consisted of a 92 amino acid length polypeptide with molecular mass of 10.2 kDa, and shared a high degree of conserved amino acids with homologues from yeast, Drosophila, cow and human.

In *Arabidopsis*, 6 ACBPs ranging from 10 to 73 kDa, which are conserved at the acyl-CoA binding domain at the amino end, but differ in motifs at the carboxyl terminus were identified (Chye, 1998; Leung et al., 2004). ACBP1 (Chye 1998; Chye et al. 1999; Chye et al. 2000) and ACBP2 (Gao et al., 2009; Li & Chye, 2003; Xiao et al., 2008) contained ankyrin repeats, whereas ACBP4 and ACBP5 (Leung et al., 2004; Li et al., 2008; Xiao et al., 2008) possessed a kelch motifs; both of which were shown to facilitate protein-protein interactions (Gao et al., 2009; Li & Chye, 2004). ACBP3 (Leung et al., 2006) and ACBP6 (Chen et al., 2008) only contained the acyl-CoA protein binding domain. ACBP6 was first identified by

Engeseth et al., (1996) in *A. thaliana* and is a 10.4 kDa protein which was shown to bind oleol-CoA and protect it from hydrolysis by microsomal acyl hydrolases.

Studies on several 10-kDa ACBPs indicated that they were involved in acyl-CoA transport within the cell and protected the acyl-CoAs from degradation by hydrolytic enzymes within the cytosol (Knudsen et al., 2000; Xiao & Chye, 2009). Specifically, ACBPs facilitate transport of acyl-CoA from the plastid, where biosynthesis of plant fatty acids primarily occurs, to the endoplasmic reticulum wherein enzymes responsible for the synthesis of nonplastidial membrane lipids and triacylglycerol are localized (Xiao & Chye, 2009). These ACBPs also play an important role in maintaining acyl-CoA pools and glycerolipid biosynthesis in the cells (Huang et al., 2005; Mandrup et al., 1993), and in the transport of acyl-CoAs for lipid biosynthesis. Larger ACBPs identified in Arabidopsis were found to be involved in developmental roles and stress response (Xiao & Chye, 2011b).

Using site-directed mutagenesis and $(His)_6$-tagged recombinant fusion proteins of the ACBPs, their binding affinity to various acyl-CoA esters were determined to be specific. Furthermore, their ability to bind phospholipids was found to be preferential based on their C-terminal domain (Chye et al., 2000; Leung et al., 2004; Leung et al., 2006; Xiao et al., 2009). The ACBPs were shown to be distributed in different locations in the cell. ACBP1 and ACBP2 were localized in the plasma membrane and endoplasmic reticulum (Chye et al., 1999; Leung et al., 2004); while ACBP3 (Leung et al., 2006) was determined to be predominantly located in the apoplast, and also associated with intracellular membranes and the endoplasmic reticulum and golgi complex. The larger ACBPs (ACBP4 and ACBP5) were localized within the cytosol (Xiao et al., 2008). Lastly, ACBP6 (Chen et al., 2008) which is the smallest among the 6 *A. thaliana* ACBPs, is also located in the cytosol. ACBP4, ACBP5 and ACBP6 were

shown to facilitate transport of cytosolic acyl-CoA and maintenance of acyl-CoA esters during production of fatty acids in the plastids (Xiao and Chye, 2011).

The ACBPs are also associated with plant development and stress response. ACBP1 and ACBP2 were detected to accumulate in different stages of seed development and maturation, during which long-chain fatty acids were actively involved (Chen et al., 2010). Disruption of ACBP1 and ACBP2 (using insertional mutants) halted development of embryos, indicating their roles in transport of lipid during embryogenesis (Chen et al., 2010; Napier & Haslam, 2010). ACBP3 showed upregulation during senescence and carbon starvation in *Arabidopsis* leaves (Xiao et al., 2010). The levels of ACBP3 and phospholipid phosphatidylethanolamine (PE) were detected to be highly correlated *in vivo*, and their interaction influenced phospholipid metabolism (Xiao et al., 2010). When overexpressed, the ACBP3 accelerated leaf senescence and when removed, senescence was delayed in *Arabidopsis* leaves. Xiao et al. (2010) determined that the interaction of ACBP3 with PE was associated with autophagy-mediated senescence. ACBP1 transformed *Arabidopsis* plants showed tolerance to Pb(II) and were able to accumulate the metal in the shoots (Xiao et al., 2008b), while transformed plants overexpressing ACBP2 were tolerant to Cd(II) and Cu(II) and oxidative stress induced by hydrogen peroxide treatment (Gao et al., 2009). These traits were associated with both ACBP1 and ACBP2 involved in phospholipid membrane repair (Xiao & Chye, 2009; Xiao et al., 2008b). Expression of ACBP6 protein and mRNA production were cold-inducible (Chen et al., 2008). When overexpressed, *Arabidopsis* plants were able to withstand freezing stress up to -8°C (Chen et al., 2008). Overexpression of ACBP6 in freezing exposed plants also induced production of phospholipase Dδ (Chen et al., 2008), which is a catalyst to the conversion of phospholipid to phosphatidic acid. Phospholipids have been known to be important in the stress response cascade (Chen et al., 2008; Li et al., 2004). Both

ACBP2 and ACBP4 were shown to interact with another *Arabidopsis* protein – the ethylene-responsive element binding protein (AtEBP), which is a transcription factor involved in abiotic and biotic stress response (Büttner & Singh, 1997). ACBP3 overexpressing *A. thaliana* plants showed resistance to *Pseudomonas syringae*, a bacterial plant pathogen (Xiao & Chye, 2011).

2.9 The Rapid-cycling *Brassica*

2.9.1 Development of the rapid-cycling Brassica *populations*

The *Brassica* 'fast plants' or rapid-cycling *Brassica* are populations of special *Brassica* developed through artificial selection for rapid completion of seed-to-seed life cycle (Friend and Helson,1966). Unlike normal populations of *Brassica* species (long-life *Brassica*), the rapid-cycling plants may start to flower within 14 days and reach seed maturity within 22 days. While most *Brassica* species are either biennals or annuals, the fast plants can reach up to 10 cycles in a year. Rapid-cycling *Brassica*s were first developed by Friend and Helson (1966) by selecting progenies from a cross between early flowering individuals.

The development of rapid-cycling *Brassica* base population was first conceived in 1970 when a germplasm collection of over 2000 accessions sourced from the United States Department of Agriculture National Plant Germplasm System was grown at the University of Wisconsin-Madison (Williams & Hill, 1986). A cultivar of *B. campestris (rapa)* 'Arlo', was previously identified as an early flowering variety, which can form an inflorescence as early as 6 days when exposed to a long day photoperiod. The rapid-cycling variety 'Arlo' has since then used by the Canadian Department of Agriculture as an experimental rape breeding material (Friend & Helson, 1966). Realising the potential of fast plants as an educational and plant biology tool, a germplasm source for rapid-

cycling *Brassica* representing the principal *Brassica* species was developed (Williams & Hill, 1986).

As accounted by Williams & Hill (1986), during the early stages of development of the rapid-cycling population, to establish a stable population of fast plants, early flowering types within each species were crossed. The plants were established in high density format using 28 by 55 centimetre pots in a 96-pot seedling tray, maintained at 24°C under 24 hour fluorescent lighting (250 μmol m^{-2} s^{-1}). Individual plants were selected based on the following traits: small plant size, early flowering, uniform flowering maturation, rapid seed maturation, absence of seed dormancy, and high female fertility. Subsequent generations were derived from 10% of the population that had the shortest time to flower, and selection was terminated until more than 50% of the population flowered within a 3-day margin. Population was increased by mass pollination. Rapid-cycling base populations (RCBPs) for *B. campestris (rapa), B. nigra, B. oleracea, B. juncea, B. napus* and *B. carinata* were established (Table 2.1). From these RCBPs, *B. campestris* (*rapa*) exhibited the most desirable combination of the traits they are selected for.

Table 2.1. The Rapid-cycling *Brassica* Collection base populations and corresponding phenotypic characteristics when grown at 24°C under continuous light.

Species	Haploid number	Cytoplasm symbol	Diploid genome	Days to flower	Length (cm)to first flower	Seeds per plant	Days for cycle	Cycles per year
B. rapa	10	A	Aaa	16	12	78	36	10
B. nigra	8	B	Bbb	20	27	69	40	9
B. oleracea	9	C	Ccc	30	23	18	60	6
B. juncea	18	AB	ABaabb	19	30	107	39	9
B. napus	19	AC	ACaacc	25	35	76	55	6
B. carinata	17	BC	BCbbcc	26	42	67	56	6

Williams & Hill (1986) also noted that in terms of flowering time and plant morphology, the RCBPs displayed variability within population for each species when isozymes were examined. Moreover, when the plants were screened for various diseases, a wide variation from susceptible to resistance was observed in between plants. Although the rapid-cycling brassicas were fixed for the specific traits of shorter life cycle and small plant morphology, they were quite highly influenced by the environment in which they were grown. When these plants were grown with more space for unrestricted growth, the plant size increased many times bigger compared when they are grown at 2500 plants per square meter while in seed production.

The rapid-cycling populations of *Brassica* have been utilized for *in vitro* regeneration techniques, bioassay and stress response studies; and as a model for plant genetics studies, and crop improvement using transgenic methods. An immortal mapping population of rapid-cycling *B. rapa* and *B. oleracea* was developed, and the molecular markers and linkage map data were made available to the public (Iniguez-Luy et al., 2009). A recombinant inbred line population of rapid-cycling *B. rapa* was also developed, and exhaustively characterized using 270 molecular markers, producing a linkage map with an average density of 4.3 cM/marker. The population was analysed for 17 traits associated with plant architecture and seed characteristics. QTL analysis of this population revealed 47 QTLs linked to the traits analysed (Bagheri et al., 2012). These advancements in the rapid-cycling *Brassica* populations will provide valuable information and be a useful tool for genomic studies.

2.9.2 The rapid-cycling **Brassica** *as model plants for teaching and research*

The RCBPs, especially *B. rapa,* have great potential for teaching and research. Tomkins and Williams (1990) used these plants for teaching Genetics at the University of Wisconsin – Madison. The utility of the fast plants to study inheritance was demonstrated by Price and Harding (1993), wherein Mendelian phenotypic traits in the progenies of a parental test cross were quantitatively predictable.

The short life cycle of the rapid-cycling *Brassica* enables breeders to rapidly select and breed for traits in a shorter time than for normal cycle *Brassica* that take many years to accomplish. In resistance breeding for *Albugo candida* (white rust) by Edwards & Williams (1987), a rapid-cycling population of *B. rapa* was used to investigate resistance derived from selection for minor genes. The rapid-cycling *B. rapa* plants were susceptible to the pathogen, and by 3 cycles of selection, minor genes were effectively accumulated to confer reduction in pathogen sporulation. In a study by Shimabuku (1991), *B. rapa* having the shortest life cycle, has been conveniently used in bioassay studies plant life cycle, and has been used to study for toxicity of the herbicide dalapon (2,2-dichloropropionic acid). In another study, toxicity to uranium was assessed in the entire life cycle of *B. rapa*, and a toxicity threshold was effectively determined; however, there was high variability between plants in terms of height and seed appearance because of the inherent variation present in the base population (Sheppard et al., 1992). To minimize variability, the *B. rapa* plants used in another bioassay study were terminalized after the first batch of siliques started to mature and before the start of axial bud development. From these studies, *B. rapa* was shown to have has a plastic phenotype, which can be easily manipulated to become more uniform by controlling light level and nutrient supply. By tweaking inherent sources of variation, *B. rapa* proved to be an ideal model plant for

bioassay of Hg and Zn sensitivity, as it responded sensitively to treatment on various life cycle stages (Sheppard et al., 1993).

A model for crop population genetics by Briggs & Goldman (2006) was established using rapid-cycling *B. rapa,* investigating response to selection, and dynamics of genetic variance and population diversity during bottlenecks. Ten cycles of recurrent selection for cotyledon size were done on populations established from 200 individuals, and from 3 bottlenecks, which originated from 2 individuals. Because of the rapid and laboratory-based nature of the rapid-cycling *B. rapa*, an artificial selection experiment was made possible whereby substantial phenotypic changes were readily observed. This process could take many years to develop in other plant systems.

2.9.3 In vitro *regeneration of rapid-cycling* Brassica

Rapid-cycling *Brassica* was first grown *in vitro* to determine their performance in an enclosed and limited space from seed to maturity (Lentini et al., 1988). The accumulation of ethylene was investigated and manipulated using ethylene chemical inhibitor 2,5 norbornadiene, and by using different kinds of tissue container caps with different porousness. The size and the life cycle of the plant make it suitable to be studied *in vitro* whereby plant morphology can easily be observed.

In vitro protocols developed for normal cycling or long life *Brassica*s specifically in regenerating new plants using protoplast culture as a prerequisite for somatic hybridization and genome transformation (Bidney et al., 1983; Glimelius, 1984; Barsby et al., 1986; Charest et al., 1988) were applied to the rapid-cycling types. Of the 6 rapid-cycling *Brassica* species (*B. napus, B. oleracea, B. rapa, B. nigra, B. carinata* and *B. juncea*), *B. napus* readily regenerated from protoplast cultures with 2% of the protoplast

developing into callus, and 5% of the calli differentiated to form shoots and subsequently into new plants (Loudon et al., 1989); while *B. oleracea* required more optimisations (Hansen & Earle, 1994; Kik & Zaal, 1993).

Production of haploid and double haploid plants to develop homozygous plants for plant breeding has been investigated by Aslam et al. (1990) using anther culture in the rapid-cycling *Brassica* populations. Rapid-cycling *B. napus* and *B. rapa* response to anther culture varied greatly based on growth condition of anther donors, specific stage of pollen growth and subsequent incubation conditions. Production of embryoids was more favourable for *B. rapa* with maximum rate of embryogenesis of 96% after 3 generations of inbreeding and selection, compared to *B. napus* (13.8%) (Aslam et al., 1990).

Successful *in vitro* regeneration of rapid-cycling *B. oleracea* with 88% percent regeneration was achieved from peduncle explants harvested from bolting plants with closed flower buds. The explants were grown on Linsmaier and Skoog (LS) medium (Linsmaier & Skoog, 1965) with amendments. Shoot regeneration occurred at 7 days in the medium and transplanting of fully elongated shoots with well-established roots were accomplished within 4 weeks (Christey & Earle, 1991).

Direct somatic embryogenesis from seeds has been used to establish *in vitro* plants of rapid-cycling *B. napus* (Koh & Loh, 2000). Embryonic potential was observed to be significantly higher in immature seeds with 41-68 % embryo produced. Somatic embryo induction was achieved without specific wounding or treatment with exogenous plant growth regulators. Plant growth regulator treatment was only done in the subsequent steps of plantlet regeneration whereby supplementation with cytokinin such as BAP and zeatin (10^{-6} to 10^{-4} M) increased regeneration of secondary embryos into plantlets to 70%. *In*

vitro flowering was induced by growing the plantlets in MS medium with controlled nitrogen, phosphate, and sucrose content (Koh & Loh, 2000).

Direct shoot regeneration from cotyledonary explants of *in vitro* grown rapid-cycling *Brassica*s proved to be a robust method for producing new plants. The method was first applied to rapid-cycling *B. rapa* (Teo et al., 1997) and with much success, applied to rapid-cycling *B. oleracea* (Cheng et al., 2001). The explants used were hypocotyl segments excised from *in vitro* grown seedlings. Explants from 3-day-old seedlings regenerated the most number of shoots with regeneration potential decreasing with age. The ethylene inhibitor 2-aminoethoxyvinyl glycine (AVG) enhanced regeneration, while $AGNO_3$ inhibited shoot formation. Root formation was promoted by the addition of 1-naphthaleneacetic acid **(**NAA) (0.5 – 2.0 μM) or the combination of indole-3-butyric acid (1.5 μM) and kinetin (4.6 μM). An average of 2.5 plants per cotyledonary explant was regenerated. In *B. oleracea*, internodal segments from the cotyledon also produced shoots. Shoot regeneration benefitted from the addition of BAP into the medium, while NAA enhanced root formation. In an effort to optimise an *in vitro* regeneration system most ideal for genetic transformation, Cogbill et al. (2010), utilized cotyledonary explants to produce adventitious shoots. The main difference of this protocol to Teo et al. (1997) and Chen et al. (2001) was that, meristematic tissues were removed by completely excluding the petiole in the cotyledon explant, thereby reducing the chance of chimaera formation after transformation.

Various approaches have been applied to the rapid-cycling *Brassica*s to attain a high regeneration frequency, and the development of consistent and reproducible culture conditions has been the main focus of research. However, the genotype of the plant species has been shown to greatly affect the success of regeneration. To attain a better

understanding of the genetic nature of regeneration, quantitative trait loci (QTL) was analysed in rapid-cycling *B. oleracea* F2 mapping population, wherein 2 QTL were identified which explained 83% of total genetic variation for plant regeneration. The QTL mapped into chromosomes 7 and 8 (Holme et al., 2004).

2.9.4 Genetic transformation of rapid-cycling Brassica

Genetic transformation of rapid-cycling *Brassica* was first reported by Berthomieu and Jouanin (1992), on rapid-cycling *B. oleracea var. capitata*, using *Agrobacterium rhizogenes,* based on the *in vitro* regeneration protocol for transformation of rapeseed (Guerche et al., 1987). The explants used for tissue culture were from leaf petioles of 10-day-old *in vitro* grown seedlings and one-month-old greenhouse plants. Shoots were regenerated from root clones excised from the inoculated petioles. Regeneration of green calli from root fragments was very low; root growth took a long time to develop, and the resulting phenotypes had a reduced apical dominance. Nevertheless, transformation of rapid-cycling *B. oleracea* was successfully achieved using both wild type and disarmed strains of *Agrobacterium tumefaciens* (Beclin et al., 1993). The *aux2* gene from *A. rhizogenes*, which converts naphthalene acetamide to auxin naphthalene acetic acid, was transformed into *B. olearacea* using a protocol developed for trees (Brasileiro et al., 1991). The inoculation was carried out by wounding the *in vitro* propagated plantlets with forceps dipped in the prepared bacterial suspension. Tumours that formed were excised 1 month after inoculation, and subsequently cultured to form shoots. Regeneration of new shoots from calli was high; however, when transgenic activity was evaluated by GUS assay, only 1.7% confirmed to be positively transformed (Beclin et al., 1993). Berthomieu et al., (1994), used a co-inoculation

technique with both wild type and disarmed nopaline types of *A. tumefaciens* to transform rapid-cycling *B. oleracea*. This approach utilized the ability of wild-type *A. tumefaciens* to induce shooty tumours, which were readily excised and grown into shoots, thereby bypassing the complications of developing an *in vitro* regeneration system. Before transformation, the plants were tested for susceptibility to the wild type *A. tumefaciens* strains. Inoculation on *in vitro* grown 20-day-old cabbage plants was performed by nipping the stem with forceps dipped into the bacterial solution. The tumours formed were harvested and subsequently cultured *in vitro*, and the plantlets were transferred into soil. Transformation efficiency (total) was 8.12% based on GUS assay. Chimaerism in the progeny was observed, and confirmed by southern analysis.

2.9.5 Biotic and abiotic response in rapid-cycling Brassica

Rapid-cycling *B. napus* was used for resistance breeding to herbicide Chlorsulfuron and leaf spot pathogen *Alternaria brassciola*, using UV mutagenesis on isolated microspores (Ahmad et al., 1991). The microspore viability was 50-70% based on fluorescein diacetate (FDA) staining of at least 100 micropsores. UV irradiation (3.3 J $m^{-2}s^{-1}$) established a LD50 at 0s, and 120 seconds exposure with 100% kills. Regeneration capacity of embryoids to new plants was unaffected, and plant morphology was the same in both irradiated and non-irradiated controls, while the majority produced normal flowers and were able to set fertile seeds. Lines resistant to chlorsulfuron and to the fungal pathogen *Alternaria brassciola* were successfully established (Ahmad et al., 1991).

There is a good potential to breed for specific traits in the rapid-cycling *Brassica* populations as they retain high genetic variability. The *Brassica* fast plants were used to

study pathogenicity for a population of *L. maculans*, the fungal pathogen responsible for 'blackleg' disease in *Brassica*s, collected from North America, Europe and Australia. The plant-to-plant variability was high within the rapid-cycling plants base population, thus the pathogenicity groups established in commercial *B. napus* cultivars were not differentiated in the same manner (Mengistu et al., 1991). Artificial selection to increase trichrome number as a defence against herbivory from cabbage butterfly *Pieres rapae* was done on *B. rapa* fast plants, indicating a substantial variation for this trait in the *B. rapa* base population (Agren & Schemske, 1992).

The rapid-cycling *Brassica* population is amenable to somatic hybridization. Resistance to blackspot disease *A. brassicicola* has been successfully introgressed from a wild relative *Camelina sativa* (L.) Crantz and *Capsella bursa-pastoris* (L.) Medik. to rapid-cycling *B. oleracea*. However, the hybrid plants did not cope well in glasshouse conditions, and had very low fertility (Sigareva & Earle, 1999). Interspecific hybridization using rapid-cycling *B. oleracea* (CC) crossed with *B. carinata* (BBCC), resulted in 8.2 ± 0.68 of ovules per silique fertilized, albeit crosses that use the CC genome as the female parent resulted in lower number of fertilized ovules, and slower embryo growth and development; survival rate of embryo rescue was very low (Rahman, 2004).

Taking advantage of the short life cycle of the fast plants, the six base populations of rapid-cycling Brassicas - *B. napus, B. rapa B. nigra, B. juncea, B. oleracea* and *B. carinata* were screened for seawater salinity tolerance. The effect of salinity on plant growth was measured based on the weight of dry matter. With increasing salt stress, there was a decline in total dry weight (shoot and root). In terms of relative growth response, the most tolerant species was *B. napus* followed by *B. rapa, B. nigra, B. juncea, B. oleracea,* and *B. carinata* which was the most salt sensitive with (Tie & Cramer, 1992).

Following this study, the extreme reactions of rapid-cyling *B. napus* and *B. carinata* were further studied, and their apparent physiological differences were demonstrated based on relative growth rate and net assimilation rate (He & Cramer, 1993b). The same response to salinity was also demonstrated in the callus stage of these plants (He & Cramer, 1993).

Rapid-cycling *B. rapa* was also evaluated for variation in waterlogging tolerance based on physiological response to root zone hypoxia. Recurrent selection was done for 7 generations to attain homogenous populations of waterlogging tolerant and sensitive plants. Indeed, a distinct difference between the tolerant and sensitive populations was observed, based on levels of alcohol dehydrogenase, chlorophyll content, leaf soluble carbohydrates, and adventitious root formation (Daugherty et al., 1994; Daugherty & Musgrave, 1994). The effect of irrigation and seed quality development in rapid-cycling *Brassica* was well studied, addressing different aspects in seed desiccation tolerance and longevity, by analysing the accumulation of stress proteins, soluble carbohydrates and heat-stable proteins (Bettey et al., 1998; Sinniah et al., 1998).

NASA studied the rapid-cycling *B. napus* as an oilseed crop considered for a program on controlled ecological life-support systems (CELSS) (Frick et al., 1994). The yield and seed oil content of rapid-cycling *B. napus* were meticulously studied based on nitrogen treatments, planting density, and carbon dioxide treatment. Seed yield and seed oil content both benefitted when nitrogen treatment was delayed and applied at the minimum amount of 15 mM. CO_2 enrichment stimulated vegetative growth, reducing the seed yield rate and harvest index. Planting density only affected seed yield response when the environment was enriched with CO_2, as the plants developed higher total shoot biomass per unit area, resulting in competition for nutrients (Frick et al., 1994).

Chapter 3

In vitro regeneration of rapid-cycling *Brassica* from cotyledon explants

3.1 Introduction

The *Brassica* 'fast plants' or rapid-cycling brassicas are populations of special genetic lines of *Brassica* developed through artificial selection for rapid completion of their life cycle (Williams & Hill, 1986). Unlike normal populations of *Brassica* species (long duration *Brassica*), the rapid-cycling plants may start to flower within 14 days and reach seed maturity within 22 days. While most *Brassica* species are either biennials or annuals, the rapid-cycling plants can reach up to multiple cycles in a year. Grown at 24°C under continuous light, the rapid-cycling *B. napus* plants have an average of 55 days to complete a life cycle, and in a year, can complete about 6 cycles; while *B. juncea* has an average of 39 days and can complete 9 cycles per year.

The small morphology and short life cycle of the rapid-cycling brassicas make them ideal for experimental use in controlled laboratory conditions. Through the establishment of Crucifer Genetics Cooperative at Madison Wisconsin (presently under the Wisconsin Fast Plants Program website http://www.fastplants.org), the rapid-cycling brassicas have been in demand for educational and research purposes. The rapid-cycling *Brassica* species have been used in studies in *in vitro* regeneration techniques (Cheng et al., 2001; Christey & Earle, 1991; Hansen & Earle, 1994; Kik & Zaal, 1993; Koh & Loh, 2000; Teo et al., 1997); in stress response studies (Agren & Schemske, 1992; Schmidt et al., 1993; Sheppard et al., 1993; Shimabuku, 1991); as a model for plant genetics (Briggs et al., 2000; Price & Harding, 1993); and in genetic transformation research (Beclin et al., 1993; Berthomieu & Jouanin, 1992).

Various methods of *in vitro* manipulation of plant tissues have been developed as a prerequisite for production of plants with improved traits. Among the rapid-cycling brassicas, *B. oleracea* and *B. rapa* have been relatively well utilized for tissue culture studies. *In vitro* regeneration of *Brassica* is genotype dependent, therefore, robust protocols established for commercial cultivars and breeding lines of *Brassica* cannot be readily applied to the rapid-cycling plants. Nevertheless, *B. rapa* rapid-cycling plants were used to study anther embryogenesis for production of haploid plants, and subsequent development of homozygous plants (Aslam et al., 1990), while *B. oleracea* rapid-cycling plants were used to regenerate shoots from peduncle explants for genetic transformation (Christey & Earle, 1991) and protoplast culture for somatic hybridization (Hansen & Earle, 1994; Kik & Zaal, 1993).

Shoot regeneration from cotyledon and hypocotyl explants was successfully achieved in *B. rapa* and *B. oleracea* rapid-cycling plants, primarily aimed for genetic transformation (Cheng et al., 2001; Teo et al., 1997). Compared to rapid-cycling *B. oleracea* and *B. campestris (rapa*), there have been very few studies into the *in vitro* regeneration of *B. napus* and *B. juncea* rapid-cycling plants, specifically tailored for genetic transformation. Loudon et al. (1989) showed that rapid-cycling *B. napus* had poor response to protoplast culture and was not very amenable to anther culture (Aslam et al., 1990). Koh and Loh (2000) successfully regenerated rapid-cycling *B. napus* plants through direct somatic embryogenesis from seeds. Shoot regeneration from cotyledon and hypocotyl explants of *in vitro* grown rapid-cycling *Brassica*s proved to be a robust method for producing new plants and presented an ideal system for *Agrobacterium*-mediated transformation. This method was first applied to rapid-cycling *B. rapa* (Teo et al., 1997) and with much success, applied to rapid-cycling *B. oleracea* (Cheng et al.,

2001). Cogbill (2010) also used cotyledon explants of rapid-cycling *B. oleracea* and successfully regenerated adventitious shoots.

The rapid-cycling *Brassica* studies of Cheng et al. (2001), Teo et al. (1997), and Cogbill et al., (2010), showed that the amount and combination of cytokinin and auxin were very crucial for successful *in vitro* regeneration of rapid-cycling brassicas. For cotyledon explants of rapid-cycling *B. oleracea,* 20 μM of N^{6}-benzyladenine (BA) was required for high-frequency shoot regeneration while 0.5-2.0 μM of α-naphthaleneacetic acid (NAA) promoted root formation of regenerated shoots (Cheng et al., 2001). Rapid-cycling *B. rapa* cotyledon explants regenerated only in media amended with 10-40 μM BA and 2 μM NAA (Teo et al., 1997) while adventitious shoots formed directly from cotyledon (without petiole) with 1.5 mg/L thiadiazuron (TDZ), a cytokinin, and 0.5 mg/L NAA (Cogbill et al., 2010). Shoot regeneration from hypocotyl and cotyledon explants of long duration *Brassica* species were highly variable, with *B. napus* requiring 10-20 μM of BA combined with 0-0.5 μM NAA (Bhalla & Singh, 2008; Moloney et al., 1989; Ono et al., 1994; Tang et al., 2003). *Brassica rapa* and *B. juncea* regenerated plants from cotyledon explants with 4.5-9 μM BA and particularly higher amount of NAA (5.4-16 μM) (Hachey et al., 1991; Mukhopadhyay et al., 1992; Pental et al., 1993).

Inherent physiological differences between long-duration and rapid-cycling brassicas were observed *in vitro*, such as varying responses to regeneration conditions and period of viability for morphogenesis (Mukhopadhyay et al., 1992; Teo et al., 1997). However, the genotype-specific nature of regeneration in *Brassica* also makes it necessary to develop an *in vitro* regeneration protocol for shoot regeneration for each rapid-cycling genotype. The short life cycle, small plant habit, high female fertility, absence of seed dormancy, and sexual compatibility with cultivated brassicas (Williams

& Hill, 1986), made the rapid-cycling *Brassica* ideal for genetic transformation and subsequent molecular and physiological characterization (Mukhopadhyay et al., 1992). The self-compatible species of rapid-cycling *B. napus* and *B. juncea* have not been studied for shoot regeneration from cotyledon explants. In this chapter, an *in vitro* regeneration protocol was developed for rapid-cycling *B. napus* and rapid-cycling *B. juncea* that can be used for *Agrobacterium*-mediated transformation.

3.2 Materials and Methods

3.2.1 Plant materials

Rapid-cycling *B. napus* base population (Stock number 5-1) and rapid-cycling *B. juncea* base population (Stock number 4-1) were acquired from Wisconsin Fast Plants RCBC, University of Wisconsin-Madison, Department of Plant Pathology at Madison, Wisconsin. In addition, long duration canola cultivar *Brassica napus* L. 'Westar' was obtained from the Australian Field Crops Collection (Horsham, Victoria), since the regeneration protocol being optimised was based on the protocol for *B. napus*. For seed production, the plants were grown in Debco® potting mix (50% composted medium bark, 40% composted coarse bark, 10% sand, SaturaidTM, lime, iron, nitrogen and other trace elements), in the glasshouse maintained at 25°C, with relative humidity (RH) of 55-75% under 16 hours of light (~100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) provided by GE Lucalox™ PSL (GE Lighting, U.S.A). The seeds were harvested in bulk.

3.2.2 In vitro *seed germination*

Seeds were surface sterilized with 20% White King® bleach solution (equivalent to < 2% active ingredient (ai) sodium hypochlorite) for 30 minutes with shaking at room temperature. The seeds were then rinsed 3 times with sterile distilled water. The seeds were planted in half-strength Murashige and Skoog (MS) (Murashige & Skoog, 1962) basal medium (SIGMA® M5519, Sigma-Aldrich U.S.A.) with 1% sucrose, and solidified with 0.4% (w/v) Phytagel™ (SIGMA® P8169 Sigma-Aldrich U.S.A.), pH 5.8. This was carried out in 250 mL polycarbonate containers with screw lids (C10065UU, Techno Plas Pty Ltd, Australia), each containing 50 mL of the medium. Approximately 20 seeds were placed in each container. The seeds were germinated at 25°C in the dark for 3-5 days.

3.2.3 Shoot regeneration

Four-day-old cotyledons were excised with 1-2 mm petioles, completely avoiding the apical meristem. The cotyledon explants were placed with the petiole end embedded in the shoot regeneration medium. The explants were immediately transferred to the medium after excision to prevent oxidation of phenolic compounds that can inhibit the regeneration process (George et al., 2008).

The shoot regeneration medium was composed of MS basal medium with 2% sucrose (MS20) and 0.4% (w/v) Phytagel™ (Sigma), with pH adjusted to 5.8; and supplemented with varying amounts and combinations of plant growth regulators: 6-benzylaminopurine (BAP) – 2-20 µM, 1-napthaleneacetic acid (NAA) – 0.5-2 µM, and gibberrellic acid (GA_3) – 0.03 µM. As an ethylene action inhibitor, $AgNO_3$ was used at a concentration of 30 µM. A total of 8 treatments included different levels and combination of BAP and NAA (Table 3.1); treatment 8 was MS20 without amendment of growth regulators, and treatment 1 contained BAP and NAA at a concentration that was optimized for long duration *B. napus* and *B. oleracea* (Bhalla and Singh, 2008). All medium supplements were filter-sterilized and incorporated into the autoclaved MS medium. The explants were cultured in sterile 100 mm x 20 mm Corning® (Corning Incorporated U.S.A.) culture plates, each with 30 mL of the regeneration medium, at a density of 8-10 explants per plate. The culture plates were sealed with porous Nexcare™ Micropore™ paper tape, and incubated at 24±1°C with photosynthetic photon flux density of ~100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied by cool white fluorescent lamp, inside an environmental chamber (Panasonic MLR-352-PE Climate Chamber, Panasonic Healthcare Co., Ltd. Japan) with relative humidity maintained at 55%.

3.2.4 Rooting and establishment of regenerated shoots

Shoots that developed to 2 cm long were excised from the explants. The shoots were carefully teased out from the prominently formed shoot base from the rest of the shoot clumps, using a micro probe or fine point forceps. The excised shoots were immediately planted into the rooting medium composed of half-strength MS basal medium with 1% (w/v) sucrose, solidified with 0.4% (w/v) Phytagel, with pH adjusted to 5.8. The medium was supplemented with 4 µM of indole-3-butyric acid (IBA).

Successfully rooted plantlets, possessing multiple roots of at least 2 cm long, were taken from the medium, and the roots were carefully washed with warm water to remove any traces of medium. The plantlets were planted in 0.5 L pots containing potting mix (Debco Seed Raising Mix) and then placed under glasshouse conditions, at 25°C, with relative humidity (RH) of 55-75% under 16 hours of light (~100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) provided by GE Lucalox™ PSL (GE Lighting, U.S.A). For the first week in the glasshouse, the plants were covered with transparent cups (punched with 3 holes at the bottom) trapping moisture in the immediate environment of the plants.

Table 3.1. Shoot regeneration medium (SRM) with varying amounts and combination of 6-benzylaminopurine (BAP) and 1-napthaleneacetic acid (NAA) plant growth regulators.

Treatment	BAP (µM)	NAA (µM)
1	13	1
2	5	0.5
3	10	0.5
4	20	0.5
5	5	2
6	10	2
7	20	2
8	0	0

3.2.5 Statistical analysis

The regeneration response of the three genotypes was measured in terms of regeneration frequency (the proportion of explants regenerating shoots to the number of explants cultured) and regeneration efficiency (the proportion of regenerated plantlets to the number of explants cultured). For each treatment, there were 5 replicate plates, containing 10 explants per plate. Analysis of variance (ANOVA) was performed to determine significant differences between the three genotypes, and the 8 treatments tested, using IBM® SPSS Statistics 22 Software. The mean values were compared using Tukey's HSD (honest significant difference).

A total of 9 selected plantlets (3 tissue culture vessels with three plantlets each) for each genotype per treatment, were transplanted to determine rooting ability. Well-rooted plantlets possessing multiple roots of at least 2 cm long were counted after 2-3 weeks in culture. ANOVA was performed to determine differences in rooting frequency.

3.3 Results

Preliminary experiments to evaluate the amenability of the rapid-cycling brassicas to regenerate *in vitro*, showed that the rapid-cycling *Brassica* plants had lower shoot regeneration frequency compared to the *Brassica napus* 'Westar' hence, the *in vitro* culture conditions were optimised.

3.3.1 Effect of culture conditions

Only the explants grown in medium supplemented with plant growth regulators produced shoot initials in the early stage, *in vitro*. Explants in MS20 with no growth regulator increased in size, but did not produce shoot initials. There were statistically significant differences for the different combination of NAA and BAP in terms of regeneration frequency $F(6,98) = 2.347,\ p < 0.05$ and regeneration efficiency $F(6,98) = 3.324,\ p < 0.05$. Among the three genotypes, there were also statistically significant differences in regeneration frequency $F(2,102) = 9.670,\ p < 0.05$ and regeneration efficiency $F(2,102) = 9.014,\ p < 0.05$. Table 3.2 shows the regeneration response of the three genotypes in different levels of BAP and NAA, with homogenous means indicated; while Figure 3.1 displays the 3-D bar graph with each combination of BAP and NAA. The ANOVA tables and descriptive statistics of the results are detailed in Appendix 1 and 2, respectively.

Among the different plant growth regulator treatments, 5 µM BAP and 2 µM NAA yielded the highest regeneration frequency of 53.3 % in rapid-cycling *Brassica napus* plants (BNFP). This also produced the highest number of regenerated plants with regeneration efficiency of 91.1 %, which was equivalent to 0.9 plant per explant. There was no significant difference in regeneration frequency with media supplemented with

0.5-2 μM NAA and 5-10 μM BAP. However, there was a considerable decline of regeneration with 20 μM BAP, with minimum regeneration frequency of 8 %. The same trend was observed in rapid-cycling *B. juncea* plants (BJFP). Maximum regeneration frequency of 43.3% was obtained in explants cultured in medium with 5 μM BAP and 2 μM NAA, yielding an efficiency of 0.7 plants per explant.

A different response was observed with the long duration *B. napus* 'Westar' (BNW). Maximum regeneration was obtained in medium supplemented with 20 μM BAP and 2 μM NAA, having 76.9% regeneration frequency and yielding an average of 1 plant per explant (regeneration efficiency of 102%). The regeneration response of 'Westar' was high in the culture medium supplemented with >10 μM BAP, regardless of the level of NAA (Figure 3.1).

Table 2.2. Regeneration frequency (top) and regeneration efficiency (bottom) of three *Brassica* genotypes [rapid-cycling *B. napus* (BNFP) and *B. juncea* (BJFP), and *B. napus* 'Westar' (BNW) in different concentrations of 6-benzylaminopurine (BAP) and 1-napthaleneacetic acid (NAA).

Regeneration frequency %					
Treatment	BAP (μM)	NAA (μM)	BNFP	BJFP	BNW
1	13	1	20.4 [ab]	14.9 [ab]	51.6 [b]
2	5	0.5	30.0 [b]	26.7 [b]	18.2 [a]
3	10	0.5	18.2 [ab]	14.0 [ab]	18.2 [a]
4	20	0.5	8.0 [a]	8.22 [a]	54.9 [b]
5	5	2	53.3 [c]	43.3 [c]	21.8 [a]
6	10	2	22.9 [ab]	18.0 [ab]	24.0 [a]
7	20	2	12.4 [ab]	10.4 [ab]	76.9 [c]
Regeneration efficiency %					
Treatment	BAP (μM)	NAA (μM)	BNFP	BJFP	BNW
1	13	1	26.4 [a]	16.9 [ab]	72.4 [bc]
2	5	0.5	38.0 [a]	32.9 [b]	24.4 [a]
3	10	0.5	16.2 [a]	14.4 [ab]	24.7 [a]
4	20	0.5	8.0 [a]	8.2 [a]	75.3 [c]
5	5	2	91.1 [b]	68.0 [c]	23.8 [a]
6	10	2	24.9 [a]	20.9 [ab]	44.0 [ab]
7	20	2	16.4 [a]	12.8 [ab]	102.0 [c]

[abcd]*Letters indicate means for groups in homogenous subsets for each genotype, based on Tukey's HSD.*

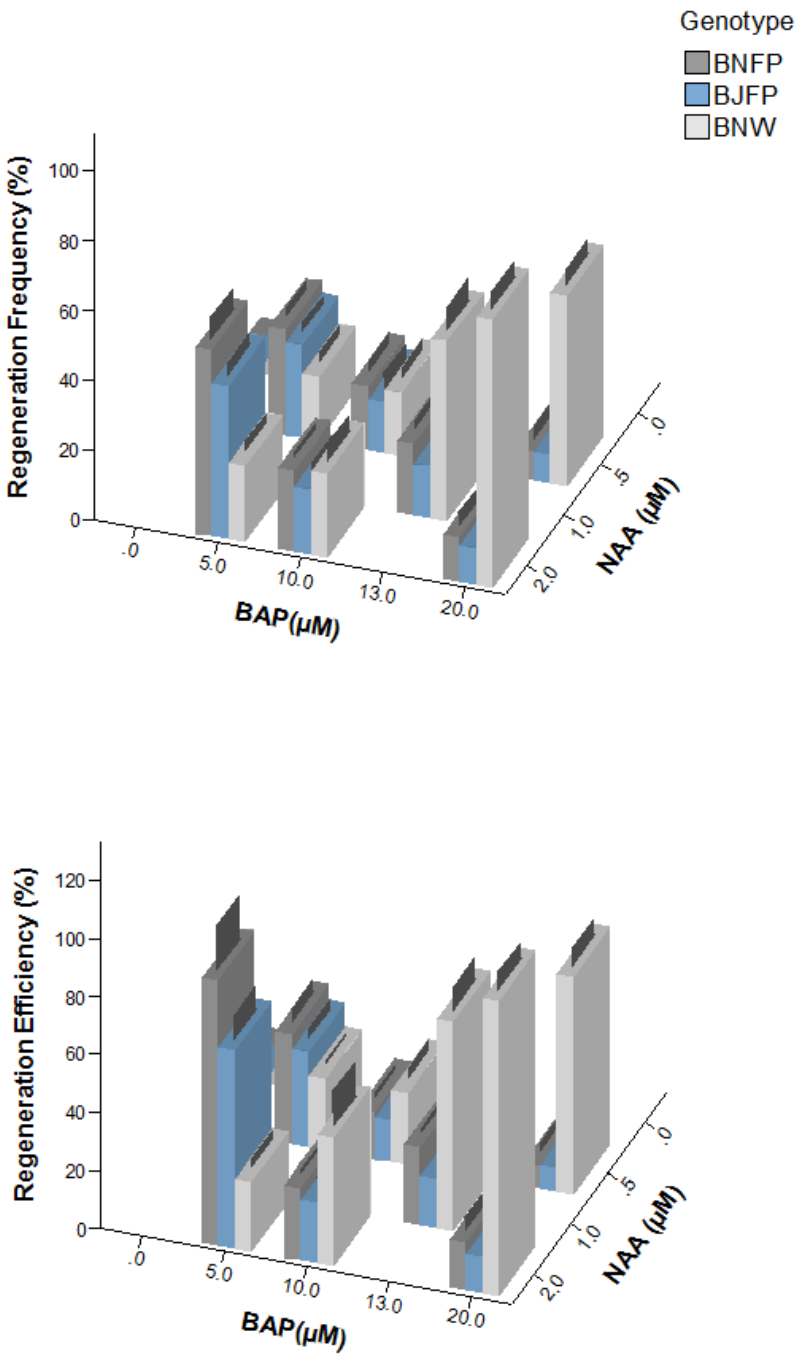


Figure 3.1. Regeneration responses of three *Brassica* genotypes [rapid-cycling *B. napus* (BNFP), rapid-cycling *B. juncea* (BJFP) and *B. napus* 'Westar' (BNW) in different combinations of 6-benzylaminopurine (BAP) and 1-napthaleneacetic acid (NAA). Error bars are shown as standard error of the means.

3.3.2 Rooting ability

Shoots that properly regenerated into well-developed plantlets under different combinations of NAA and BAP showed no significant difference in rooting ability when transferred to the rooting medium, $F(6, 56) = 1.305, p = 0.270$. However, there was statistically significant differences among the 3 genotypes in rooting ability $F(2,60)=18.497, p < 0.005,$ with rapid-cycling *B. juncea* plants having significantly lesser plants that formed roots. BNW showed the highest number of rooted plants, followed by BNFP and BJFP (Table 3.3). Statistical results for rooting ability are displayed in Appendix 3.

Table 3.3. Frequency of root formation of *Brassica* plantlets regenerated with different combinations of 6-benzylaminopurine (BAP) and 1-napthaleneacetic acid (NAA).

	Frequency of root formation (%)*		
Treatment	BNFP	BJFP	BNW
1	88.9	44.4	88.9
2	88.9	77.8	100.0
3	66.7	55.6	100.0
4	88.9	77.8	100.0
5	100.0	77.8	100.0
6	100.0	66.7	100.0
7	88.9	77.8	100.0

**Frequency of root formation was based on 3 tissue culture vessels, containing 3 regenerated plantlets each.*

3.3.3 General response to culture

A considerable increase in size of the cotyledon explants (Figure 3.2A) was observed after 3 days in the culture media containing plant growth regulators, with the petioles swelling and forming shoot initials. There was no characteristic callus formation

observed at the petiole ends of the cotyledon explants. Explants with the petiole end not embedded in the medium did not regenerate shoots, and formed adventitious roots instead. While the growth of BNFP and BJFP in medium containing 5 μM BAP and 2 μM NAA was very active, not all shoots were viable. As shown in Figure 3.2, 3 weeks into culture, BJFP (C) expressed a high volume of shoot growth; however, irregularity in shoot morphology was very apparent. There was a high amount of vitrified or hyperhydric shoots in *B. juncea*, which appeared elongated and stringy. Also, profuse rooting was observed in the explants. With rapid-cycling *B. napus* (B) and 'Westar' (D), growth of new shoots had more pronounced rounded leaf structure. Organogenesis in the rapid-cycling brassicas occurred earlier compared to 'Westar'. At 10-14 days in the medium, shoots started to elongate and form new leaves; whereas for 'Westar' 20-25 days was required for shoot elongation.

When transplanted into a culture container, rapid vertical growth was observed in BNFP and BJFP due to an apparent apical dominance. The stem elongated and no further leaf growth occurred; and at 20-25 days *in vitro*, flower buds formed (Figure 3.2D). With BNW, upon subculture to fresh medium in a culture container, horizontal growth was more apparent, as more leaves were produced, and the first leaves started to expand. A major problem of establishment of plantlets *in vitro* was that root formation was very slow, or absent. However, shoots that regenerated into proper plantlets, with well-developed leaves and elongated stems, readily established roots when transferred to the rooting medium.

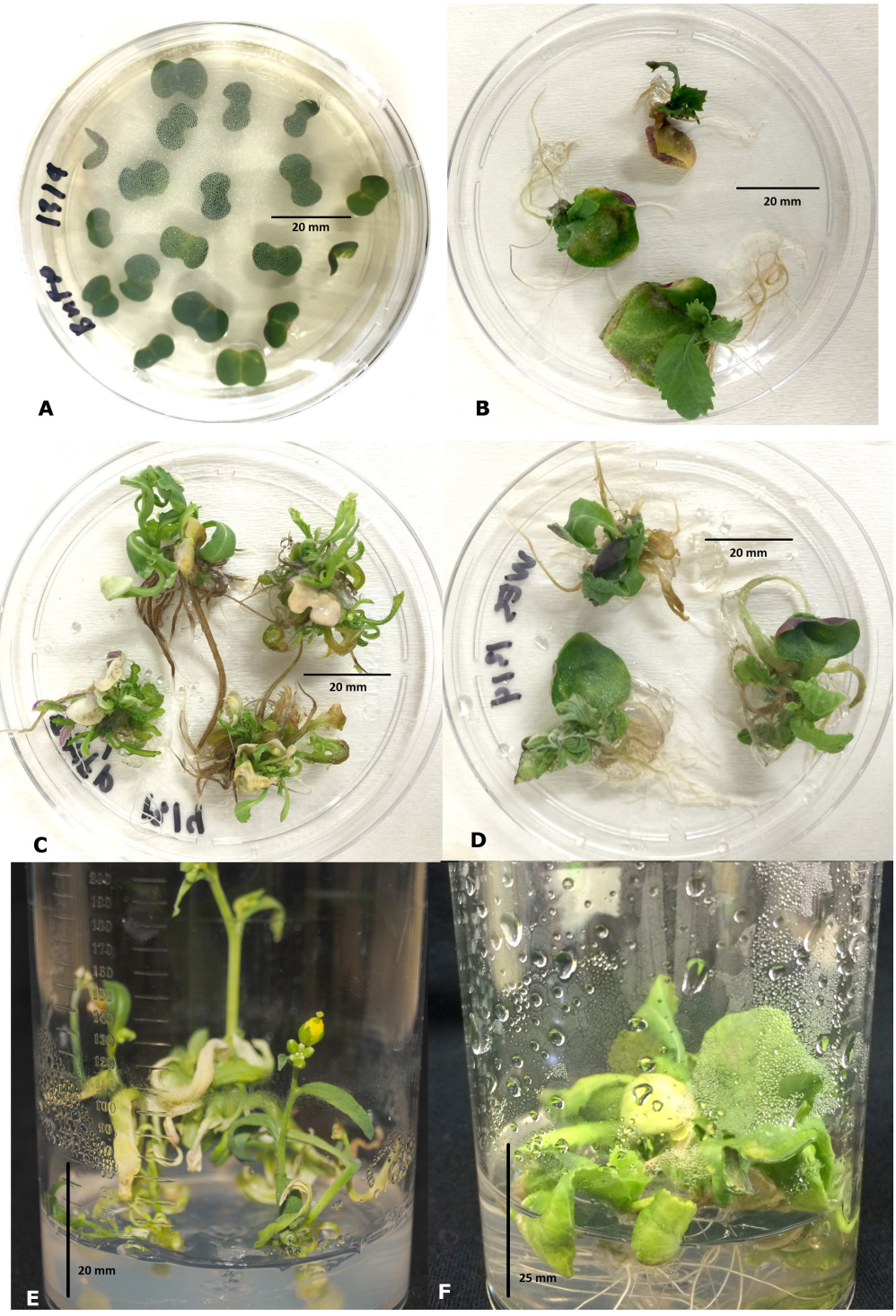


Figure 3.2. Growth of the cotyledon explants in the shoot regeneration medium. Enlargement of explants 3 days after co-cultivation with *Agrobacterium* (A). Shoot growth after 3 weeks in SRM of rapid-cycling *Brassica napus* (B) and rapid-cycling *Brassica juncea* (C) and *Brassica napus* 'Westar' at 4 weeks (D); and regenerated plantlets of rapid-cycling *B. juncea* showing flowering (E) and rooted plantlets of 'Westar' (F).

3.4 Discussion

Cotyledon explants from rapid-cycling *B. napus* and *B. juncea* exhibited high competency to induction of morphogenesis *in vitro* with the addition of optimum concentration of BAP and NAA. Regeneration of shoots was rapid and did not undergo a prolonged callus phase. These characteristics are ideal for production of transgenic plants through *Agrobacterium*-mediated transformation.

The 'fast plant' characteristic of the rapid-cycling brassicas was evident during shoot regeneration in comparison to that of 'Westar'. The rapid-cycling brassicas started shoot elongation 1-2 weeks before 'Westar'. Similar observations were also noted in rapid-cycling *B. rapa* cotyledon explants which formed shoots at 10 days and maximised shoot formation within 10-17 days in culture (Teo et al., 1997); and *B. oleracea* leaf petioles which also formed shoots at 10 days in culture (Cheng et al., 2001). With long duration *B. napus*, the first shoots formed after 14 days in culture (Moloney et al., 1989), and morphologically distinct shoots appeared after 30 days (Khehra & Mathias, 1992).

Manipulation of tissues in the culture was quite intricate with the rapid-cycling plants. The cotyledons of the *Brassica* rapid-cycling plants were much smaller (~2 mm) than for *B. napus* 'Westar' plants (3-5 mm), hence considerable care and accuracy was required during the excision of the 4-day-old cotyledons of the rapid-cycling plants. There was a challenge of excising at the proper point, such that the apical meristem was completely avoided. The physical trauma in manipulating delicate shoots during shoot clump separation (or wounding of the shoot base and improper separation of shoots) was a major stress factor that may have contributed to non-formation of roots of rapid-cycling *B. juncea* when the elongated shoots were transferred to rooting medium. Wounding was

identified by Gaj (2001) as one of the stress factors that affected embryogenic competence of plant tissues *in vitro*.

The cotyledon explants of rapid-cycling *B. napus* and rapid-cycling *B. juncea* responded differently compared to *B. napus* 'Westar' when grown in MS medium with different concentrations of BAP and NAA. Plant regeneration of different *Brassica* tissues have been shown to be highly genotype-dependent; while explant tissues may vary in their capacity to respond to induction for morphogenesis. These characteristics have been widely observed in long-duration diploid and amphidiploid brassicas (Akasaka-Kennedy et al., 2005; Bhalla & Singh, 2008; Khehra & Mathias, 1992; Narasimhulu & Chopra, 1988; Ono et al., 1994; Pavlovic et al., 2010). Ono et al. (1994) surveyed the potential of 100 cultivars of *B. napus* for shoot regeneration from cotyledon explants and found an apparent disparity of regeneration potential of the different cultivars, ranging from 0 to 97%. Regeneration protocol for protoplast culture that worked for commercial brassica cultivars failed with the rapid-cycling *Brassica* species with the exception of *B. napus* (Loudon et al., 1989).

Four-day-old cotyledons were used as explants, based on the results of previous studies that determined this stage to be the optimum to produce the highest regeneration potential and amenability for *Agrobacterium*-mediated transformation in *B. napus* long duration genotypes (Bhuiyan et al., 2009; Cogbill et al., 2010; Hachey et al., 1991; Ono et al., 1994; Tang et al., 2003). Cotyledons as the source of tissue culture explants have been proven to regenerate new plants in all 6 long duration *Brassica* species (Narasimhulu & Chopra, 1988). Hachey et al. (1991), observed that the cut surface of the cotyledon along the petiole side was lined with cells with very high morphogenic potential. The histology of shoot primordia of cotyledon explants of *B. rapa* placed in shoot regeneration

medium for 2 days, underwent active random cell division at the cut end of the petiole. At 5 days, rapid cell division gave rise to meristematic nodules, which subsequently grew into shoot bud meristems by day 7. Within 15 days in the shoot regeneration medium, shoot buds became visible.

Optimum concentration of BAP and NAA for regeneration of explants for rapid-cycling *B. napus* and rapid-cycling *B. juncea* was 5 µM of BAP and 2 µM of NAA, whereas for *B. napus* 'Westar', higher concentration of BAP (20 µM) was required for regeneration. The optimum concentration of auxin was similar to a previous study in rapid-cycling *B. rapa* but, the level of cytokinin was 4 times lower (Teo et al., 1997). Previous studies showed that the optimal concentration of auxin and cytokinin varied depending on the species/genotype and the kind of explant used. As a cytokinin, BAP has been more widely used in regeneration studies in *Brassica*, while NAA was proven to be more effective compared to other auxins (Narasimhulu & Chopra, 1988). Rapid-cycling *B. oleracea* obtained high regeneration frequency with the addition of 5 µM BAP only (Cheng et al., 2001).

The regeneration response of 'Westar' was more likely influenced by the amount of BAP rather than NAA. In long duration *B. juncea* (Sharma et al., 1990), the addition of 5 uM BAP alone produced the highest shoot bud differentiation from cotyledon explants and produced an average of 8 shoots per explant, in comparison to other cytokinins (kinetin, 2-ip and zeatin). The addition of NAA encouraged more callus growth but reduced differentiation of calli into shoot buds. Previous studies with *B. napus* also showed that addition of BAP (without NAA) was critical for shoot regeneration at 18 uM producing 70% regeneration (Ono et al., 1994); and at 20 uM there was 80% shoot regeneration of *B. napus* 'Westar' (Moloney et al., 1989). *In vitro* regeneration of

Agrobacterium-transformed *Brassica* explants was shown to benefit from the addition of NAA to the shoot regeneration medium (Jin & Liu, 2000; Kong et al., 2009; Kuvshinov et al., 1999).

Chapter 4

Agrobacterium-mediated transformation of *Brassica napus* with the *Arabidopsis ACYL-COA-BINDING PROTEIN6* (*ACBP6*)

4.1 Introduction

Transformation of *Brassica* using *Agrobacterium tumefaciens* has been well studied and numerous protocols have been developed for the different species and cultivars, using different types of explants. Although these protocols have been optimised for individual genotypes, the steps for obtaining a transformed plant are essentially similar.

The preparation of explant is a critical step to guarantee regeneration while the optimum concentration of *Agrobacterium* inoculum and duration of infection and co-cultivation of *Agrobacterium* with the explants ensure plant cells are transformed (Poulsen, 1996). Explants are usually prepared from sterile *in vitro* grown seedlings. Pre-culturing of seedling cotyledon and hypocotyl explants in the co-cultivation medium promoted survival of tissues after *Agrobacterium* inoculation (Metz, et al., 1995; Pental et al., 1993).

Following explant preparation, the target cells are inoculated with *Agrobacterium* and co-cultivated in a nutrient medium, commonly composed of solidified MS, sucrose, and a combination of plant growth regulators. Co-cultivation period of 2-3 days was generally optimum for most *Brassica* species (Bhuiyan et al., 2011; Jin & Liu, 2000; Moloney et al., 1998). After this step, the explants were subcultured in callus induction medium with antibiotics to eliminate *Agrobacterium.* The binary plasmid contains a marker gene, such as the antibiotic resistant gene for kanamycin, thus incubation of

transformed tissue on medium containing the antibiotic will result in the selection of transformed cells and death of non-transformed plant cells. Delaying the transfer of the transformed explants to the medium containing the selective antibiotic has been found to significantly increased regeneration of transgenic shoots (Bhalla & Singh, 2008; Jin et al., 2000).

Transformed cells then undergo organogenesis in the presence of the selection agent, such as kanamycin, depending on the transgene marker. In this step, the type and combination of plant growth regulators, and the concentration of the selection agent, are highly critical for optimum shoot regeneration. Well-established and elongated shoots can then be separated from the shoot clump and subcultured to induce rooting. In some protocols, a final step of selection with increased concentration of the selection agent was added, and effectively eliminated the non-transformed tissues (Bhalla & Singh, 2008; Radke et al., 1992). The addition of $AgNO_3$, which is an ethylene antagonist, was found to be essential for stimulating *B. napus* and *B. oleracea* explants to regenerate transformed shoots, especially in the presence of kanamycin (de Block et al., 1989; de Block, 1988).

Oilseed rape *Brassica napus* 'Westar' has been widely used as a model genotype for *Agrobacterium*-mediated transformation of canola plants (Bhalla & Singh, 2008; Cardoza & Stewart, 2004; Charest et al., 1988; Fry et al., 1987; Khehra & Mathias, 1992). This cultivar has been shown to successfully produce transgenic plants derived from different explant sources such as the hypocotyl, stem segments and cotyledonary petioles as reviewed by Poulsen (1996). Tissue regeneration and transformation frequency in *Brassica* is highly genotype-dependent and may vary from 0 to 90%, depending on the genotype (Cardoza and Stewart, 2004). In contrast to the normal long duration cultivars

of *B. napus*, the rapid-cycling *Brassica napus* and *B. juncea* (Rapid-cycling *Brassica* Collection; http://www.fastplants.org), developed through artificial selection for rapid completion of seed-to-seed cycle, have very short life cycle and small plant habit. These traits make these genotypes ideal as a platform to test gene function especially for traits that are of agronomic value to oilseed rape.

Rapid-cycling *B. napus* has been successfully regenerated *in vitro* through direct somatic embryogenesis from seeds (Koh and Loh, 2000). However, this genotype has not been assessed for shoot regeneration from vegetative tissues which are needed for *in vitro* based *Agrobacterium*-mediated transformation. There has not been any report of utilising rapid-cycling *B. juncea* for *in vitro* shoot regeneration, nor genetic transformation. Among the rapid-cycling *Brassica* species, only *B. oleracea* has been successfully genetically transformed, through *A. tumefaciens* (Beclin et al., 1993; Berthomieu et al., 1994; Berthomieu & Jouanin, 1992) and *A. rhizogenes* or Ri-mediated transformation (Christey et al., 1997).

Frost is a major abiotic stress for oilseed rape production in Australia, which affects yield and oil quality (GRDC, 2009). In *Arabidopsis thaliana*, the ACYL-COA-BINDING PROTEIN6 (ACBP6) confers freezing tolerance (Chen et al., 2008). The ACBP6 is a 10-kDA protein involved in the transport of cytosolic acyl-coA and maintenance of acyl-coA esters following production of fatty acids in the plastids. The expression of ACBP6 protein and the production of mRNA in *A. thaliana* were cold-inducible. When overexpressed, *Arabidopsis* plants were able to withstand freezing stress up to -8°C (Chen et al., 2008). Freezing tolerance in *Arabidopsis* rosettes over-expressing the ACBP6 was independent of the induction of cold-regulated *COLD-RESPONSIVE*

(COR) gene expression (Chen et al., 2008), which are mainly involved in cold-acclimated frost tolerance observed in most *Brassica* species (Thomashow, 1999).

In this chapter, the rapid-cycling *B. napus* and *B. juncea,* and *B. napus* 'Westar' were used for *Agrobacterium*-mediated transformation with the *A. thaliana ACYL-COA-BINDING PROTEIN6 (ACBP6)* for freezing tolerance.

4.2 Materials and Methods

There were two sets of experiments carried out. Experiment 1 consisted of preliminary studies to adapt an established protocol (Bhalla and Singh, 2008) for *Agrobacterium*-mediated transformation of *B. napus* 'Westar' over-expressing ACBP6. Experiment 2 involved the application of the optimised *Agrobacterium*-mediated transformation protocol to produce ACBP6-overexpressing *B. napus* 'Westar' and rapid-cycling *B. napus* and *B. juncea* transgenic plants.

4.2.1 Experiment 1: Initial transformation studies on B. napus *'Westar'*

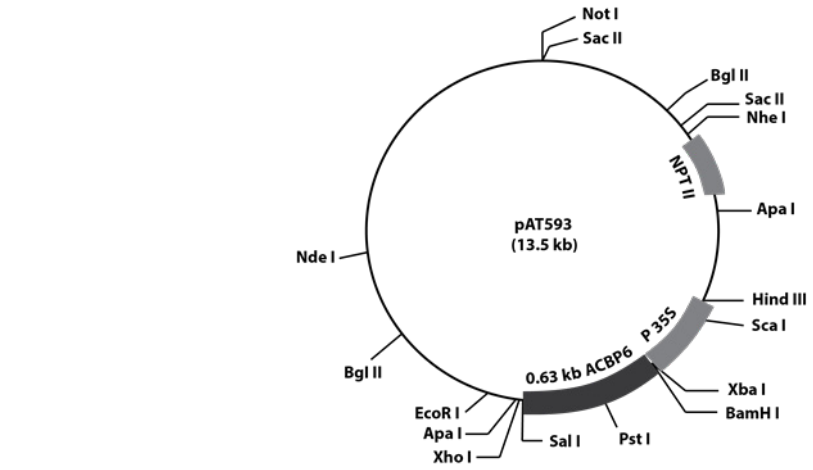
4.2.1.1 Explant preparation

The canola cultivar *Brassica napus* 'Westar' obtained from the Australian Field Crops Collection (Horsham, Victoria), was used as source of explants for *in vitro* regeneration following *Agrobacterium*-mediated transformation with plasmid pAT593 containing *ACBP6*. Cotyledon explants were obtained from *in vitro* germinated seeds, as previously described in Chapter 3.

4.2.1.2 *Agrobacterium* preparation

The *Agrobacterium tumefaciens* strain used was GV3101::pMP90 carrying the binary plasmid pAT593 (source: Dr. Wei Meng and Prof. Mee Len Chye, HKU; unpublished data). The binary plasmid pAT593 (Figure 4.1) was constructed by W. Meng (HKU; unpublished) by cloning the 0.63 kb *Bam*H I - *Sal* I fragment of the *ACBP6* cDNA from pAT332 (Chen et al., 2008) into vector pSa13 (Gao et al., 2009) which originated from pBI121. The plasmid contained CaMV 35S promoter driving *ACBP6*, a neomycin phosphotransferase II (*NPTII*) encoding kanamycin resistance, and nopaline synthase

(nos) terminator. The *Agrobacterium* host strain GV3101::pMP90 was maintained in 50 µg/mL kanamycin and 50 µg/mL gentamicin.



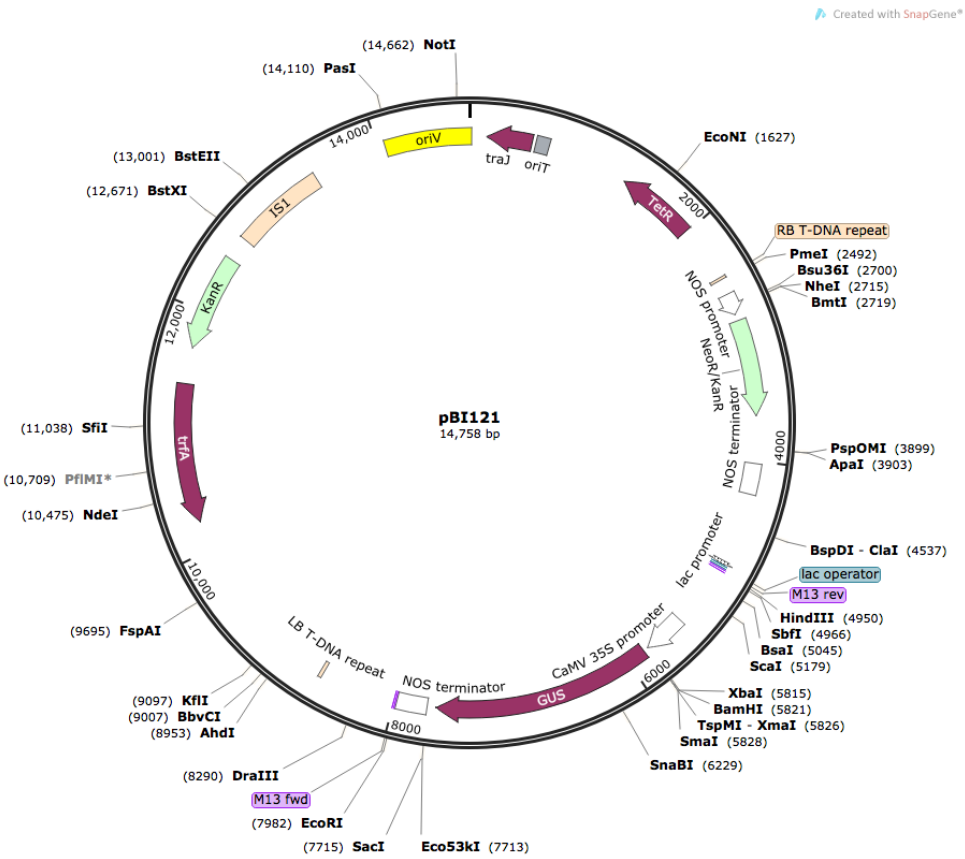


Figure 9. Plasmid pAT593 harbouring the *ACBP6* gene (W. Meng, HKU; unpublished data) and the original vector backbone pBI121 (Chen, 2003).
Source: www.snapgene.com/resources.

Frozen stock of *Agrobacterium* culture was streaked on Luria-Bertani (LB) plates containing 50 µg/mL gentamicin and 50 µg/mL kanamycin, and incubated at 28°C for 48 hours. A single colony growth was inoculated into 10 mL LB broth with 50 µg/mL kanamycin and 50 µg/mL gentamicin. The bacteria cells were allowed to proliferate for 36 hours at 28°C with shaking (250 rpm) using an orbital shaking incubator (Ratek Instruments Pty. Ltd., Australia). The cells were centrifuged at 5,000 rpm for 10 minutes at room temperature. The pellet was rinsed once and resuspended with MS20 liquid medium. The optical density (OD) of the bacterial suspension was adjusted to 0.05 at 650 nm.

4.2.1.3 Explant infection and co-cultivation with *Agrobacterium*

The *Agrobacterium*-mediated transformation followed the general procedures in the transformation protocol of Bhalla and Singh (2008). The MS medium was commercially prepared Murashige and Skoog (MS) basal medium (SIGMA® M5519, U.S.A.). The cotyledons were excised from the seedlings as described in Chapter 3.2.3, and immediately placed on a Petri plate containing the prepared *Agrobacterium* solution for 30-60 seconds. Three seedlings, which provided 6 cotyledon explants, were processed each time. The cut ends of the explants were lightly touched on a sterile filter paper to absorb excess bacterial solution and placed with the cut end embedded in the co-cultivation medium at a density of 20-25 explants per plate. The co-cultivation medium was composed of solidified MS20 with pH adjusted to 5.8 (described in chapter 3.2.3) containing 3.3 µM BAP, 0.2 µM NAA, 30 µM AgNO3, and 0.03 µM gibberellic acid (GA_3) set on a 90 mm x 15 mm Petri plates. The culture plates were sealed with porous

Nexcare™ Micropore™ paper tape and wrapped in foil. The cotyledon explants and *Agrobacterium* were co-cultivated for 2 days at 24±1°C.

4.2.1.4 Regeneration of transgenic shoots

After 2 days of co-cultivation in the dark, the explants were transferred to the callus induction medium, which had similar composition to the co-cultivation medium but contained 250 µg/mL of the antibiotic carbenicillin to eliminate the *Agrobacterium*. The cultures were sealed with the porous tape, wrapped in foil, and incubated at 24±1°C for 7 days. After this period, the explants were transferred to 30-40 mL shoot initiation medium set on 100 mm x 20 mm Corning® (Corning Incorporated U.S.A.) culture plates, composed of the same growth regulators and additives but with 250 µg/mL carbenicillin and 25 µg/mL kanamycin, with a density of 10-12 explants per plate, and sealed with the porous tape. The explants were grown at 24±1°C under 16 hours of light at photosynthetic photon flux density of ~100 µmol·m-2·s-1 in an environmental chamber (Panasonic MLR-352-PE Climate Chamber, Panasonic Healthcare Co., Ltd. Japan) with relative humidity maintained at 55%. After a period of 4 weeks, explants that formed shoots were subsequently transferred to 50 mL of shoot outgrowth medium set in 250 mL polycarbonate containers, with 6 explants per container, and grown in the same conditions in the environmental chamber. The containers were loosely capped. The shoot outgrowth medium was composed of solidified MS20 (pH 5.8) with the addition of 40 µg/mL adenine hemisulfate and 500 µg/mL PVP40k. After autoclaving the media, it was supplemented with 250 µg/mL carbenicillin, 25 µg/mL kanamycin, and 0.5 µM of BAP. The shoots were allowed to grow for 4 weeks until distinct leaves were formed.

4.2.1.5 Selection of transgenic shoots

Explants with shoots that elongated to at least 2 cm long, possessing well-developed nodes, internodes, leaves and apical buds were removed, and well-developed shoots were individually excised with extreme care. To eliminate non-transgenic or false positives, shoots were transplanted into the transformant selection medium which was the same as the shoot outgrowth medium but the sucrose was reduced to 10% (w/v), and the kanamycin concentration was increased to 50 µg/mL. The containers were wrapped with foil, and placed back into the growth chamber for another 3 weeks.

This final step of selection was omitted in Experiment 1, batch B.

4.2.1.6 Rooting and establishment

The shoots that remained green and continued to develop in the transformant selection medium were transplanted into the rooting medium composed of half-strength MS basal medium with 1% (w/v) sucrose, solidified with 0.4% (w/v) Phytagel, and pH adjusted to 5.8. The medium was supplemented with 4 µM of indole-3-butyric acid (IBA). After 3 weeks, successfully rooted plantlets were taken from the medium, the roots washed with warm water, transferred into potting mix (Debco Seed Raising Mix, Debco Pty. Ltd., Australia) and then placed under glasshouse conditions, at 25°C, under 16 hours of light. For the first week in the glasshouse, the plants were covered with inverted transparent cups with 5 mm holes at the bottom to maintain humidity.

4.2.2 Experiment 2: Agrobacterium-*mediated transformation of rapid-cycling* Brassica spp. *and* B. napus *'Westar'*

4.2.2.1 Explant source

Rapid-cycling *Brassica napus* from the base population (Stock number 5-1) and rapid-cycling *B. juncea* from the base population (Stock number 4-1) and canola variety *Brassica napus* 'Westar', were used as sources of explants for *in vitro* regeneration following *Agrobacterium*-mediated transformation with plasmid pAT593 containing *ACBP6*. Cotyledon explants were obtained from *in vitro* germinated seeds, as previously described in Chapter 3.

4.2.2.2 Effect of kanamycin on shoot regeneration

To investigate the effect of kanamycin on shoot formation of rapid-cycling *B. napus* and *B. juncea,* four-day old cotyledon explants were excised from *in vitro* grown seedlings as previously described in Chapter 3. The shoot regeneration medium optimised specifically for the rapid-cycling *B. napus* and *B. juncea*, and *B. napus* 'Westar' (as described in Chapter 3) were supplemented with kanamycin at 0, 10, 20, 30 and 50 mg/L and set in100 mm x 20 mm Corning® (Corning Incorporated, U.S.A.) culture plates, with an average of 25 explants per plate. There were 3 replicate plates for each concentration of kanamycin tested. The cultures were incubated at 24±1°C under 16 hours of light at photosynthetic photon flux density of ~100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in an environmental chamber with relative humidity maintained at 60%. The regeneration response was evaluated under selection conditions after 4 weeks *in vitro*. Explants that regenerated green shoots were counted.

4.2.2.3 *Agrobacterium* preparation

The same *Agrobacterium* strain and culture preparations were used as previously described in section 4.2.1.2. There were three *Agrobacterium* concentrations evaluated for infection, with cell suspension OD_{650} of 0.05, 0.1 and 1.5. There were approximately 25 explants used in each culture plate, with 3 replicate plates, for each *Agrobacterium* concentration tested. The number of explants that regenerated shoots under selection condition was recorded after 4 weeks *in vitro*.

4.2.2.4 Transformation procedure

The transformation procedure was essentially similar as previously described in section 4.2.2, following the process of explant infection and co-cultivation with *Agrobacterium*, regeneration of transgenic shoots, selection of transgenic shoots, and rooting and establishment. However, the concentrations of BAP and NAA in the shoot regeneration medium that was optimised in chapter 3 specifically for the rapid-cycling *B. napus* and *B. juncea* (5 µM BAP and 2 µM NAA) and *B. napus* 'Westar' (20 µM BAP and 2 µM NAA) were used. The *Agrobacterium* concentration was adjusted to OD_{650} of 0.1.

The growth period in the shoot initiation medium for both rapid-cycling genotypes was reduced to 2-3 weeks, and shoot outgrowth medium for 2-3 weeks. Regenerated plantlets were further selected in the transformant selection medium for 2 weeks.

4.2.2.5 Development of advanced transgenic generations (T_1 to T_3)

Putatively transformed T_0 plants were analysed for the presence of transgenes *35S*::*ACBP6* and *NPTII*. Harvested seeds from T_0 plants were sown *in vitro* in 50 mL MS20 with 100 mg/L kanamycin, set in 250 mL polycarbonate containers with a density of 20 seeds per container. After 3 weeks, properly rooted green seedlings that developed green true leaves and stems, were rated as kanamycin resistant. These seedlings were washed with water and transplanted in soil in normal growing conditions in the glasshouse (as previously described in 4.2.1.6). T_1 plants were confirmed for the transgenes through PCR analysis before they were self-pollinated for T_1 seeds production. The same process was done until T_3 generation was attained. Methods for PCR analysis are described in section 4.2.3.

4.2.3 Molecular analysis of transgenic lines

4.2.3.1 DNA Extraction

Leaf samples of putative transformants were harvested from seedlings or from the youngest leaves or side shoots of mature plants in Experiments 1 and 2. DNA extraction from leaves harvested from mature plants was carried out using a modified CTAB method protocol, specifically for plant samples with high polyphenol and polysaccharide content (Porebski et al., 1997). Harvested leaves were stored at -80°C until use. Twenty to 50 mg of leaf tissues were pulverized in liquid nitrogen into very fine powder with a mortar and pestle, and transferred into 1.5 mL microcentrifuge tubes. Four hundred µL of 60°C extraction buffer composed of 100mM Tris, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 2% CTAB and 0.3% β-mercaptoethanol, and 5 mg of PVP (polyvinyl pyrrolidone) were added into each sample. The samples were mixed by inversion and incubated in 60°C

water bath for 60 minutes; mixing was done every 15 minutes. The samples were cooled down to room temperature for 5 minutes, and 500 µL of chloroform:isoamyl alcohol (24:1) was added, and mixed by inversion for approximately 2 minutes, and spun at 10,000 rpm for 10 minutes at room temperature using a table top centrifuge (Eppendorf 5414D Benchtop Microcentrifuge). The aqueous layer was carefully transferred to a new microcentrifuge tube and the chloroform:isoamyl alcohol step was repeated to completely remove PVP. Two volumes of cold (-20°C) isopropanol were added into the recovered aqueous phase; the samples were mixed by inversion. The samples were spun at 10,000 rpm for 10 minutes at room temperature, and the supernatant was discarded. The white pellets were soaked in 1 mL wash 1 solution composed of 76% ethanol and 0.2 M NaOAc (sodium acetate) for 15 minutes with occasional mixing, followed by rinsing with 500 µL wash 2 solution composed of 76% ethanol and 10 mM NH_4OAc (ammonium acetate). The pellets were air dried at room temperature (~ 1 hour) and resuspended in 50-100 µL TE (10 mM Tris and 1 mM EDTA, pH 8.0) buffer with 1% v/v RNase A (10 mg/ml). The samples were mixed and incubated at 37°C for 15-30 minutes, until the DNA pellets were dissolved.

Routine DNA extraction of very young seedlings was carried out using the rapid and simplified extraction protocol of Edwards et al. (1991), with modifications. Twenty mg leaf tissue samples were placed in individual microcentrifuge tubes. Four hundred µL of cold (4°C) extraction buffer composed of 200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5% SDS (sodium dodecyl sulphate) were added into each sample. The tissue samples were macerated on ice with a small amount of sterile silica sand (Anachemia) using pellet pestle. The slurry was spun at 10,000 rpm for 5 minutes. Each supernatant was transferred to a new centrifuge tube, and 500 µL of ice cold isopropanol

was added, mixed and incubated on ice for 15 minutes. The samples were spun at 10,000 rpm for 5 minutes, the supernatant liquid removed, and the pellets were air-dried at room temperature. The pellets were resuspended in 100 µL TE buffer, followed by another round of centrifugation to remove remaining impurities and stored at -20°C.

The DNA samples were analysed on 1% agarose gel electrophoresis and stained with ethidium bromide solution (Sambrook and Russell, 2001). The quantity and quality of the extracted DNA were determined by visual inspection of the electrophoretic bands of the samples against known standards of uncut ʎ DNA (Thermo Fisher Scientific Inc., U.S.A.) diluted with TE buffer to final concentrations of 10 ng, 50 ng, 100 ng, and 200 ng.

4.2.3.2 PCR amplification

The PCR was performed using the optimized amplification conditions for each primer. Optimized conditions among the different primers varied in terms of the annealing temperature. The PCR conditions were as follows: total reaction volume of 10 µl containing 30 ng template DNA, 1X colored Mango *Taq*™ Reaction PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Bioline dNTP Mix), 0.2 mM each of forward and reverse primers and 1 U of Mango *Taq*™ DNA polymerase enzyme (Bioline GmBH, Germany). Amplification was carried out using Eppendorf Mastercycler® (Applied Biosystems, U.S.A.) using the following profile: initial denaturation, 94°C for 5 minutes; 35 cycles of 94°C for 1 minute denaturation, 55°C-65°C for 1 minute annealing (depending on the primer) and 72°C for 2 minutes extension, and final extension at 72°C for 7 minutes. The temperature was held at 4°C after the cycle finished. The PCR product was then loaded on 1.5% agarose gel on a horizontal gel electrophoresis in 1X TAE

running buffer at 100 V for 60 minutes. The electrophoresis gel was visualized by staining with ethidium bromide solution (10 mg/mL) and viewed under UV light using the Bio-Rad Gel Doc™ (Bio-Rad Laboratories, Inc., U.S.A.) system.

The PCR primers (Table 4.1) were targeted to amplify the *ACBP6* cDNA, the CaMV 35S promoter and the *NPTII* coding region. The relative primer locations amplifying the *ACBP6* and *NPTII* are presented in Figure 4.2. The gene-specific primers used were designed from *Arabidopsis thaliana ACBP6* mRNA (NCBI Reference Sequence: NM_102916.3) and from Chen et al. (2008); the CaMV 35S promoter and *NPTII* are from published sequences GenBank accession numbers BAL46513.1 and S51061.1, respectively (Appendix 5).

Table 4.1. List of primers used to screen putatively transformed *Brassica* plants.

Primer Code	Sequence (5' to 3')	Target
ML750	ATATGGATCCCACGCGTTGTCCTCGTCTTCT	*ACPB6*
ML751	AATATATCATCTTGAATTCAACTG	*ACBP6*
ML838	CAGGATCCTGAAGCCTTGGAAGCAGCAACT	*ACBP6*
6acbp01	AGCAAGCCAAGTTTGGGCCTGT	*ACBP6*
6acbp02	CAGGTTGAAGCCTTGGAAGCAGCA	*ACBP6*
6acbp05	GAGGAGCACGCTGAGAAAGT	*ACBP6*
6acbp06	ATTCATGGCTTCTTCCGATG	*ACBP6*
35S-1F	GCTCCTACAAATGCCATCA	CaMV 35S promoter
35S-1R	GAT AGT GGG ATT GTG CGT CA	CaMV 35S promoter
35SB	CAATCCCACTATCCTTCGCAAGACC	CaMV 35S promoter
NPT II-2F	CTGCAGACCATGAT	*NPTII*
NPT II-2R	GTCAGCCACTCC	*NPTII*
NPT II-3F	AGACAATCGGCTGCTCTGAT	*NPTII*
NPT II-3R	TCATTTCGA ACCCCAGAGTC	*NPTII*

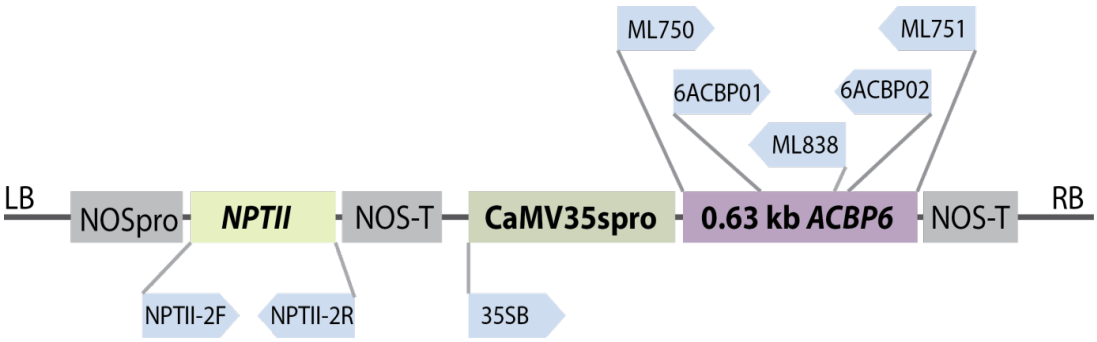


Figure 10. Relative location of primers amplifying *NPTII*, CaMV *35S* promoter, and *ACBP6*. The construct contains LB - left border, NOSpro - nopaline synthase promoter, NOS-T – nopaline synthase terminator, CAMV35Spro – CAMV *35S* promoter, *ACBP6* gene, NOS terminator and RB – right border.

4.2.3.3 RNA Extraction

Total RNA was extracted from seedling leaves of putatively transformed plants in Experiment 2. Upon harvesting, the leaf samples were immediately placed in liquid nitrogen. The leaf samples (approximately 50 mg) were ground into very fine powder in liquid nitrogen, and RNA was extracted using the Trizol® (Thermo Fisher Scientific, U.S.A.) method. The extracted RNA samples were evaluated using agarose gel electrophoresis containing formaldehyde (Sambrook & Russell, 2001) and quantified using NanoDrop UV-Vis spectrophotometer (Thermo Fisher Scientific, U.S.A) and diluted into 200 ng working stock with RNase-free water.

4.2.3.4 Reverse-transcription PCR (RT-PCR)

In vitro first-strand complementary DNA (cDNA) synthesis from RNA was carried out with Omniscript® Reverse Transcription kit (Qiagen Pty. Ltd., Germany), using oligo-dT primer following manufacturer's instructions. The reverse transcription (RT) reaction had a final volume of 20 µL composed of 1x RT buffer, 0.5 mM each of dNTP, 1 µM of oligo-dT primer, 10 units of RNase inhibitor, 4 units of Omniscript Reverse Transcriptase, and 200 ng RNA. Reaction was carried out at 37°C for 60 minutes. The cDNA product (2 µL) was then used as the PCR template for a 20 µL PCR reaction; PCR conditions were as previously described in section 4.2.3.2.

4.2.3.5 Western blot analysis

Leaf samples of 4-week-old seedlings of *ACBP6*-transformed and untransformed plants in Experiment 2 were harvested for protein extraction. Fifty mg of leaf tissues were ground into very fine powder in liquid nitrogen and transferred into 2 mL microcentrifuge

tubes. The extraction buffer was prepared by mixing 150 mM NaCl, 1 mM EDTA, 1% β-mercaptoethanol, 1 mM PMSF, and Tris-HCl pH 7.5, in 5% glycerol in water (Shewry & Fido, 1996). Pre-cooled (-4°C) 700 µL of the extraction buffer was immediately added into the samples and incubated on ice for 30 minutes, with mixing for 3 seconds on a vortex mixer every 5 minutes. The samples were spun at 10,000 for 30 minutes at 4°C. The protein supernatant from each sample was carefully transferred to a new tube and kept on ice.

The extracted protein samples were quantified using the Total Protein Kit, Micro Lowry, Peterson's Modification (SIGMA® TP0300 AND L 3540, Sigma-Aldrich, U.S.A.) following manufacturer's instructions. Working stock solutions were prepared by diluting the samples in SDS reducing buffer (Laemmli, 1970) in 1:2 sample to buffer ratio. Prior to use, 50 µL of β-mercaptoethanol was added to 950 µL of each sample buffer. The protein samples were denatured at 95°C for 4 minutes.

Total proteins were separated with polyacrylamide gel electrophoresis using the Mini-PROTEAN® 3 Cell (Bio-Rad Laboratories, U.S.A.), following the SDS-PAGE Laemmli Buffer system (Laemmli, 1970). The stacking and resolving gels were made up of 6% and 15% acrylamide/bis, respectively, and prepared from 30% acrylamide/bis (37.5:1) solution (Bio-Rad 1610158). The polyacrylamide gels were casted using a 0.75 mm spacer, and 10-well comb. A total of 40 µg of each protein sample were loaded into each well; a protein marker (Novex® Sharp Pre-stained Protein Standard, Invitrogen U.S.A.) was also included. Electrophoresis was carried out at 150 V until the samples passed the stacking gel, and lowered down to 60 V until electrophoresis was completed. A duplicate gel was included in the same electrophoresis run to determine proper

separation of the protein samples. The duplicate gel was stained with Bio-Safe™ Coomassie Stain (Bio-Rad Laboratories 1610786) and photographed.

The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, U.S.A.). Before the transfer, the membrane was pre-wet by immersing in 100% methanol for a few seconds until the membrane turned translucent. The membrane was then equilibrated in the transfer buffer, ensuring that it was fully submerged. All ensuing steps followed the instructions included in the Mini Trans-Blot® electro blotter manual. Transfer was carried out at 30 V, 90 mA overnight with 25 mM Tris pH 8.3, 192 mM glycine transfer buffer. The membrane was sent to Prof. ML Chye (HKU) for hybridization with *ACBP6*-specific antibodies generated from a synthetic peptide (VEGKSSEEAMNDY) (Chen et al., 2008) that corresponded to amino acids 63 to 75 of the *ACBP6* protein.

4.2.4 Statistical analysis

The regeneration and transformation response of the three genotypes was measured in terms of *regeneration frequency*, which is the proportion of explants regenerating green shoots under selection pressure to the number of explants cultured; and *transformation efficiency*, which is the proportion of transgenic plants recovered to the number of explants cultured. Analysis of variance (ANOVA) was performed to determine if there were significant differences in the three genotypes using IBM® SPSS Statistics 22 Software.

4.2.5 Progeny segregation test

T_1 and T_2 seeds from transgenic rapid-cyling *B. napus* and *B. napus* 'Westar' were germinated in MS medium containing 100 mg/L kanamycin. After 10 days, the number of seedlings that showed resistance (rooted green seedlings, with green true leaf and stem) and susceptibility (purple and pale or white seedlings) to kanamycin were counted. Chi-square goodness of fit was used to test against 3:1 ratio for Mendelian inheritance of a single dominant gene. The T_1 and T_2 progeny were PCR-screened using both *NPTII* and *ACBP6* specific primers.

4.3 Results

4.3.1 Experiment 1: Initial transformation studies on B. napus *'Westar'*

Initial transformation experiments showed very low transformation efficiency (Table 4.2). The first batch (A) of these transformation experiments had only 11 plants from 961 explants survive the final selection step and develop roots. Total transformation efficiency was only 1.14%. There were 1452 explants used for the second batch (B) of transformation experiments, wherein the final step of selection was omitted. There were 55 rooted plants contributing to a transformation efficiency of 3.79%.

Table 4.2. Preliminary transformation of cotyledon explants of *B. napus* 'Westar'.

Experimental batch	No. of explants	No. of explants regenerated shoots	Regeneration frequency (%)	Rooting plantlets	Transformation efficiency (%)
A	961	46	4.7	11	1.14
B	1452	270	18.6	55	3.79

Experiments A and B represent 2 experimental batches of transformation of B. napus *'Westar' cotyledon explants with pAT593 using* A. tumefaciens *GV3101::pMP90.*

PCR screening results using primers specific for *NPTII* and 35S::*ACBP6* (Table 4.3) showed that out of the 11 putative transformants in Batch A, 7 were positive for both transgenes (*35S::ACBP6* and *NPTII)*, 1 was positive for *35S::ACBP6* but negative for *NPTII*, and 1 positive for *NPTII* but negative for 35S::ACBP6. Two plants were negative for both of the 2 transgenes analysed, which indicated that these 2 were untransformed but survived kanamycin selection (escapes). For batch B, there was a very high proportion of escapes (38 out of 55) that were negative for *NPTII* and 35S::*ACBP6* DNA. Two plants were confirmed for the presence of both *NPTII* and 35S::*ACBP6*, with only 14 positive for the 35s::*ACBP6*, and 1 positive for *NPTII*.

Table 4.3. PCR screening results of putative transformants in the initial transformation experiments A and B.

Experimental batch	PCR Result	*35S::ACBP6* (+)	*35S::ACBP6* (-)	Total number of plants
A	*NPTII* (+)	7	1	11
	NPTII (-)	1	2	
B	*NPTII* (+)	2	1	55
	NPTII (-)	14	38	

Experiments A and B represent 2 experimental batches of transformation of B. napus 'Westar' cotyledon explants with pAT593 using A. tumefaciens GV3101::pMP90.

Analysis of PCR-confirmed T_0 adult plants established in the glasshouse showed inconsistent PCR results compared to when they were *in vitro* regenerated plantlets. PCR results from 2 sampled branches of the same plant were not consistent. Table 4.4 shows the PCR results for both *NPTII* and *35S::ACBP6* primers.

Table 4.4. PCR analysis on established mature T_0 plants analysed on separate branches (a and b).

Plant Number*	Branch	*NPTII*	*35S::ACBP6*
AT0_1	a	-	+
	b	+	-
AT0_19	a	+	-
	b	-	-
AT0_21	a	+	+
	b	-	+
BT0_8	a	-	+
	b	-	-
BT0_15	a	+	+
	b	-	-
BT0_16	a	+	+
	b	-	-

**Three plants from each batch were analysed. Plants number labels starting with 'A' comes from experiment A, and 'B' from experiment B. Experiments A and B represent 2 experimental batches of transformation of* B. napus *'Westar' cotyledon explants with pAT593 using* A. tumefaciens *GV3101::pMP90.*

4.3.2 Experiment 2: Agrobacterium-*mediated transformation of rapid-cycling* Brassica spp. *and* B. napus *'Westar'*

4.3.2.1 Sensitivity of *Brassica* explants to kanamycin

The three genotypes – rapid cycling *B. napus* (BNFP), rapid-cycling *B. juncea* (BJFP) and *B. napus* 'Westar' (BNW) were tested for kanamycin sensitivity at concentrations of 0, 10, 20, 30 and 50 mg/L. Addition of 10 mg/L kanamycin reduced the regeneration frequency by almost 50% (Figure 4.3). As shown in Figure 4.4, the regenerating explants grew and expanded, almost comparable to that of the untreated explants; remained green, and were able to produce shoot buds; however, development of viable shoots was greatly inhibited, such that they did not further elongate. At 20 mg/L, there was an increase in size of the cotyledon explants, but they become bleached and were unable to produce new growth. Majority of the explants produced callus but did not develop further and no viable shoots were able to be harvested from the explants. At 30 mg/L kanamycin the explants initially expanded, but died within 2 weeks *in vitro*. All the explants died within 1 week when grown in SRM with 50 mg/L kanamycin. The three genotypes exhibited the same degree of sensitivity in terms of regeneration frequency when tested in varying levels of kanamycin ($p=0.931$).

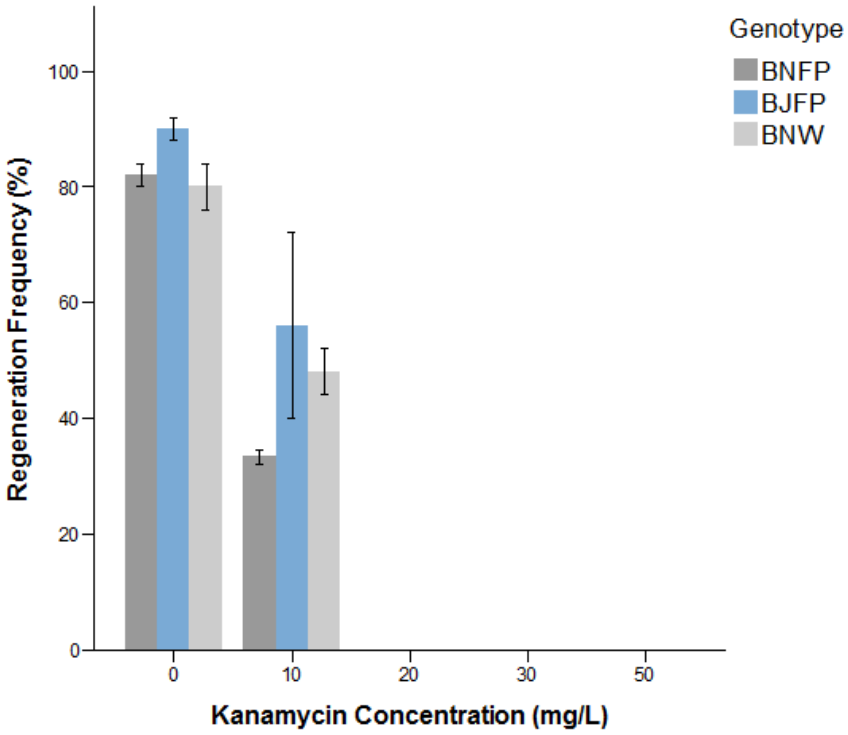


Figure 11. Sensitivity of rapid-cycling *B. napus* (BNFP), rapid-cycling *B. juncea* (BJFP) and *B. napus* 'Westar' (BNW) cotyledon explants to varying concentrations of kanamycin. There was no tissue regeneration observed from 20 - 50 mg/L of kanamycin.

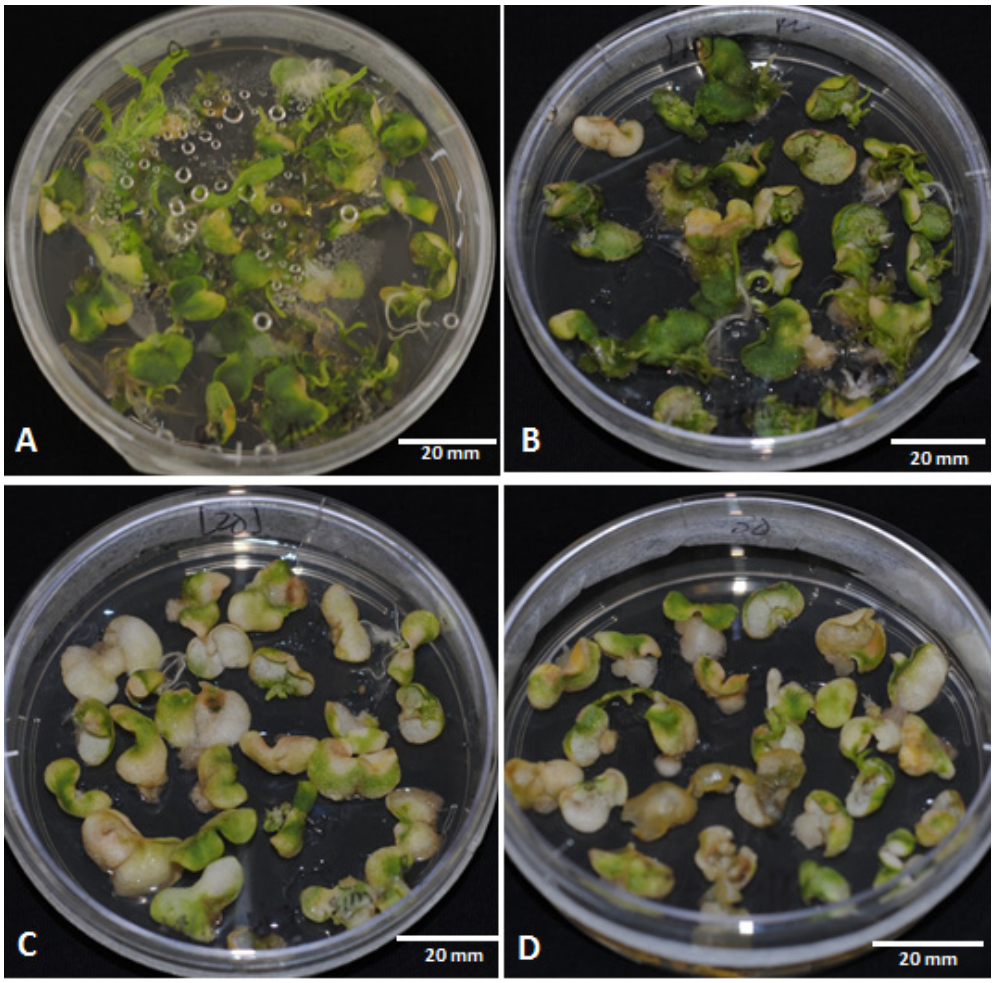


Figure 12. Cotyledon explants grown in shoot regeneration medium with varying concentration of kanamycin: (A) no kanamycin, (B) 10 mg/L, (C) 20 mg/L and (D) 30 mg/L. Regeneration of green tissues can be observed in 0 and 10 mg/L of kanamycin, while at 20 and 30 mg/L, the explants were bleached and did not regenerate.

4.3.2.2. Effect of *Agrobacterium* concentration on shoot regeneration of cotyledon explants

Over all one-way analysis of variance for all cotyledon explants evaluated showed significant difference in terms of regeneration frequency for the three *Agrobacterium* concentrations evaluated (Appendix 4), with OD_{650}=0.1 having the highest average regeneration of 53.9%. When evaluated for each genotype (Table 4.5), 54.1% BNFP explants produced shoot initials in the shoot initiation medium with 25 mg/L kanamycin when inoculated with OD_{650}=0.10 *Agrobacterium*, followed by 26.3% at OD_{650}=0.5 and at OD_{650}=0.05, 24.27% was recovered with shoot initials. In Westar, both OD_{650}=0.05 and OD_{650}=0.1 showed high regeneration frequency of 41.3% and 53.7%, respectively. At high *Agrobacterium* concentration (OD_{650}=0.5), the explants of BNFP exhibited necrosis at the petiole end of the cotyledon after 2 days in the co-cultivation medium.

Table 4.5. Regeneration response of cotyledon explants of *B. napus* 'Westar' (BNW) and rapid-cycling *B. napus* (BNFP) to varying concentrations of *Agrobacterium*.

	BNW			BNFP		
Agrobacterium concentration (OD_{650})	No. of explants*	No. of explants with shoots	Regeneration frequency (%)	Total no. of explants*	No. of explants with shoots	Regeneration frequency (%)
0.05	27.0	11.0	41.3[b]	25.3	6.3	24.2[a]
0.1	24.3	13.0	53.7[b]	21.0	11.3	54.1[b]
0.5	24.0	4.7	19.7[a]	23.0	6.0	26.3[a]

**Mean number of explants from 3 culture plates.*
[ab]Letters indicate means for groups in homogenous subsets based on Tukey's HSD.

4.3.2.3 Regeneration of transgenic shoots

The cotyledon explants of rapid-cycling *B. napus* (BNFP), rapid-cycling *B. juncea* (BJFP) and *B. napus* 'Westar' (BNW) showed varying shoot regeneration rates *in vitro* after infection and co-cultivation with *Agrobacterium* harbouring the 13.5-kb plasmid pAT593. After two days of co-cultivation of cotyledon explants of rapid-cycling genotypes, there was an apparent increase in size of the cotyledon explants. Callus formation in the petiole end of the cotyledons and presence of shoot primordia were observed after 7 days in the callus induction medium, which contained carbenicillin to kill *Agrobacterium* cells. Profuse shoot bud formation was observed at 5 days after the explants were transferred to the shoot initiation medium (SRM with 250 μg/mL carbenicillin and 25 μg/mL kanamycin). After 10 days at in this medium, three kinds of phenotypes were observed: explants producing green shoots, explant with greenish and purple shoots, and explants with completely white shoots. Figure 4.5 shows the regeneration stages of cotyledon explants of rapid-cycling *B. napus*. Hyperhydricity or vitrification was prevalent in the rapid-cycling *B. juncea* shoots, with elongated, brittle, and translucent shoots. In 'Westar' there was extensive callus proliferation and explant enlargement up to 14 days at which time shoot initiation occurred.

From 10-14 days in the shoot initiation medium, BNFP and BJFP maximized shooting potential with the shoot explants being transferred to shoot outgrowth medium. Developed shoots with defined leaves were excised from the growth clump. In contrast, the explants of Westar were transferred to the shoot outgrowth medium around 20-25 days. The regeneration frequencies of the potentially transformed explants were not significantly different among the three *Brassica* genotypes, with BNFP having 12.9 %, BJFP 15.5% and 'Westar' 14% regeneration frequency (Table 4.6). Hyperhydric shoots

of rapid-cycling *B. juncea* did not develop true leaves and did not express proper morphogenesis.

Table 3. *In vitro* regeneration of cotyledon explants of rapid-cycling *B. napus* (BNFP), rapid-cycling *B. juncea* (BJFP), and *B. napus* 'Westar' (BNW), transformed with pAT593.

Genotype	No. of explants	No. of explants with shoots	Regeneration Frequency (%)	No. of plantlets produced	No. of rooting plantlets	Transformation efficiency (%)
BNFP	247	32	12.9	27	15	6.1
BJFP	252	39	15.5	32	0	0
BNW	406	57	14	46	20	4.9

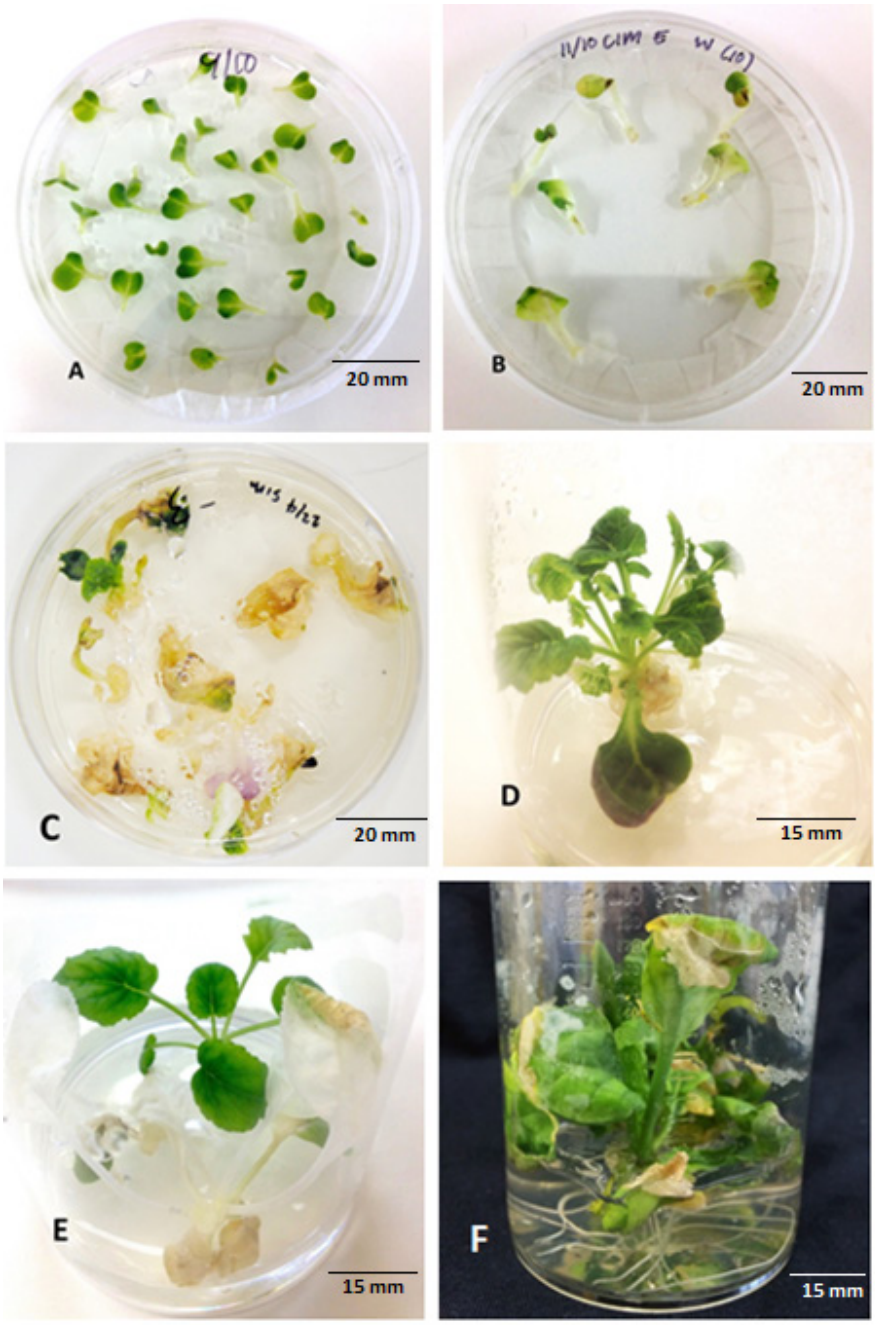


Figure 13. Stages of plant regeneration from cotyledon explants of rapid-cycling *Brassica napus* transformed with *ACBP6* through *Agrobacterium* infection. (A) Explants after 2 days of co-cultivation with *Agrobacterium*. (B) Explants in callus induction medium showing swelling and callus formation at the petiole end. (C) A transformed explant producing green shoots after 10 days in shoot initiation medium. (D) Transformed shoots developing in shoot outgrowth medium. (E) Well-developed shoots excised and transferred to the selection medium containing 50 mg/L kanamycin. Non-transformed shoots became bleached in the medium. (F) Transformed plantlets grown in the rooting medium to develop roots before transplanting to soil.

4.3.2.4 Recovery of transgenic plants

At 7-10 days, non-transformed shoots started to turn white, and at 21 days non-transformed shoots completely died. There was a huge reduction in the number of putatively transformed shoots subjected to 50 mg/L kanamycin concentration, with 'Westar' being reduced by 56% and BNFP by 44%, while BJFP plantlets were completely killed. The transformation efficiencies of BNFP and 'Westar' were 6.1% and 4.9%, respectively. After transfer of the green shoots to the rooting medium, not all of the transformed shoots developed roots with 15% of the shoots developing callus instead. The roots that appeared originated from the callus. Constant checking and subculturing to scrape off the growing callus resulted in the shoots finally producing true roots. Out of the 20 transformed 'Westar', 17 established roots; while 10 out of 15 BNFP rooted. When transplanted to the soil, shoots with well-established roots readily grew in the soil (Figure 4.6). The transgenic plants developed into normal mature plants and produced fertile seeds.

Figure 14. Transgenic *B. napus* 'Westar' (left) and rapid-cycling *B. napus* (right) at reproductive stage, growing in the glasshouse.

4.3.2.5 PCR analysis of T_0 plants

The *35S::ACBP6* region and *NPTII* gene were amplified in all T_0 BNFP plants. In 'Westar', 24 plants were positive for *35S/ACBP6* and 25 were positive for *NPTII* out of a total of 29 plants. Figure 4.7 shows the amplification of the 0.47 kb *35S/ACBP6* fragment in selected T_0 BNFP and Westar plants. Figure 4.8 is an example of PCR screening of T_0 plants for *NPTII*.

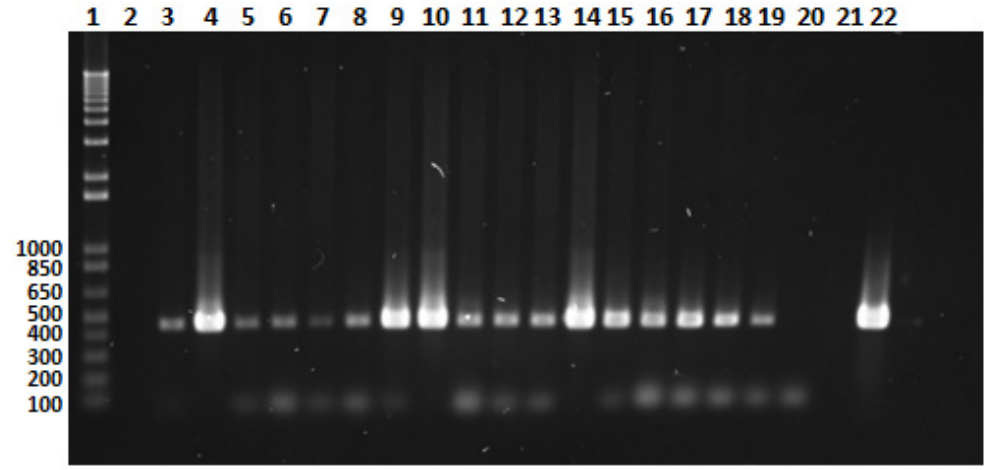


Figure 15. PCR of independent T_0 transgenic plants established in the glasshouse, amplifying 35s::*ACBP6* transgene region (0.47 kb) of pAT593 using primer pairs 35SB/ML838. Lane 1- 1 kb Plus DNA ladder, lane 2- empty, lanes 3 to 9 *B. napus* 'Westar' independent T_0 plants (BNW01, BNW02, BNW03, BNW04, BNW05, BNW06, and BNW07, respectively); and lanes 10-14 rapid-cycling *B. napus* (BNFP01, BNFP02, BNFP03, BNFP04 and BNFP05, respectively). Lane 20 is 'Westar' untransformed control, lane 21 empty and lane 22 plasmid control.

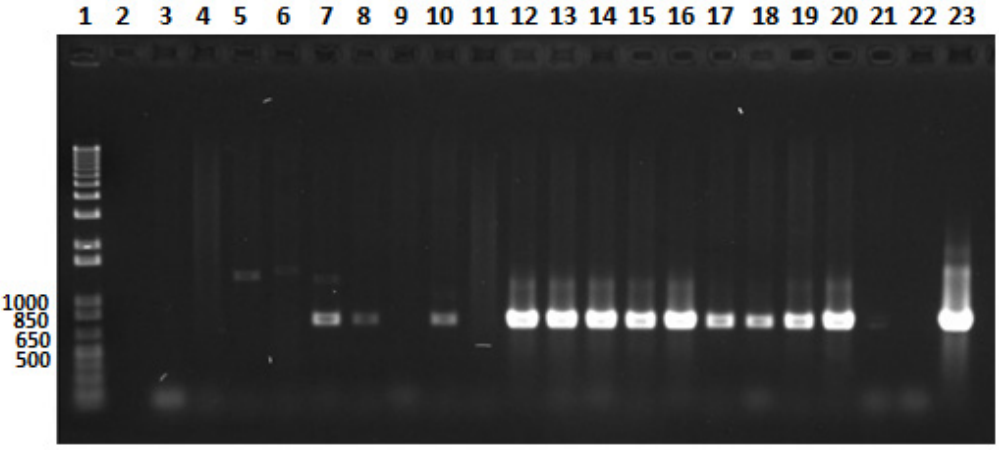


Figure 16. PCR of independent T_0 transgenic plants using primer pairs NPTII-2F/NPTII-2R amplifying the *NPTII* transgene (0.72 kb). Lane 1- 100 kb Plus ladder, lane 2- empty, lanes 3-20 T_0 plants, and lane 22 untransformed 'Westar' DNA. Lanes 12-6 are rapid-cycling *B. napus* plants (BNFP01, BNFP02, BNFP03, BNFP04 and BNFP05, respectively); and lanes 17-20 *B. napus* 'Westar' plants (BNW01, BNW02, BNW03 and BNW04, respectively).

4.3.2.6 Expression of ACBP6

Reverse-transcription PCR using both the *ACBP6* specific primer pair ML750/ACBP02 which amplified 0.36 kb of the *ACBP6* gene and *NPTII* primer which amplified a 0.72 kb fragment showed that 12 out of 20 properly established T_0 plants were consistently positive for the two genes (Figure 4.9).

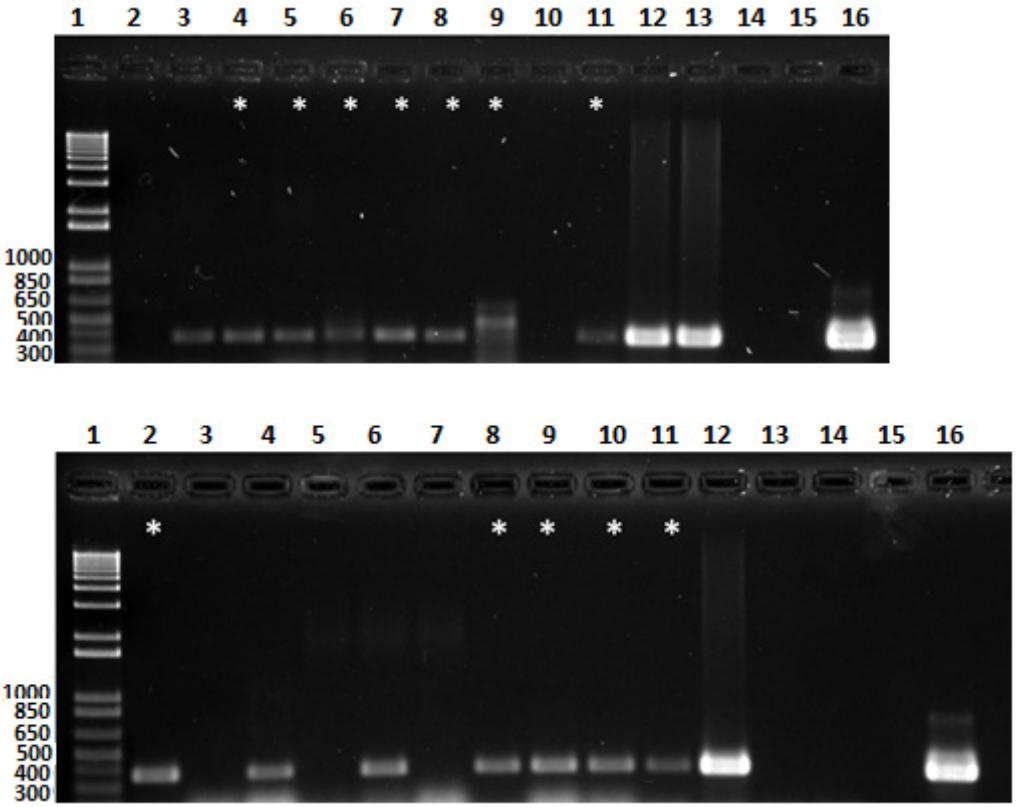


Figure 17. Reverse transcription PCR (RT-PCR) on T_0 transgenic plants using ML750/ACBP02 primers, amplifying the 365 bp *ACBP6* DNA. Top: Lane 1 – 1 kb Plus DNA Ladder, lane 2- empty, lanes 3-11 cDNA samples of T_0 plants, lanes 12 and 13 genomic DNA PCR control, lane 14 wild type *B. napus*, lane 15 no template control, and lane 16 pAT593. Bottom: Lane 1 – 1 kb Plus DNA Ladder, lanes 2-11 cDNA of T_0 plants, lane 12 genomic DNA PCR control, lane 13 wild type *B. napus*, lane 14 no template control, lane 15 empty, and lane 16 pAT593. The 12 selected T0 plants were indicated by asterisks.

Western blot analysis of 13 T_0 plants that were positive for ACBP6 in RT-PCR analysis showed that 12 plants expressed 10.4 kDa ACBP6 protein in the leaves (Figure 4.10).

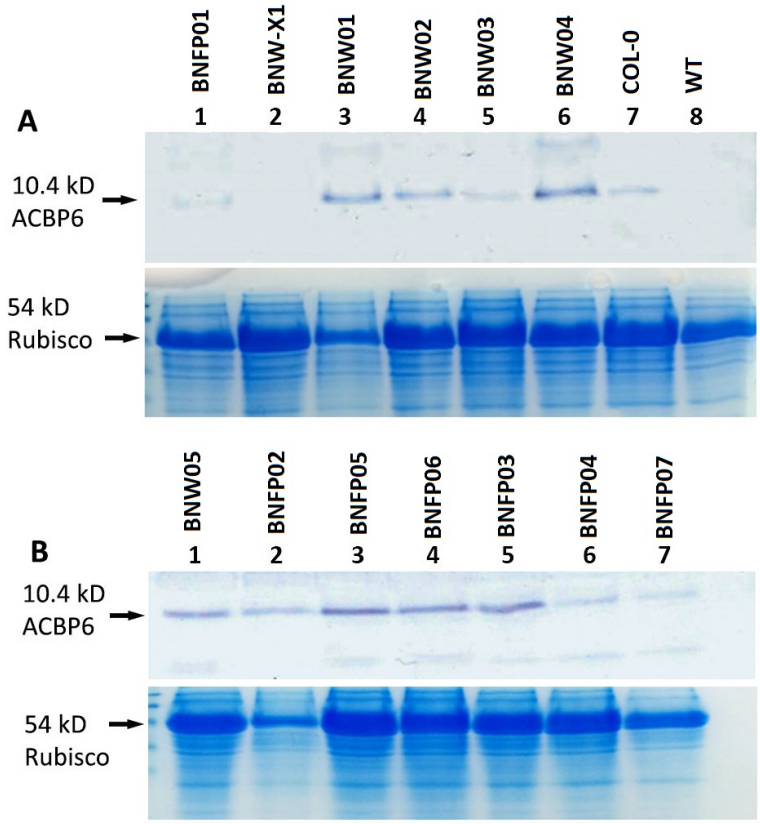


Figure 4.10**.** Western blot analysis using ACBP6-specific antibodies in T_0 plants of rapid-cycling *B. napus* and *B. napus* 'Westar'. Total protein (20 µg per lane) was extracted from 13 T_0 plants. The top panel shows the Western blot with 10.4 kD ACBP6 protein, and the bottom panel shows an identically loaded gel and stained with Coomassie blue. (A) Lanes 1-6 T_0 plants, lane 7 *A. thaliana* Col-0, and lane 8 wild type *B. napus*. (B) Lanes 1 to 7 all T_0 plants. Only 1 T_0 plant (A, lane 2) showed no expression of the *ACBP6*.

4.3.3 Segregation of transgene in the progeny

4.3.3.1 Kanamycin screening of T_1 and T_2 progeny

Seven of the 10 T_1 transgenic lines tested, followed the 3:1 (kanamycin-resistant, Kan^R:kanamycin-sensitive, Kan^S) ratio for Mendelian inheritance of a single dominant gene, $p > 0.05$ (Table 4.7). Three lines, BNFP03, BNW04 and BNW06, did not fit the expected segregation ratio, $p < 0.05$. All transgenic lines in the T_2 generation inherited the kanamycin resistance transgene in 3:1 ratio. All transgenic lines in the T_2 generation also inherited the kanamycin resistance transgene in 3:1 ratio (Table 4.8).

Table 4. Segregation analysis of T_1 progeny of pAT593-transformed *B. napus* plants in MS medium containing 50 mg/L kanamycin, tested at 3:1 ratio for Mendelian monogenic inheritance.

T1 Progeny	Total (*n*=3)	*Mean*		χ^2
		Kan^R	Kan^S	
BNFP01	119	25.0	14.7	3.0
BNFP02	96	27.3	4.7	1.9
BNFP03	112	22.3	15.0	4.6*
BNFP04	101	21.7	12.0	2.0
BNFP05	96	20.7	11.3	1.9
BNW03	92	25.0	5.7	0.7
BNW04	112	20.7	16.7	7.7*
BNW05	92	21.3	9.3	0.5
BNW06	100	19.3	14.0	5.1*
BNW07	92	21.7	9.0	0.3

**Not significant, P value (0.05) = 3.841, df=1.*

Table 5. Segregation analysis of T_2 progeny of pAT593-transformed *B. napus* plants in MS medium containing 50 mg/L kanamycin tested at 3:1 ratio for Mendelian monogenic inheritance.

T_2 Progeny	Total (*n*=3)	Mean		χ^2*
		Kan^R	Kan^S	
BNFP01	104	22.7	12.0	1.7
BNFP02	88	20.3	9.0	0.5
BNFP03	116	24.0	14.7	3.4
BNFP04	92	21.3	9.3	0.5
BNFP05	98	25.7	7.0	0.2
BNW06	121	27.3	13.0	1.1
BNW07	101	21.3	12.3	2.4

**All chi-square values, χ^2, are significant at P value (0.05) = 3.841, df=1.*

4.3.3.2 T_1 and T_2 progeny screening for *ACBP6* and *NPTII*

PCR screening of T_1 progeny of the 10 selected independent transgenic events (5 BNFP and 5 BNW) revealed that all followed the predicted 3:1 Mendelian ratio when genotyped for the presence of *ACBP6* $p > 0.05$ (Table 4.9 and Figure 4.11). However, when genotyped for the *NPTII*, two lines, BnFP05 and BnW04, deviated from the tested ratio, $p < 0.05$.

T_2 progeny were bulked for each transgenic line for PCR analysis, and showed that all lines tested for inheritance of *ACBP6* (5 BNFP and 2 BNW) fitted the 3:1 Mendelian ratio, with the exception of BNFP05. Transmission of the NPTII gene deviated from the tested ratio in progenies of BNFP01 and BNFP03 (Table 4.10). Figure 4.12 shows the segregation of *ACBP6* transgene in BNFP05 T_2 progeny.

Table 6. Segregation analysis for *ACBP6* and *NPTII* in T_1 progeny of pAT593-transformed rapid-cycling *B. napus* (BNFP) and *B. napus* 'Westar' (BNW), tested at 3:1 ratio for Mendelian monogenic inheritance.

T_1 Progeny	*ACBP6*		χ^2	*NPTII*		χ^2
	Positive	Negative		Positive	Negative	
BNFP01	35	14	0.33	32	17	2.46
BNFP02	17	1	3.63	17	1	3.63
BNFP03	29	6	1.15	29	6	1.15
BNFP04	11	6	0.96	10	7	2.37
BNFP05	15	8	1.17	13	10	4.19*
BNW03	14	1	2.69	14	1	2.69
BNW04	16	7	0.36	11	12	9.06*
BNW05	7	3	0.13	7	3	0.13
BNW06	17	8	0.65	16	9	1.61
BNW07	11	3	0.10	8	6	2.38

**Not significant, P value (0.05) = 3.841, df=1.*

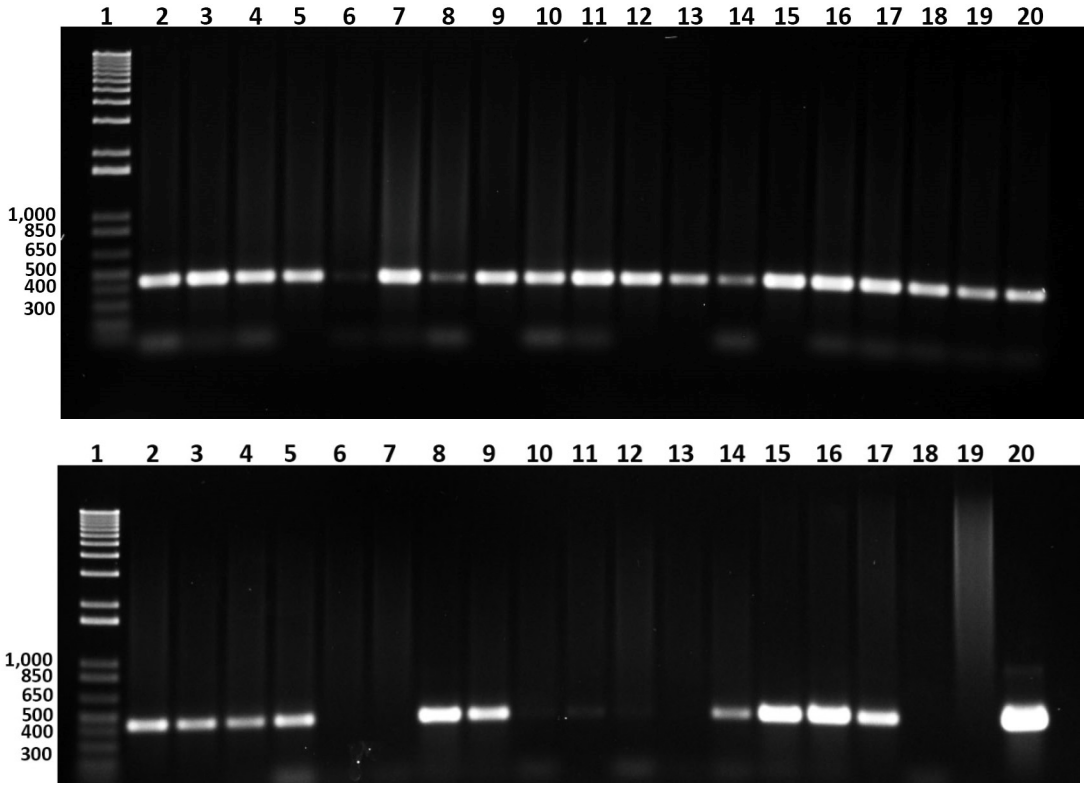


Figure 4.11. An example of PCR screening for *ACBP6* in T_1 progeny of BNFP03, using primers for *35S::ACBP6* amplifying 0.47 kb region of the transgene. Top gel: Lane 1- 1 Kb Plus DNA Ladder, lanes 2-20 BNFP03 T1 progeny. Bottom gel: Lane 1-1 Kb Plus DNA Ladder, lanes 2-17 BNFP03 T_1 progeny, lane 18 empty, lane 19 untransformed BNFP, and lane 20 pAT593 positive control.

Table 7. Segregation analysis for *ACBP6* and *NPTII* in 7 transgenic lines of the T_2 progeny of pAT593-transformed rapid-cycling *B. napus* (BNFP) and *B. napus* 'Westar' (BNW), tested at 3:1 ratio for Mendelian monogenic inheritance.

T_2 Progeny	*ACBP6*		χ^2	*NPTII*		χ^2
	Positive	Negative		Positive	Negative	
BNFP01	48	22	1.54	45	25	4.29*
BNFP02	14	2	1.33	13	3	0.33
BNFP03	22	10	0.67	19	13	4.17*
BNFP04	15	4	0.16	11	8	2.96
BNFP05	30	19	4.96*	34	15	0.82
BNW06	13	6	0.44	16	3	0.86
BNW07	19	10	1.39	21	9	0.45

**Not significant, P value (0.05) = 3.841, df=1.*

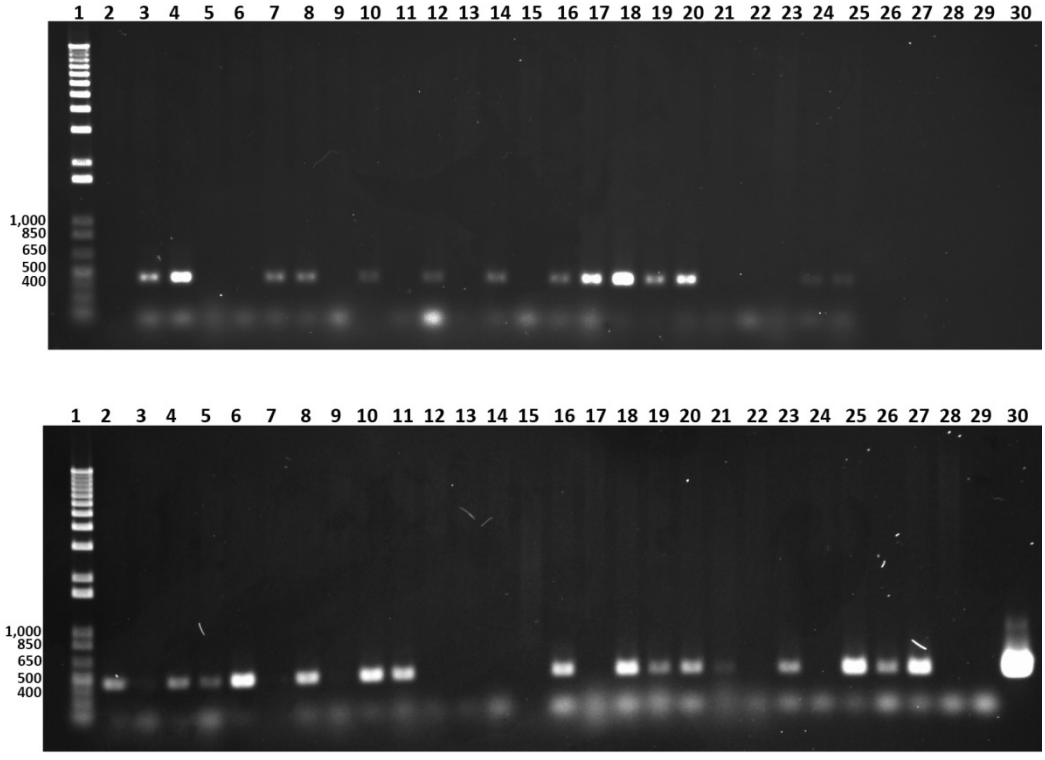


Figure 18. PCR screening for *ACBP6* in T_2 progeny of transgenic line BNFP05, using primers for *35S::ACBP6* amplifying 0.47 kb region of the transgene. Top gel: Lane 1 is 1 Kb Plus DNA Ladder, lane 2 empty, lanes 3-25 BNFP05 T_2 plants, and lanes 26-30 empty. Bottom gel: Lane 1 is 1 Kb Plus DNA Ladder, lanes 2 – 27 T_2 plants, lane 28 and 29 are untransformed control, and lane 30 is plasmid positive control.

4.4 Discussion

Brassica napus 'Westar' and rapid-cycling *B. napus* were successfully transformed with pAT593 through *Agrobacterium*-mediated transformation; while transformation of rapid-cycling *B. juncea* was unsuccessful. The transformation efficiency of 4.9% and 6.1% for rapid-cycling *B. napus* and *B. napus* 'Westar', respectively, was lower than the transformation efficiency of 33% reported by Bhalla and Singh (2008) and 55% reported by Moloney et al. (1989). Other transformation efficiencies reported for Westar varied from 4% to 25% (Cardoza and Stewart, 2003) and maximum of 8.6% by Hao et al. (2010); while Damgaard et al. (1997) reported varying transformation efficiencies of Westar cotyledons from 4% to 12%, depending on the *Agrobacterium* strain and vectors used.

Agrobacterium-mediated transformation of *Brassica* species, and *in vitro* regeneration, has been widely known to be genotype-dependent (Christey et al., 1997; De Block et al., 1989; Poulsen, 1996). The transformation procedure and *in vitro* regeneration system of transformed shoots were based on a multi-step approach similar to that of De Block et al. (1989); Metz et al. (1995); Moloney et al. (1989). Cotyledon as an explant source has been reported numerous times to be ideal for transformation and regeneration of long duration *Brassica* genotypes (Bhalla & Singh, 2008; Bhuiyan et al., 2011; Hachey et al., 1991; Moloney et al., 1989; Zhang et al., 2000).

The rapid-cycling *B. juncea* showed higher tendency for hyperhydricity or vitrification in the media from early to later stages *in vitro*, after co-cultivation with *Agrobacterium* resulting to loss of apical dominance and lack of development of true leaves. These hyperhydric shoots had very poor chance of establishing once subcultured to a new medium, for example from shoot initiation medium to shoot elongation medium. Also, the regenerated shoots were extremely brittle and difficult to separate from the shoot

clump. Hyperhydricity of micropopagated shoots is caused by numerous stress factors such as wounding, high humidity, build-up of gasses in the tissue culture container, concentration of the gelling agent, and hormonal imbalance, among others (Kevers et al., 2004). The apparent tendency of the rapid-cycling *B. juncea* to be hyperhydric in the same media that both rapid-cycling *B. napus* and 'Westar' successfully regenerated new plants, would require a tissue culture protocol optimised solely for this genotype. In *B. oleracea*, hyperhydricity was reversed by reducing sucrose, removing ammonium ion sources from the nutrient medium, and increasing the gelling agent concentration (Yu et al., 2011).

There was a large decrease and low regeneration frequencies of *Agrobacterium* co-cultivated explants, for both rapid-cycling *B. napus* and *B. napus* 'Westar', compared to the regeneration frequencies of non-inoculated explants (Chpater 3). The reduction in regeneration frequencies may have been due to the non-susceptibility of the highly morphogenic meristematic cells to transformation (Mukhopadhyay et al., 1992), thereby non-survival of the untransformed cells during selection with kanamycin. Wounded cells along the petiole side of the cotyledon explants have very high morphogenic potential, as histologically demonstrated by Hachey et al. (1991), hence, shoots readily formed in the optimum medium when the explants were tested for their regeneration ability. However, this primary layer of cells may not be readily amenable for transformation. Mukhopadhyay et al. (1992) elucidated that vascular parenchyma cells approximately 500 μm behind the cut surface of a cotyledon explant directly regenerated into shoots, but genetic transformation in these cells were rare and yielded chimeras. Meanwhile, hypocotyl explants that underwent an intermediary callus phase were readily transformed. The presence of auxin and cytokinin in the media after *Agrobacterium* infection induced callus formation of the wounded cotyledon. Transformed shoots from the cotyledon

explants may have originated from both direct regeneration of vascular parenchyma cells, and through the cells that underwent callus phase. Nevertheless, regeneration decreased once kanamycin selection was applied, because not all of the target cells were transformed.

The transformation protocol followed a 2-day co-cultivation step, which was essential to enable the *Agrobacterium* to infect and transform the wounded actively growing cells. The *Agrobacterium* concentration of OD_{650}=0.1 produced more explants with green shoots ($p < 0.05$) than at 0.05, while increasing to OD_{650}=0.5 resulted in the inoculated petiole ends of the cotyledon explants becoming necrotic after 2 days of co-cultivation. Forty-eight hours of co-cultivation in *Agrobacterium* was determined optimum in most transformation studies in *Brassica* including *B. napus* 'Westar' (Bhuiyan et al., 2011; Khan et al., 2003; S. Radke et al., 1988).

After co-cultivation, the explants developed in the medium without application of kanamycin for 7 days. During this period, callus formed on the cut surface of the petiole ends of the cotyledon explants, indicating high cell division activity. The period in which application of kanamycin was withheld allowed regeneration of both transformed and untransformed cells. However, when kanamycin was applied, only the transformed cells divided and differentiated to form shoots. Other *Brassica* transformation studies (Bhuiyan et al., 2011; Tang et al., 2011) showed that by delaying application of kanamycin until after shoot primordia formation, the transformation efficiency of *B. juncea* and *B. napus* greatly improved.

The number of green putatively transformed regenerated plantlets of the three genotypes was less than that of the initial number of explants producing green shoots. This meant that not all shoots harvested from the explants survived or developed further,

although they were maintained at the same selection pressure (25 mg/L of kanamycin). The separation of shoots from the shoot clump was critical for the success of putatively transformed regenerated shoots to develop further. Excision of the shoots was undertaken so as not to damage the shoot base thus ensuring that a complete shoot was separated. The overall vigour of the putatively transformed shoots growing in the presence of 25 mg/L of kanamycin allowed these to develop into plantlets. Improperly developed shoots, or damaged shoots from excision were more unlikely to further develop into plantlets. Untransformed control explants growing on 25 mg/L of kanamycin failed to develop further because the kanamycin inhibited cell division and photosynthesis.

A large proportion of the regenerated well-developed shoots died during the final selection step treatment with 50 mg/L, and some were unable to establish roots. From the initial transformation studies in Experiment 1, the final selection step was shown to be necessary to remove non-transformed shoots (escapes or false positives), but the high concentration of kanamycin may also have been toxic as reflected in the extremely low recovery of rooted plantlets. The decrease in survival of regenerated shoots could have been due to the low expression of NPTII, and inability of the shoots to establish roots, a common occurrence *in vitro* transformed plants (Maheshwari et al., 2011). The effect of kanamycin in *Brassica* species has been shown to vary with 20 mg/L preventing growth of untransformed shoots but also inhibiting shoot maturation and rooting of transformed shoots (Berthomieu & Jouanin, 1992; Radke et al., 1992).

PCR analysis of independent transgenic plants (T_0) in Experiment 2 confirmed the presence of the transgenes *ACBP6* and *NPTII*. However, in 'Westar', 5 plants amplified the *NPTII* and not the *ACBP6* transgene. Also, 4 plants only amplified the *NPTII* and not the *ACBP6*. The inconsistency in the detection of the transgenes may have

indicated a possible rearrangement, loss or incomplete integration of the genes into the plant genome. Such occurrences may result in the loss or mutation of the specific binding sites for the PCR primers, resulting in no amplification of the transgene detected. T-DNA rearrangements and truncation may spontaneously occur during transfer and commonly cause non-detection of the entire half portion of the T-DNA towards the right border (Kohli et al., 2010).

Twenty well-established T_0 plants in the glasshouse from Experiment 2 were analysed by RT-PCR and 12 were consistently positive for transcription of *NPTII* and *ACBP6* mRNA. These lines were also positive for the expression of the ACBP6 protein in leaf tissues as analysed by Western blot. Segregation analysis of T_1 progeny of 10 selected lines grown in MS medium with 100 mg/L of kanamycin revealed that 7 out of 10 lines were highly likely to contain a single copy of the *NPTII* transgene as it followed the 3:1 ratio for Mendelian inheritance of a single dominant gene. Three T_1 lines (2 BNFP and 1 BNW) deviated from the tested ratio, instead having a ratio of 1.2-1.5:1 kanamycin-resistant to kanamycin-sensitive plants. PCR analysis of the T_1 generation revealed inconsistency of the segregation of *NPTII* in the progeny. These results however, need to be validated with Southern blot for determination of the exact copy number of the integrated transgenes of each transgenic line.

In the T_2 generation of selected lines, all progeny segregated in the 3:1 Mendelian manner for seedling kanamycin resistance, however PCR analysis showed deviation for the *ACBP6* and *NPTII* transgenes. Transgene integration is commonly inherited as a single Mendelian locus regardless of its copy number. The transgene locus in the primary transformants (T_0) are considered hemizygous and the transgenes are expected to be transmitted as a dominant gene, segregating in 3:1 ratio when self-pollinated (Campbell

et al., 2000). The inconsistent segregation of the transgene from T_1 to T_2 may have been partly attributed to the T_1 flowers not bagged and self-pollinated for T_2 seed production. Although *B. napus* is predominantly self-pollinating, a small chance of cross-pollination with non-transgenic flowers may have occurred. *Brassica napus* flowers have very high pollen load thus pollens from neighbouring flowers have a small chance to successfully cross pollinate. Nevertheless, outcrossing with adjacent plants does occur at approximately 30% under field conditions (Damgaard & Loeschcke, 1994; Hoyle et al., 2007; Simard et al., 2009). Non-Mendelian inheritance may have also occurred due to instability of the transgene during transmission to the next generation; or because it was poorly expressed. There are many possible reasons why a transgene can become unstable in terms of expression and inheritance. Unstable transmission of the marker genes (i.e. *NPTII*) has been widely observed in *Brassica* and tobacco (Potrykus et al., 1985; Sikdar, 1999).

Results of the initial transformation studies on *B. napus* 'Westar' (Experiment 1), also indicated evidence of poor marker gene expression, and highly unstable transmission of both *NPTII* and *ACBP6* in the T_1 progeny. There was a phenotypic variation observed in kanamycin resistance in the T_1 generation, with some phenotypes showing extreme and slight sensitivity to kanamycin. Moreover, chimaerism was observed in 4 T_0 plants, wherein inconsistent PCR results were obtained when the plants were sampled during young stage *in vitro*, and mid-maturity stage in the glasshouse. When 2 lateral branches were sampled in the mature plants, the PCR results were inconsistent. The dosage effect or the variation in *NPTII* gene expression, reflected as phenotypic variation in the kanamycin resistant plants, was also observed by Sikdar (1999). Chimerism and weaker expression of the resistant phenotype was also observed by De Block et al. (1989) as non-

consistency of kanamycin activity and/or phosphonitricin resistance with enzymatic assay results. Another evidence for chimerism was also observed by Sikdar (1999) wherein GUS was differentially expressed in different tissues of the transformed plants. When chimerism occurred, there was a very high chance that the T_1 generation would not reflect the expected gene inheritance. This was also reported by Mathews et al. (1990) in *B. juncea* where there was a very high proportion of kanamycin sensitive plants in T_1 generation.

A number of transformation events in T_0 plants in both Experiments 1 and 2 showed amplification of either *NPTII* or *ACBP6* but not both sequences, which may have indicated that the T-DNA was not fully integrated into the plant genome. Introduced DNA could contain deletions as it is being integrated into the genome (Potrykus et al., 1985). The transgene could be undetected or appear to be lost in various stages of growth. Transgene integration can be very variable in different generations. For example in wheat, *bar* and *gus* genes were expressed in R_0, while in R_1, expression were lost, however DNA was detected, and finally in R_3, the transgenes were not detected at all. These patterns of inheritance may have been caused by certain sites in the plant genome which were unfavourable for stable and heritable transgene integration (Srivastava et al., 1996). Positional effect in transgene insertion site such as sites with highly repetitive sequences, were observed in petunia and rice (Matzke & Matzke, 1998). Modification of the transgene by methylation commonly occurred in plant cells, causing transgene silencing. Certain silencing effects occur in allotetraploid plants when the transgenes were being integrated into one or more subgenomes. In plants, polyploid genomes such as the *Brassica napus*, are inherently composed of repetitive DNA sequences and abundant transposable elements which tend to aggregate into specific component subgenomes

(Iglesias et al., 1997; Matzke & Matzke, 1998). Cross-infiltration of these elements in the intergration site could render the transgene unstable. The presence of transposable elements, incompatible sequences, high GC content are the usual sites for DNA methylation (Kohli et al., 2010; Matzke & Matzke, 1998). Instability may be observed more when the genes are homozygous with the other dominant allele behaving as an epigenotype of the other such that one is active and the other inactive (Iglesias et al., 1997). Also, unstable integration of DNA may be caused by the inherent instability of a newly established foreign DNA into the genome (Kohli et al., 2010). Loss of the transgene could appear in further generations due to genetic flux, wherein a gene could be lost if the selective pressure was removed (Matzke & Matzke, 1998).

In summary, rapid-cycling *B. napus* and *B. napus* 'Westar' were successfully transformed with plasmid pAT593. Transformed rapid-cycling *B. napus* plants developed faster when compared to transformed long duration Westar plants. The shoot outgrowth stage for rapid-cycling *B. napus* was shorter compared to Westar. In four weeks after callus induction, the rapid-cycling plants produced well-developed shoots that were readily separated as individual regenerated plants. Recovery of transgenic plants was faster with the rapid-cycling plants compared with Westar. Moreover, because of the fast cycle nature of the rapid-cycling plants, advanced generations were attained at a short period, compared to Westar. Evidence of unexpected transgene expression and segregation, and chimeric integration was observed in some of the transgenic progenies.

Chapter 5

Freezing response of ACBP6-overexpressing *Brassica napus*

5.1 Introduction

Frost stress is a major agronomic risk in growing canola and other field crops in Australia. In Australian field conditions, the temperature can drop below zero at night time (-2 to -5°C) and frost forms through radiation, wherein heat conserved in the ground is lost to the atmosphere, commonly occurring during clear and still nights (Chen et al., 2009; www.bom.gov.au, 2016). Yield can be greatly affected when frost affects the grain-filling stage, when seeds contain approximately 60% moisture resulting in poor seed fill and poor oil quality (Johnson-Flanagan et al., 1990; GRDC, 2009).

Most studies into cold and freezing tolerance in canola or oilseed rape were done on winter types focusing on winter survival mechanisms necessary to survive winter environments in the temperate zone where the temperature reaches -25°C. Winter survival is a complex trait that involves vernalisation response, cold-acclimation and freezing tolerance (Rife & Zeinali, 2003; Teutonico et al., 1995). Australian canola varieties are bred for the short days of winter-spring seasons, based on flowering responses. Canola is sown in late autumn or early winter (April to June) during the onset of the rainy season. Australian winter ranges from minimum of 4-5°C to a maximum of 14-18 °C, on average. Harvest is ideally during late spring and early summer in the months of November and December when the seeds contain approximately 30-40% moisture and the plants are ready for swathing (Burton et al., 2003; Potter et al., 1999).

Canola plants respond to freezing temperature differently based on their growth stages. Maturing seeds actively produce storage lipids and proteins until the moisture content drops to around 50% (Crouch & Sussex, 1981). When exposed to freezing temperature at this stage, seed maturation will be affected, disrupting the degreening process and promoting synthesis of green pigments (Johnson-Flanagan & Mclachlan, 1990); while seeds with more than 80% moisture content are unable to develop and germinate (Johnson-Flanagan et al., 1990). Johnson-Flanagan et al. (1991) reported that after a freezing event, seeds desiccated at a high rate.

The electrolyte leakage method has been used to determine injury to plant tissues as early as 1915 and was based on the measurement of the electrical conductance of a solution resulting from increased concentration of electrolytes or leaked ions (Merrill, 1915). This method has been applied to determine hardiness of plants exposed to various stresses such as freezing, pathogen damage, chemical exposure, and mechanical injury (Dexter et al., 1932). Numerous studies on freezing stress have measured electrolyte leakage of exposed tissues to estimate freezing injury, in conjunction with other methods such as chlorophyll fluorescence and gas exchange parameters, and has been proven to be an effective method to measure freezing tolerance in many plant species such as conifers (Mckay, 1992; Peguero-Pina et al., 2008; Wulff et al., 1994), cereals (Clement & vanHasselt, 1996; M. Rapacz, 2007; M. Rapacz et al., 2011), and *Brassica*s (Jung et al., 2014; Manley & Hummel, 1996a, 1996b; Prasil & Zamecnik, 1998).

The primary response of the plant during exposure to environmental stress is usually detected in the leaves. During photosynthesis, when light enters the plant's chloroplast, the energy will be absorbed by chlorophyll molecules and will be used for production of glucose. Depending on the efficiency of the plant's photochemistry, some

of this energy will not be utilized for photosynthesis and instead, re-emitted as light or released as heat. The measurement of the re-emitted light (photon) or chlorophyll fluorescence gives an indication of the state of the plant's photochemistry and ability to dissipate heat. In general, maximum fluorescence yield is attained if photochemical efficiency and heat dissipation is at minimum (Bose, 1982; Briantais et al., 1986; Reece et al., 2011).

Chlorophyll fluorescence has been used in various studies to assess stress response including low temperature injury (De Faria et al., 2013; Fracheboud et al., 1999; Griffith et al., 1994; Klosson & Krause, 1981; Melcarek & Brown, 1977; Ottander & Oquist, 1991; Smillie & Hetherington, 1983). In another aspect of photosynthesis separate to the light-dependent reactions in photosystem II, a plant's photosynthetic efficiency can also be measured based on the absorption of CO_2 and transpiration. Through the stomata, CO_2 molecules in the air are absorbed by the plant and incorporated into the chloroplast, initiating the process of the Calvin Cycle. Together with the energy produced from the light reaction, CO_2 is converted into carbohydrates. Oxygen molecules, the by-product of the light reaction, are liberated through the stomata. With sufficient water supply, adequate exchange of CO_2 and O_2 for photosynthesis is maintained. Meanwhile, transpiration – the loss of water vapour by diffusion and evaporation, also occurs in the stomata. In response to the changing environmental conditions, the stomata can open and close to conserve water for photosynthetic requirements (Reece et al., 2011). In most plants, photosynthesis can be disrupted by stomatal closure, brought about by a limited CO_2 supply for Calvin cycle which can be detected by gas exchange systems as a depletion of intercellular CO_2 (Von Caemmerer & Farquhar, 1981).

In *Arabidopsis thaliana,* ACYL-COA-BINDING PROTEIN6 (ACBP6), a 10-kDa protein, was found to confer freezing tolerance (Chen et al., 2008). The expression of ACBP6 was induced by cold treatment, and ACBP6-overexpressing *A. thaliana* plants can withstand freezing stress up to -8°C. ACBP6 was found to facilitate transport of acyl-coA and maintain acyl-coA esters for lipid biosynthesis in the cell (Chen et al., 2008). The *ACBP6* gene has been successfully integrated into the *B. napus* 'Westar' and rapid-cycling *B. napus* genomes, through *Agrobacterium*-mediated transformation (Chapter 4).

The aims of this chapter were to optimise the freezing tolerance trial and evaluate *B. napus* 'Westar' and rapid-cycling *B. napus* that were transformed with *ACBP6* using electrolyte leakage assay, chlorophyll fluorescence and gas exchange parameters. Injury to developing seed embryos after freezing was also determined by tissue viability staining.

5.2 Materials and Methods

5.2.1 Optimisation of freezing trial for frost tolerance evaluation

5.2.1.1 Plant Materials

Brassica napus germplasm of oilseed rape sourced from Australia and China (Appendix 6) were used to test and optimize freezing conditions and to screen for potential frost-tolerant cultivars. The germplasm accessions were part of the joint project Oilseed *Brassica improvement in China, India and Australia – ACIAR/GRDC* (Salisbury & Gurung, 2011*)*. The seeds were germinated in the dark (23°C) using Debco Seed Raising Mix (Debco Pty. Ltd., Australia). After emergence the seedlings were transplanted into individual 7.5 cm pots with a mixture of soil, sand and peat (1:1:1, v/v/v) and amended with Osmocote™ controlled release fertilizer (NPK 16-3.9-10). Fifteen plants from each cultivar were in the glasshouse maintained at 25°C, with relative humidity of 55-75% under 16 hours of light (~100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) provided by GE Lucalox™ PSL (GE Lighting, U.S.A.) in late July to September 2012.

5.2.1.2 Freezing trial

Freezing treatment was carried out in a 609.6 x 736.6 x 1473.2 mm upright fan-forced freezer with an electronic microprocessor (CAREL® ir33) temperature controller and an overhanging temperature probe located at the centre shelf of the freezer. To ensure accuracy and proper timing of the temperature cycle, a separate portable electronic temperature logger (MicroLite, Fourier Systems) was used to monitor temperature.

Freezing treatment followed the protocol used in *Arabidopsis* (Chen et al, 2008) with modifications based on the protocol to test cold tolerance in barley, which simulated

Australian frost conditions (Chen et al., 2009). The freezing cycle started at 4°C and the temperature was lowered down to -2°C at a rate of 1°C per hour. At -2°C, approximately 20 ml of ice crystals (amorphous granular flake chipped ice) were placed at the base of the stem of each plant to induce ice formation in the plant tissues and the temperature was maintained for 2 hours. This was followed by a successive decrease in temperature at a rate of 1°C per hour until -4°C was achieved. The plants remained at -4°C for an hour and, and returned straight to 4°C and maintained at this temperature to thaw for 24 hours. After thawing at 4°C, the plants were placed back to normal growing condition at 25°C, with relative humidity of 55-75% under 16 hours of light (~100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) provided by GE Lucalox™ PSL (GE Lighting, U.S.A.).

Two experimental trials were performed, testing slightly different freezing regimes and lay-out of the plants inside the freezing chamber. In trial 1, plants were at 6-7 - leaf stage, while in trial 2, plants were younger at 5-6 - leaf stage.

5.2.1.3 Electrolyte leakage assay

The plant tissue sampling was based on the protocol by Rapacz (1999) and the electrolyte leakage estimation was based on relative conductivity (Dexter, 1956; Prasil & Zamecnik, 1998). The second leaf next to the youngest fully expanded leaf from each plant were collected for sampling before the plants were returned to normal growing condition. Three leaf discs were sampled from each leaf using a 14 mm circular borer. The leaf discs were separately placed in 50 mL plastic tubes containing 15 mL of Milli-Q® purified water. The tubes were shaken at 80 rpm using an orbital shaker, for 30 minutes at room temperature (23°C) before the initial electrolyte conductivity reading was measured using a Eutech PC 700 conductivity meter. To obtain the final electrolyte

conductivity, the leaf disc samples were autoclaved at 121°C, 103 kPa for 15 minutes. Electrolyte leakage (%) was computed as the ratio of the initial electrical conductivity (before autoclaving) to the final conductivity (after autoclaving).

5.2.1.4. Statistical analysis

In trial 1, the freezer was divided into 3 horizontal plots, with each plot consisting of 1 replicate of all 15 cultivars randomly arranged. A total of 6 replicates were evaluated, and split into 2 freezing experiments with a one-day difference between each experiment. In trial 2, there were a total of 21 cultivars evaluated, with 9 replicates each. Three freezing experiments were performed, with a one-day difference between each experiment. A two-way ANOVA was carried out to determine interaction effects of the cultivars and replications on the electrolyte leakage values after freezing treatment. Using the over-all error term, differences in the electrolyte leakage among the different cultivars were determined. Post hoc analysis was performed using Tukey's HSD (honest significant difference) test. All statistical tests were carried out using IBM® SPSS® Statistics Version 22 software.

5.2.2 Screening of ACBP6-overexpressing B. napus *for freezing tolerance*

5.2.2.1 Plant materials

There were 7 ACBP6-overexpressing lines of *B. napus* 'Westar' at the second transgenic generation (T_2), and 4 ACBP6-overexpressing lines of rapid-cycling *B. napus* at the third transgenic generation (T_3), evaluated for freezing tolerance (Table 5.1). These lines were confirmed for presence of plasmid pAT593, based on PCR analysis using

primers amplifying *NPTII*, *35S* and *ACBP6*. Only the seedlings that survived kanamycin screening and were positive for the transgene were forwarded to the next generation.

The test plants were grown in Debco® potting mix (50% composted medium bark, 40% composted coarse bark, 10% sand, SaturaidTM, lime, iron, nitrogen and other trace elements), in 50 mm wide x 120 mm deep pots, with once a week fertilization with Aquasol® Soluble Fertiliser. The plants were grown to appropriate stage in the glasshouse maintained at 25°C, supplemented with 16 hours of Philips Agro Plant Light.

Table 8.1. List of independent transgenic lines of *B. napus* 'Westar' and rapid-cycling *B. napus*.

Transgenic Lines	Background
BNW01	*B. napus* 'Westar'
BNW02	*B. napus* 'Westar'
BNW03	*B. napus* 'Westar'
BNW04	*B. napus* 'Westar'
BNW05	*B. napus* 'Westar'
BNW06	*B. napus* 'Westar'
BNW07	*B. napus* 'Westar'
BNFP01	Rapid-cycling *B. napus*
BNFP02	Rapid-cycling *B. napus*
BNFP03	Rapid-cycling *B. napus*
BNFP04	Rapid-cycling *B. napus*

5.2.2.2 Freezing treatment

The freezing treatment followed the same protocol as described in section 5.2.1.2. There were two sets of experiments performed for freeze screening of ACBP6-overexpressing *Brassica* lines: (i) T_2 *B. napus* 'Westar' lines and (ii) T_3 rapid-cycling *B.*

napus. The T_2 'Westar' lines were at 7-8 leaf stage, while the T_3 rapid-cycling lines were at late flowering stage, during the freezing trials. The T_2 consisted of 12 plants (replicates) for each line, while the T_3 consisted of 7 plants for each line. The plants were arranged in a randomized complete block design (RCBD) inside the freezer with the blocks serving as replicates. Due to space limitation inside the freezer, replicates were divided into two batches, with 24- hour difference between freezing times for each batch. The trial set-up inside the freezer and the corresponding lay-out of samples are shown in Figure 5.1.

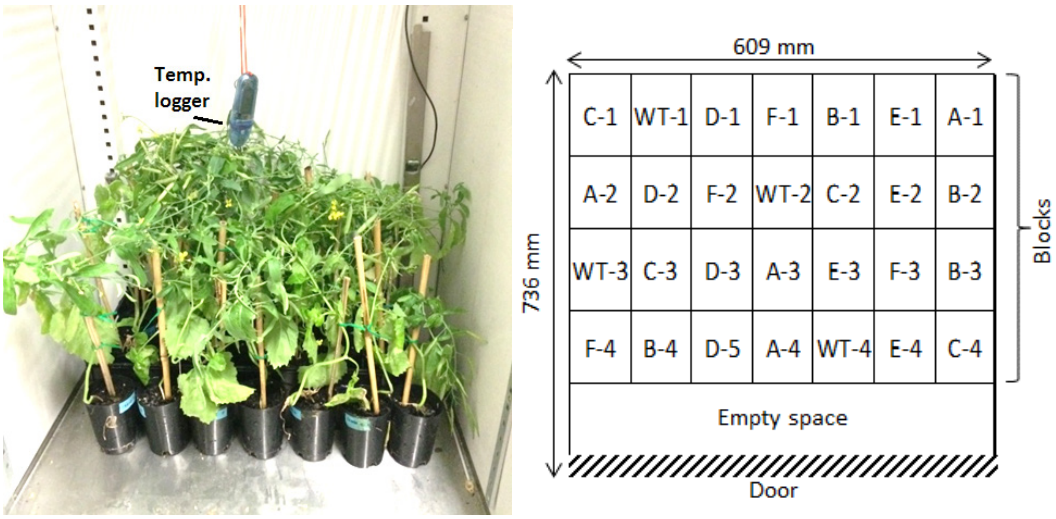


Figure 5.1. Experimental set-up inside the freezer during freezing treatment, showing the rapid-cycling *B. napus* T_3 lines at late flowering stage and the graphic representation of the freezer and test plants lay-out. The test plants were arranged in randomized complete block design. A to F are codes for the T_3 lines (treatment), WT is wild-type control, and the numbers correspond to blocks, which also serve as replicates. The space closest to the door of the freezer was left empty.

5.2.2.3 Electrolyte leakage assay

Leaf tissues were analysed for electrolyte leakage after freezing as described in section 5.2.1.3.

5.2.2.4 Evaluation of freezing injury by chlorophyll fluorescence and gas exchange

After thawing at 4°C for 24 hours, the plants were transferred to a growth chamber maintained at 25°C, with relative humidity (RH) of 55-75% under 16 hours of light (~100 $\mu mol \cdot s^{-1} \cdot m^{-2}$) provided by GE Lucalox™ PSL (GE Lighting, U.S.A). The plants were allowed to settle for an hour before measurements were taken. Gas exchange and chlorophyll fluorescence were measured in the third fully expanded leaf of the test plants using Li-COR® 6400 Portable Photosynthesis System (LI-COR Biosciences, U.S.A.) and Walz® MINI-PAMI Photosynthesis Yield Analyzer (Walz, Germany).

5.2.2.4.1 Chlorophyll fluorescence measurements

Using the MINI-PAM Leaf-Clip Holder 2030-B, the third youngest leaf was measured following the manufacturer's instruction. The leaf-clip holder uses a 60° fibre optics attachment which records photosynthetically active radiation (PAR) and temperature simultaneously. The measurements were taken under steady-state light source at photosynthetic photon flux density of ~150 µmol·m-2·s-1. Sampling was performed using MODE 1, which is the standard mode of measurement for YIELD parameter (ΔF/Fm). AUTO-ZERO function mode (MODE 2) was called every 6 samplings to clear any false signal. The quantum yield of photosystem II was based on the equation by Genty (1989):

$$\Phi_{PSII} = \frac{Fm' - Ft}{Fm'}$$

where Fm' is the maximum fluorescence when actinic light is applied. From this equation, the proportion of light that entered Photosystem II is being quantified. Φ_{PSII} is also used to estimate the linear electron transport rate (J) as proposed by Genty et. Al. (1989) in the following equation:

$$ETR = \Phi_{PSII} \times PFDa \times (0.5)$$

where PFDa is the incident light (μmol photon m-2 s-1) and 0.5 is a factor accounting for the energy distribution between the Photosystem II and Photosystem I.

The effective quantum yield of PSII, notated as Y(II), and the ETR were the main parameters used in this study. The parameter Y(II) was also referred to as 'PSII operating efficiency', based on Baker (2008).

5.2.2.4.2 Gas exchange measurements

Measurement of leaf gas exchange parameters were conducted in the same leaf and carried out in tandem with chlorophyll fluorescence measurement. The leaf gas exchange parameters were measured using a portable gas exchange system Li-COR® 6400 Portable Photosynthesis System (LI-COR Biosciences, U.S.A), at photosynthetic photon flux density of ~150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Molar air flow ($CO_2$ concentration) inside the leaf chamber was set to 400 $\mu mol\ mol^{-1}$, with temperature of 30°C, and relative humidity maintained at 25-50%. At the start of measurement and every 15 minutes in the course of the measurement, sample IRGA (infrared gas analyser) was matched with the reference IRGA. The net leaf photosynthetic CO_2 uptake or assimilation rate of CO_2 (*A*), transpiration rate (*E*), stomatal conductance (g_{sw}) and intercellular CO_2 concentration (Ci) were measured.

5.2.2.5 Evaluation of freezing injury to seeds by image analysis

Seeds from transformed T_3 rapid-cycling *B. napus* exposed to freezing stress were analysed for frost damage using image analysis of fluorescence signal. Siliques were harvested after thawing and the seeds were removed. A total of 20-30 seeds per plant were analysed. The seed coat was removed and mature embryos (10-15 days after pollination) were stained with fluorescein diacetate (FDA) dissolved in acetone and 10% sucrose solution. The stained embryos were viewed with a stereomicroscope (Leica® M205A, Leica Microsystems, Germany) under fluorescence light provided by an external light source (Leica® EL6000) through a Green Fluorescence Pigment (GFP) filter. Photos were captured with Leica® DMC2900 digital microscope camera.

The fluorescence signals captured in the images were analysed with MATLAB® (MathWorks, U.S.A.) image processing algorithm based in the method used in quantification of berry tissue vitality (Fuentes, Sullivan, Tilbrook, & Tyerman, 2010). Each embryo in the image was defined based on the cotyledon area and excluding the radicle (Figure 5.2). Relative luminance was computed for the corresponding area of embryo. Percent vitality or percent living tissue for each embryo was computed based on the proportion of fluorescence over the total area defined.

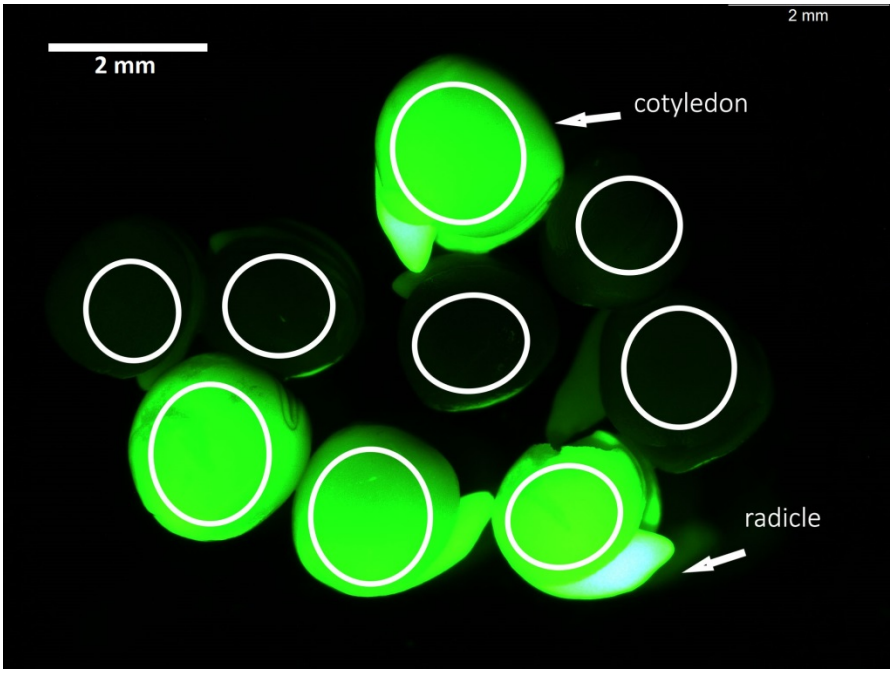


Figure 5.2. Embryos stained with fluorescein diacetate (FDA), displaying varying fluorescence. Relative luminance analysed was defined within the cotyledon area (encircled) and excluded the radicle.

5.2.2.6 Statistical analysis

All statistical analyses were performed using IBM® SPSS® Statistics Version 22 (IBM Corporation, U.S.A) software. Interaction effects between blocks and treatments were determined with a two-way analysis of variance (ANOVA) using a general linear model. For the analysis of T_2 lines, the interaction terms were kept and the main effects were analysed using type III sum of squares. For the T_3 lines, one-way ANOVA was performed to compare measurement means. Student's t-test was used to compare the baseline and post-freezing measurements.

Boundary line analysis based on Jarvis (1976) was used to examine the effect of exposure to freezing temperature on the photosynthetic responses of the ACBP6-overexpressing plants and controls. Boundary layer modelling was performed using a

customised code developed in Matlab 2016b (Fuentes et al., unpublished). This method primarily fits a general non-linear model to all the data points from an x and y graph. All residual data below the curve was disregarded to fit a new model of the remainder data. These steps were iterated automatically in a loop until the boundary points were found, with the final model representing the boundary layer information for the specific dataset.

5.3 Results

5.3.1 Optimisation of freezing trial for frost tolerance evaluation

5.3.1.1 Freezing conditions

The two trials carried out to screen differences in response of different *B. napus* cultivars to freezing, had the same target temperature of -4°C, however, as shown in Figure 5.3, there were slightly different freezing regimes. The first trial (left) showed a shorter (8 hours) stepwise decrement of 2°C, with and extended time at 0°C for introduction of ice crystals. The second trial (right) had a longer duration (11 hours) and had a decrement of 1°C per hour, with an extended time at -2°C where ice crystals were introduced. Moreover, the temperature record showed differences in fluctuations. In trial 1, the lowest mean temperature reached was -4.4 ± 0.33°C, while the set and actual temperature had a mean difference of 0.37 ± 0.33°C. In trial 2, the lowest mean temperature was -5.29 ± 0.56°C, while the actual temperature was 1.29± 0.56°C lower than the set temperature.

5.3.3.2 Electrolyte leakage of *B. napus* cultivars

A statistically significant interaction was observed between the cultivars and the replications or plots assignment inside the freezing chamber, $F(68, 164)=5.16$, $p<0.001$, partial $n^2=0.68$. Analysis on the simple main effects of the cultivars and replications showed that in replications 1 and 4, which are both located in plot 1 in the freezing chamber, the mean electrolyte leakage of the 15 cultivars were not significantly different. Moreover, the mean electrolyte leakage for replications 1 and 4 (Figure 5.4) were 9.8% and 18.7%, respectively, indicating an extremely minimal damage to the sampled leaves.

Since replicates 1 and 4 were not significantly affected by the freezing treatment, these two replicates were not included in the analysis of variance of leaf electrolyte leakage of the different cultivars.

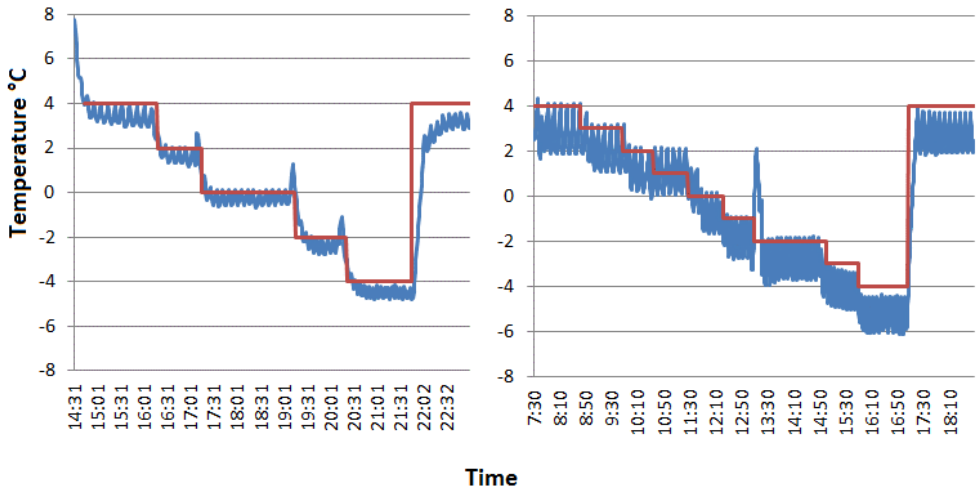


Figure 5.3. Temperature regimes used in trial 1 (left) and trial 2 (right) of frost tolerance screening of different *B. napus* cultivars. The freezing regime in trial 1 was a 2°C decrement to -4°C, with 2- hour period at 0°C; while the freezing regime in trial 2 was a 1°C decrement, with a 2-hour period at -2°C. Temperature was recorded every 30 seconds.

Considering replicates 2, 3, 5 and 6, there was statistically significant difference in leaf electrolyte leakage in the cultivars evaluated $F(14, 108) = 12.5$, $p < 0.001$. There was significant interaction between cultivars and replications for electrolyte leakage measurement after freezing, $F(41, 108) = 6.54$, $p<0.001$, partial $n^2=0.46$, and the mean electrolyte leakage between the different cultivars in all replicates were significantly different $F(13, 108)=6.829$, $p<.001$, partial $n^2= 0.45$. The four replications did not have statistically significant effect on the electrolyte leakage of the different cultivars except for P30830, 30851, Zhongshuang, FAN168, FAN189, 20045, and 20047. Trial 1 (Figure

5.5) showed generally lower electrolyte leakage with mean minimum of 26.92% by Spectrum, followed by Zhongyuoza (28.09%). Maximum electrolyte leakage was 73.36% by cultivar 06-63737.

With trial 2, differences in the mean leaf electrolyte leakage were statistically significant $F(20, 163) = 373.75$, $p < 0.001$, partial $n^2 = 0.98$. There was a significant interaction between cultivars and replication for electrolyte leakage $F(134,52)=319.58$, $p<0.001$, partial $n^2 = 0.99$. Leaf electrolyte leakage values were higher ranging from 49.6 % to 77.8 %. The top 4 performing cultivars were 20048 (49.6%), Spectrum (51.9%), Zhongyouza (54.67%) and P30830 (54.74%). Details of the statistical analyses are presented in Appendix 7 and 8.

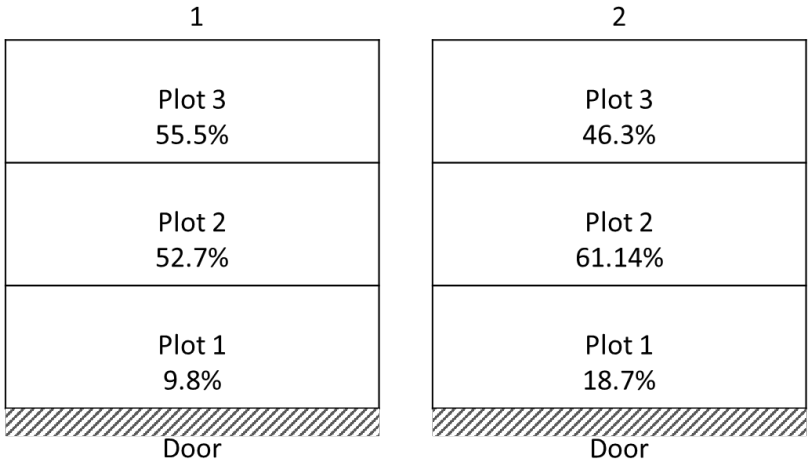


Figure 5.4. Mean leaf electrolyte leakage (%) of *B. napus* cultivars evaluated in the corresponding plot assignment inside the freezing chamber. Two freezing experiments (1 and 2) were carried out, with 24-hour difference on each experiment to accommodate 6 replications. Plot 1 which is located along the freezer door resulted to very minimal mean leaf electrolyte leakage after freezing treatment.

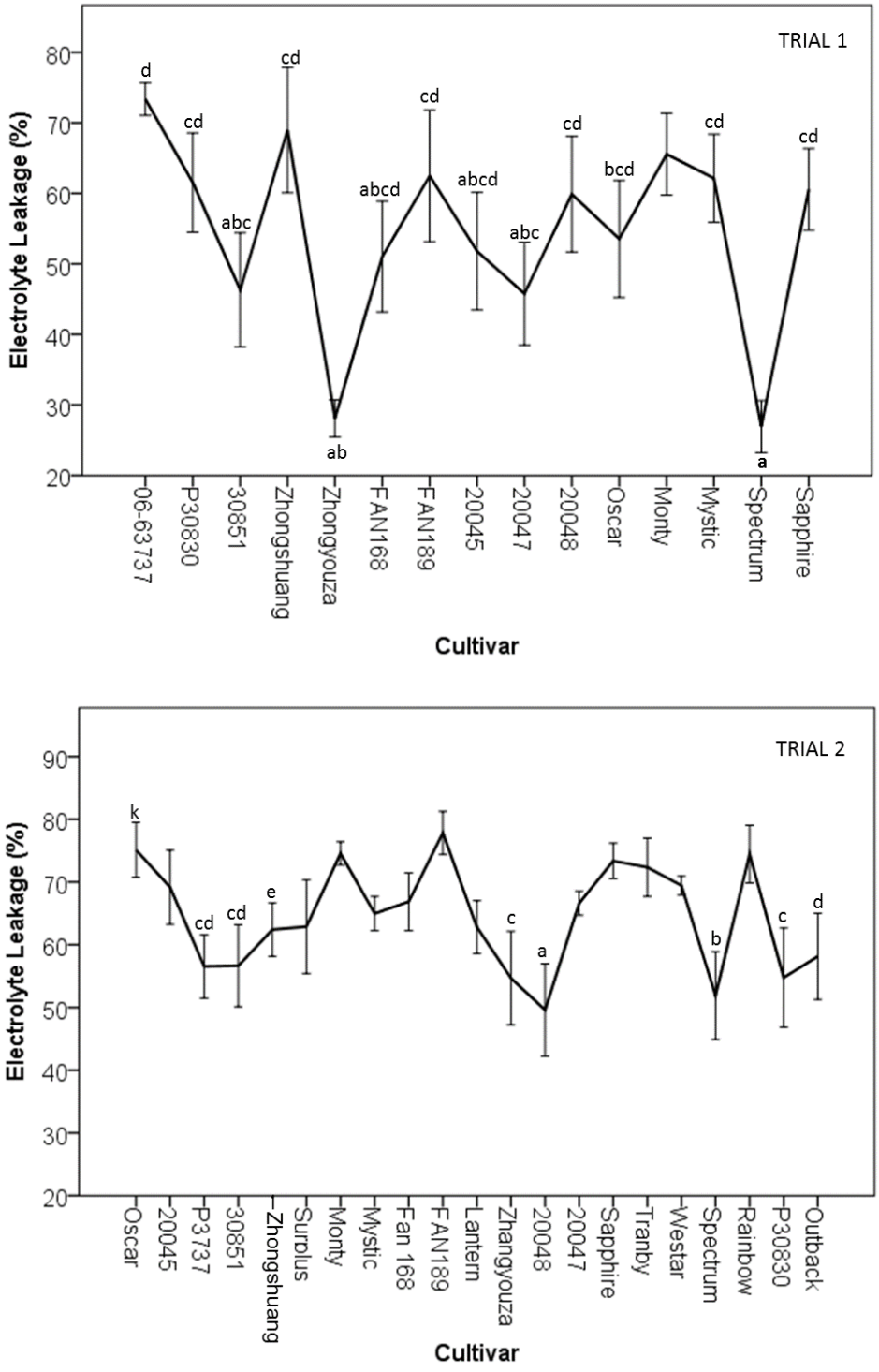


Figure 5.5. Leaf electrolyte leakage (%) of different *B. napus* cultivars after exposure to freezing temperature. Trial 1 (top) showed the cultivars Zhongyouza and Spectrum to have the least electrolyte leakage, and the relative hardiness of these two cultivars were confirmed in Trial 2 (bottom) with the addition of 20048 and P30830.

5.3.2 Frost tolerance screening of ACBP6-overexpressing B. napus

5.3.2.1 Freezing condition

There was a discrepancy between the set temperature and the actual temperature, recorded by a separate digital temperature logger which took record every 30 seconds. The fluctuation of the actual temperature over an 11-hour period of freezing trial is shown in Figure 5.6. For the two batches of T_2 trials the recorded mean temperatures were -5.3°C ± 0.55 and -5.2°C ± 0.79, respectively. In the T_3 trials mean temperatures were -5.1°C ± 0.93 and -5.0°C ± 0.57. From the intended testing temperature of -4°C, there was a mean decrease of -1.15 ± 0.02°C in the actual temperature in the freezer during the trials. Despite the decrease, the temperatures were maintained consistently, except in brief opening of the freezer door, shown as increase in temperature peaks.

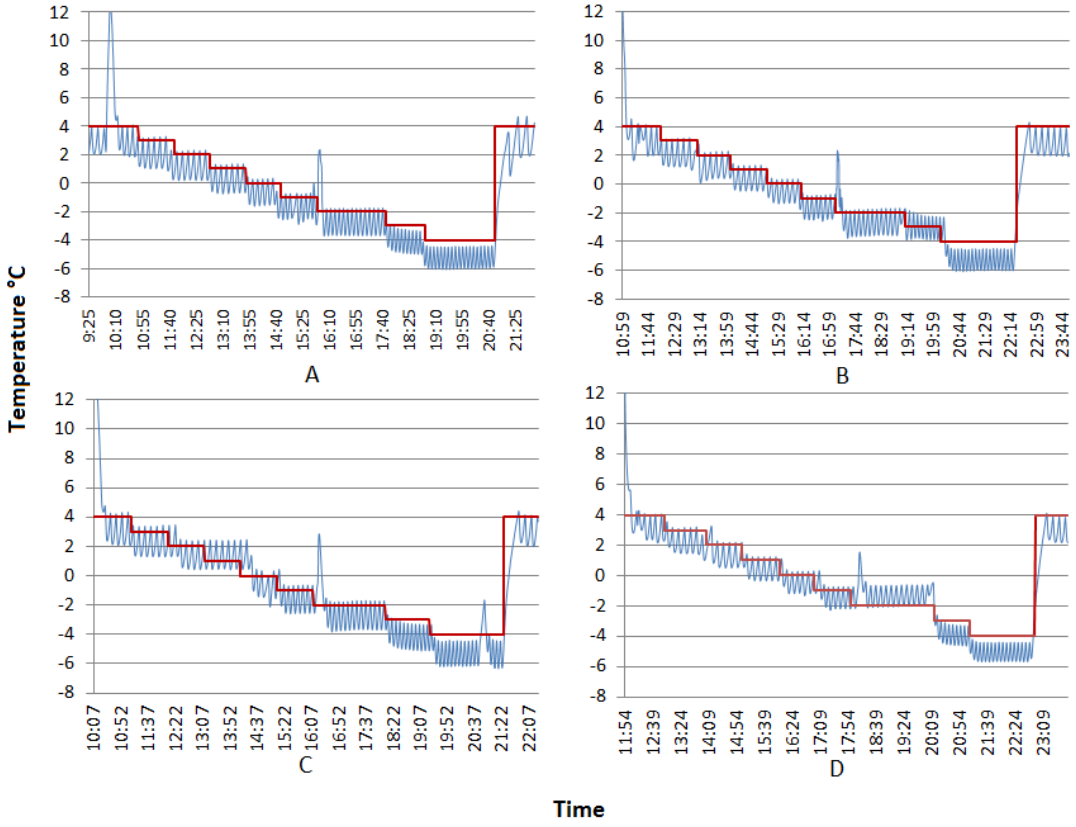


Figure 5.6. Temperature record during freezing trials of T_2 and T_3 ACBP6-overexpressing transgenic plants. The red line indicates the set temperature (-4°C), while the blue line was the actual temperature recorded by a digital temperature logger. Graphs A and B were the temperature record for the two batches of T_2 trials, while C and D were the 2 batches of T3 trials.

5.3.2.2 Frost tolerance screening of *Brassica napus* 'Westar' T_2 in vegetative stage

Two-way ANOVA to examine the effect of experimental blocks inside the freezer on the freezing response of the T_2 lines and WES control, showed that there was no statistically significant interaction between experimental blocks and ACBP6-overexpressing plants for electrolyte leakage measurement (EL, %), $F(21, 48) = 1.69$, $p = 0.07$, partial $\eta^2 = 0.43$; net photosynthetic rate (A) $F(20,47) = 1.061$, $p = 0.418$, partial $\eta^2 = 0.311$; effective photochemical quantum yield of PSII [Y(II)], $F(21, 46) = 0.677$, p=0.833, partial $\eta^2 = 0.24$. The effect of blocks was the same for all lines tested, based on the three major parameters measured from three different methods (electrical conductivity, gas exchange, and chlorophyll fluorescence). An analysis of the main effects (Appendix 9) revealed statistically significant difference among the 7 T_2 ACBP6-overexpressing 'Westar' lines and wild-type control (WES) based on electrolyte leakage EL $F(7, 48) = 6.08$, $p < 0.05$, partial $\eta^2 = 0.47$; net photosynthetic rate *A* $F(7, 47) = 3.17$, *$p < 0.05$* partial $\eta^2 = 0.32$; stomatal conductance g_{sw} $F(7, 47) = 3.26$, $p < 0.05$, partial $\eta^2 = 0.33$; transpiration rate *E F(7, 47) = 3.91, p < 0.05, partial n2 = 0.37;* effective photochemical quantum yield of PSII Y(II) $F(7, 46) = 4.32$, $p < 0.05$, partial $\eta^2 = 0.39$; and electron transport rate ETR $F(7, 46) = 4.41$, $p < 0.05$, partial $\eta^2 = 0.4$. Minimal fluorescence yield, F_0 $F(7, 46) = 3.65$, $p < 0.05$, partial $\eta^2 = 0.36$ and maximal fluorescence yield F_m, $F(7, 46) = 5.2$, p <0.05, partial $\eta^2 = 0.44$, also showed significant differences. There was no statistically significant difference observed in intercellular CO_2 concentration (Ci) and water use efficiency (WUE) among T_2 lines and WES control. Table 5.2 shows the freezing response measurements on leaves of T_2 ACBP6-overexpressing lines of *B. napus* 'Westar' (WES) and wild-type untransformed 'Westar' based on electrolyte leakage assay, chlorophyll fluorescence-based parameters.

Table 5.2. Freezing response measurements on leaves of T_2 ACBP6-overexpressing lines of *B. napus* 'Westar' (WES) and wild-type untransformed 'Westar' (WES) based on electrolyte leakage assay, chlorophyll fluorescence-based parameters - effective quantum yield of PSII [Y(II)] and electron transport rate (ETR); and gas exchange parameters – net photosynthetic rate (*A*), stomatal conductance (g_{sw}), transpiration rate (*E*) and intercellular CO_2 concentration (Ci), and water use efficiency (WUE – calculated as A/E).

T2 Lines	Electrolyte leakage (EL, %)	Net photosynthetic rate (*A,* µmol $CO_2m^{-2}S^{-1}$)	Stomatal conductance ($g_{sw,}$ mol $H_2Om^{-2}s^{-1}$)	Transpiration rate (*E,* mmol $H_2Om^{-2}s^{-1}$)	Intercellular CO_2 conc. (Ci, µmol CO_2mol^{-1})	Effective quantum yield of PSII [Y(II)]	Electron transport rate (ETR, $\mu molm^{-2}S^{-1}$)	Minimal fluorescence (F_0')	Maximal fluorescence (F_m')	Water use efficiency $(WUE)^{ns}$
BNW01	47.53^{ab}	1.95^{ab}	0.06^{ab}	1.21^{ab}	329.60	0.54^{b}	18.01^{ab}	473.5^{ab}	1349.57^{b}	1.45
BNW02	44.25^{ab}	3.19^{b}	0.06^{b}	1.26^{b}	297.43	0.55^{b}	22.97^{b}	479.11^{ab}	1302.44^{b}	2.57
BNW03	36.79^{a}	2.34^{b}	0.05^{ab}	1.13^{ab}	302.50	0.62^{b}	27.71^{b}	549.46^{b}	1528.92^{b}	2.22
BNW04	37.15^{a}	2.41^{b}	0.07^{b}	1.48^{b}	337.20	0.52^{b}	20.82^{b}	506.39^{b}	1380.09^{b}	1.45
BNW05	56.95^{ab}	1.89^{ab}	0.06^{ab}	1.26^{b}	330.82	0.47^{b}	20.24^{b}	466.88^{ab}	1174.30^{b}	1.51
BNW06	48.74^{ab}	2.54^{b}	0.07^{b}	1.45^{b}	338.70	0.52^{b}	21.19^{b}	489.07^{ab}	1422.09^{b}	1.43
BNW07	71.84^{bc}	1.38^{ab}	0.04^{ab}	0.80^{ab}	335.27	0.48^{b}	17.81^{ab}	503.42^{ab}	1128.90^{b}	1.98
WES	93.96^{c}	0.27^{a}	0.02^{a}	0.47^{a}	332.55	0.14^{a}	6.61^{a}	364.18^{a}	455.10^{a}	0.78

^{abc} *Means for groups which are homogenous have the same letters, based on Tukey's post hoc test for highly significant difference.*
^{ns} *Means are not significantly different from each other.*
All details of statistical analysis are displayed in Appendix 9.

Westar (WES) T_2 showed the highest percentage of leaked electrolytes (EL) of 93.96% indicating an almost complete kill of the leaf tissue sampled. BNW03 and BNW04 showed the least EL of 36.79% and 37.15%, respectively. WES also consistently expressed the lowest performance after freezing based on gas exchange (*A,* g_{sw}, and *E*) and chlorophyll fluorescence parameters [ETR, Y(II), F_0 and F_{max}], with the exception of Ci, where the measurements were homogenous in all plants. Net photosynthetic rate (*A*) derived from gas exchange measurements showed BNW02, BNW03, BNW04, and BNW06 to have significantly higher readings above 2.3 µmol CO_2 $m^{-2}S^{-1}$. Stomatal conductance (g_{sw}) in the transgenic T_2 lines ranged from 0.036 – 0.07 mol H_2O $m^{-2}s^{-1}$, with BNW02, BNW04 and BNW06 having significantly higher readings. Transpiration rate (*E*) expectedly showed the same trend with values ranging from 1.26 – 1.48 mmol H_2O $m^{-2}s^{-1}$ for T_2 lines having significantly higher readings against the wild-type control, WES (0.47 mmol H_2O $m^{-2}s^{-1}$).

The PSII operating efficiency Y(II) was significantly higher in the ACBP6-overexpressing T_2 lines compared with the wild-type control WES, with values ranging from 0.48 to 0.62. The electron transport rate (ETR) was significantly lower in WES (6.61 $\mu molm^{-2}S^{-1}$) compared with T_2 lines BNW02, BNW03, BNW04, BNW05 and BNW06 with values ranging from 20.2 – 27.9 µmol m^{-2} S^{-1}. The initial fluorescence (F_0') of the leaf samples and the maximum fluorescence (F_m') emitted by the leaf samples after a saturating actinic light was applied, were also significantly higher in the T_2 plants.

A one-way ANOVA was also conducted to determine if chlorophyll fluorescence and gas exchange parameters (Appendix 11) before treatment varied for each T_2 ACBP6-overexpressing lines and WES. Results showed that T_2 lines and WES control had homogenous means of *A* $F(7, 83) = 1.374$, $p = 0.23$, g_{sw} $F(7, 83) = 2.1$, $p = 0.53$, Ci $F(7,$

83) = 1.7, p = 0.125 and Y(II) $F(7, 83) = 2.0$, p = 0.06. A significant variation among means was observed in E $F(7, 83) = 2.3$, p = 0.36 and ETR $F(7, 83) = 2.4$, p = 0.03.

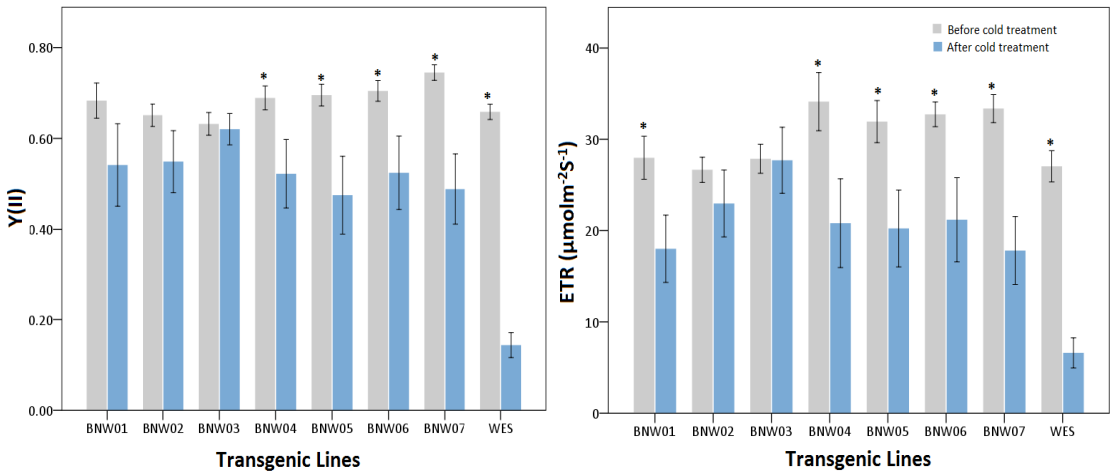


Figure 5.7. Chlorophyll fluorescence parameters measured before and after freezing or cold treatment on the 7 ACBP6-overexpressing T_2 lines of *B. napus* 'Westar' (BNW) and wild-type control (WES). The PSII operating efficiency Y(II) and electron transport rate (ETR) are shown. Bars represent standard errors, and asterisks indicate significant difference ($p<.05$) between before and after freezing treatment values.

Y(II) and ETR of the ACBP6-overexpressing T_2 lines and wild-type control before and after freezing treatment are shown in Figure 5.7. Y(II) on 3 T_2 lines – BNW01, BNW02 and BNW03 did not significantly decrease after freezing, p=0.06. There was a significant decrease in Y(II) for BNW04, BNW05, BNW06, BNW07 and WES. However, the maximum decrease in Y(II) among T_2 lines before and after freezing treatment, such as in BNW07, was 0.26, 95% CI [0.08 to 0.43] as determined by T-test. With WES, Y(II) was 0.51, 95% CI [0.45 to 0.58], higher before it was exposed to freezing stress. There was no statistically significant difference on ETR means of BNW02 and BNW03. Highest decrease in ETR among T_2 lines was observed in BNW07, with

difference of 15.55, 95% CI [6.78 to 24.31]. WES had a mean difference of 20.43, 95% CI [15.48 to 25.38] in ETR before and after freezing treatment.

The freezing responses of the 7 T_2 lines and control based on gas exchange parameters *A*, g_{sw}, *E* and Ci, before and after freezing are shown in Figure 5.8. After freezing, *A* significantly decreased in all T_2 lines and control WES. Among the 7 T_2 lines, BNW07 showed the highest decrease in *A* after freezing with mean difference of 2.63, CI 95% [1.62 to 3.63]. The least mean difference was observed in BNW02 at 1.32, CI 95% [0.11 to 2.53]. Among all samples, WES had the highest difference of means of 3.75, CI 95% [3.29 to 4.21]. Significant decrease in g_{sw} was observed in BNW03 with mean difference of 0.04, CI 95% [0.01 to 0.07], BNW07 with mean difference of 0.02, CI 95% [0.005 to 0.04], and WES with mean difference of 0.06, CI 95% [0.04 to 0.08]. The same trend was observed with *E* means in both T_2 lines and WES. In contrast to all other measurements, there was a significant increase in Ci after freezing in almost all samples, except for BNW02 and BNW03.

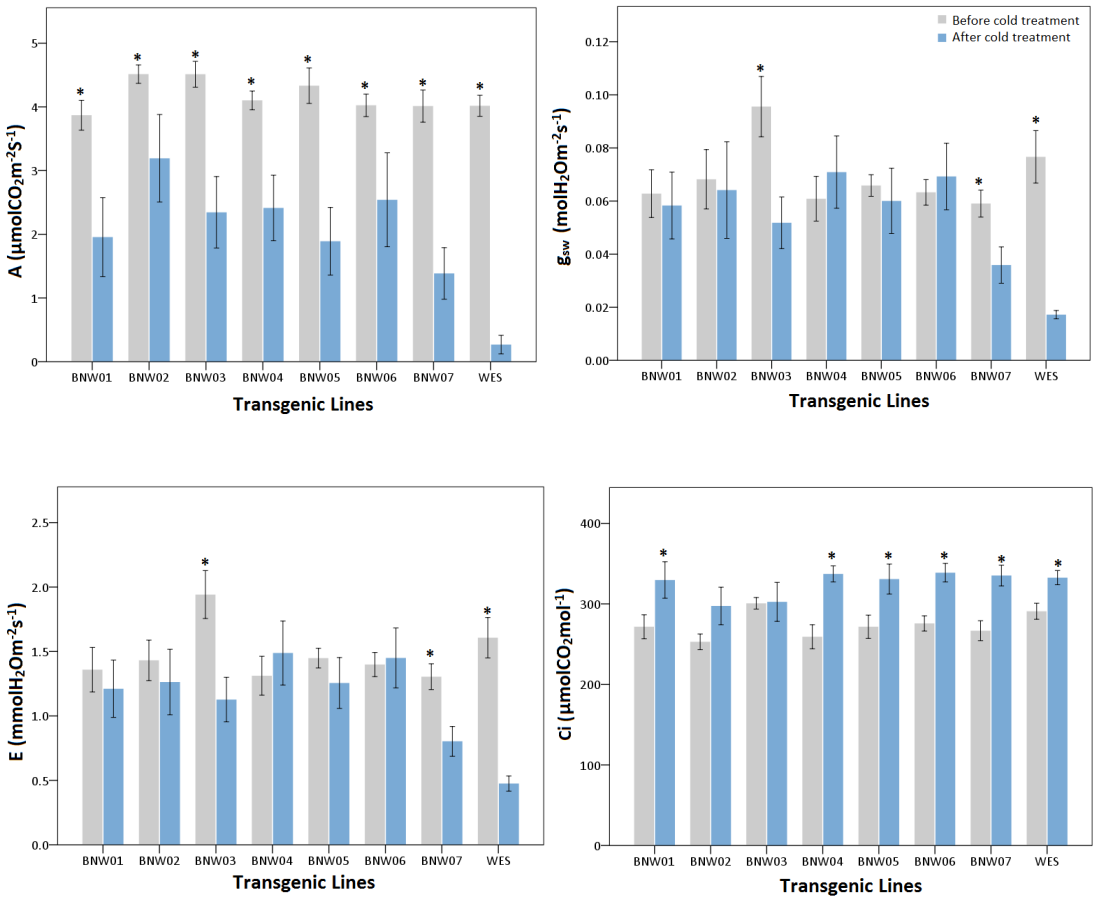


Figure 5.8. Gas exchange parameters — net photosynthetic rate (*A*, µmolCO_2m^{-2}S^{-1}), stomatal conductance (g_{sw}, molH_2Om^{-2}s^{-1}), transpiration rate (*E*, mmolH_2Om^{-2}s^{-1}), and intercellular CO_2 concentration (Ci, µmolCO_2mol^{-1}) measured before and after freezing treatment of 7 T_2 ACBP6-overexpressing lines of *B. napus* 'Westar' and untransformed Westar control (WES). Bars represent standard errors, and asterisks indicate significant difference ($p<.05$).

5.3.2.3 Frost tolerance screening of rapid-cycling *Brassica napus* T_3 transgenic lines

A one-way ANOVA was conducted to determine if a variation existed in the freezing stress response of the T_3 ACBP6-overexpressing lines of rapid-cycling *B. napus* (BNFP) together with the untransformed or wild-type control (WT), based on electrolyte leakage, gas exchange and chlorophyll fluorescence measurements (Appendix 13). There were statistically significant differences between the T_3 lines and control tested in terms of leaf electrolyte leakage (EL, %) $F(6, 29) = 8.139$, $p < 0.05$; net photosynthetic rate A $F(6, 27) = 3.9$, $p < 0.05$, PSII operating efficiency Y(II) $F(6, 29) = 11.26$, $p < 0.05$; and electron transport rate ETR $F(6, 29) = 10.03$, $p < 0.05$. There were no statistically significant differences in freezing response of the T_3 lines and WT in terms of stomatal conductance g_{sw} $F(6, 28) = 0.41$, $p = 0.86$; transpiration rate E $F(6, 29) = 0.47$, $p = 0.82$ and initial fluorescence reading F_0 $F(6, 29) = 2.4$, $p = 0.06$. Table 5.3 shows the freezing response measurements on leaves of ACBP6-overexpressing T_3 lines of rapid-cycling *B. napus* and wild-type control based on electrolyte leakage assay (EL), chlorophyll fluorescence-based parameters

Table 5.3. Freezing response measurements on leaves of ACBP6-overexpressing T_3 lines of rapid-cycling *B. napus* (BNFP) and wild-type control (WT) based on electrolyte leakage assay (EL), chlorophyll fluorescence-based parameters – PSII operating efficiency Y(II) and electron transport rate (ETR); and gas exchange parameters – net photosynthetic rate (*A*), stomatal conductance (g_{sw}), transpiration rate (*E*), intercellular CO_2 concentration (Ci) and water use efficiency (WUE - calculated as *A/E*).

T3 lines	Electrolyte leakage (EL, %)	Net photosynthetic rate (*A,* µmol $CO_2m^{-2}S^{-1}$)	Stomatal Conductance ^{ns} ($g_{sw,}$ mol $H_2Om^{-2}s^{-1}$)	Transpiration rate ^{ns} (*E,* mmol $H_2Om^{-2}s^{-1}$)	Intercellular CO_2 concentration (C_i, µmol CO_2mol^{-1})	Effective quantum yield of PSII [Y(II)	Electron transport rate (ETR, $\mu molm^{-2}S^{-1}$)	Minimal fluorescence (Fo) ^{ns}	Maximal fluorescence (Fmax)	WUE
BNFP01_5	56.07^{a}	2.71^{ab}	0.04	0.75	267.75^{ab}	0.39^{b}	24.28^{b}	572.00	945.6^{abc}	4.68^{ab}
BNFP01_8	62.83^{a}	3.58^{b}	0.05	0.89	261.23^{ab}	0.47^{b}	31.67^{b}	881.67	1639.00^{c}	3.47^{ab}
BNFP02_17	59.51^{a}	3.35^{b}	0.03	0.72	212.00^{a}	0.50^{b}	28.65^{b}	553.00	1173.00^{abc}	2.79^{ab}
BNFP02_40	65.41^{a}	2.65^{ab}	0.04	0.86	263.80^{ab}	0.44^{b}	27.15^{b}	857.83	1561.83^{bc}	5.28^{ab}
BNFP03_15	50.73^{a}	3.62^{b}	0.03	0.80	230.00^{a}	0.34^{b}	24.28^{b}	553.75	866.25^{ab}	4.62^{ab}
BNFP04_12	72.58^{ab}	2.27^{ab}	0.03	0.59	258.00^{ab}	0.42^{b}	25.77^{b}	536.75	939.5^{abc}	3.93^{ab}
WT	96.06^{b}	0.06^{a}	0.04	0.86	385.80^{b}	0.08^{a}	4.67^{a}	628.70	694.00^{a}	0.08^{a}

^{abc}*Means for groups which are homogenous have the same letters, based on Tukey's post hoc test for highly significant difference.*
^{ns} *Means are not significantly different from each other.*
All details of statistical analysis are shown in Appendix 13.

The electrolyte leakage (EL) means after exposure to freezing stress was highest in WT (96.06%) and significantly lower in majority of the ACBP6-overexpressing T_3 lines, ranging from 50.73 % to 65.41% (Table 5.3). The transgenic T_3 lines (BNFP01_8, BNFP02_17 and BNFP03_15) had significantly higher mean net photosynthetic rate *A,* ranging from 3.35 to 3.62 μmol CO_2 $m^{-2}S^{-1}$. Stomatal conductance g_{sw} and transpiration rate *E*, were the same for all samples. Intercellular CO_2 concentration ($C_{i)}$ was highest in WT (385.8 μmol CO_2 mol^{-1}) and lowest in BNFP02_17 (212.0 μmol CO_2 mol^{-1}). For chlorophyll fluorescence parameters, PSII operating efficiency Y(II) was homogenous in the T_3 lines with means ranging from 0.34 to 0.5, and statistically significantly higher compared to that of the WT (0.08); while electron transport rate (ETR) was also significantly higher in all T_3 lines with means ranging from 24.28 to 31. 65 $\mu molm^{-2}S^{-1}$, compared to WT (4.67 $\mu molm^{-2}S^{-1}$). The initial fluorescence reading F_0', was the same for all samples, while F_m', the maximal fluorescence reading after a pulse of saturating light was applied, was different with highest mean value of 1,639 in BNFP01_8 and lowest in WT (694). There was also a significant difference in water use efficiency between ACBP6-overexpressing lines (with highest WUE observed in BNFP02_40 with 5.28) and the wild type control (0.08).

Measurement means before and after freezing treatment were compared using t-test. The graph for chlorophyll fluorescence measurements are shown in Figure 5.9 while the gas exchange parameters are shown in Figure 5.10. There was a significant reduction in Y(II) in all T_3 lines and WT. Among T_3 lines, largest decrease was observed in BNFP03_15 with mean difference of 0.37, CI 95% [0.19 to 0.56] and the smallest decrease on BNFP02_17 by 0.15, CI 95% [0.02 to 0.27]. WT decreased the most with a mean difference of 0.55, CI 95% [0.41 to 0.69]. With ETR means, there was a slight

increase after freezing treatment in 2 of the T_3 lines, however, this was not significant. Four T_3 lines decreased in ETR but were only significant for BNFP03_15, which has a mean difference of 10.1, CI 95% [0.44 to 19.7]. WT had the highest reduction with a mean difference of 18.7, CI 95% [13.7 to 23.7].

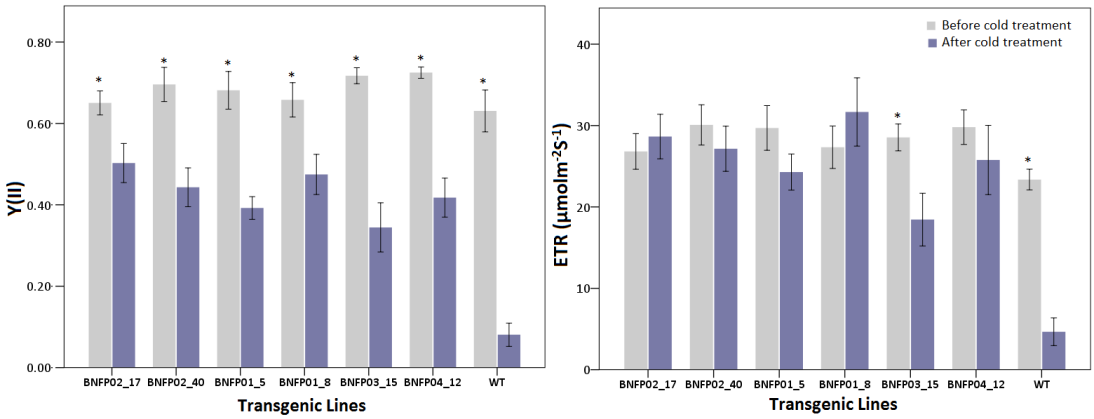


Figure 5.9. Chlorophyll fluorescence parameters measured before and after freezing treatment on the 6 T_3 transgenic lines of rapid-cycling *B. napus* (BNFP) and untransformed control (WT). The PSII operating efficiency Y(II) and electron transport rate (ETR) are shown. Bars represent standard errors, and asterisks indicate significant difference ($p<.05$) before and after freezing treatment values.

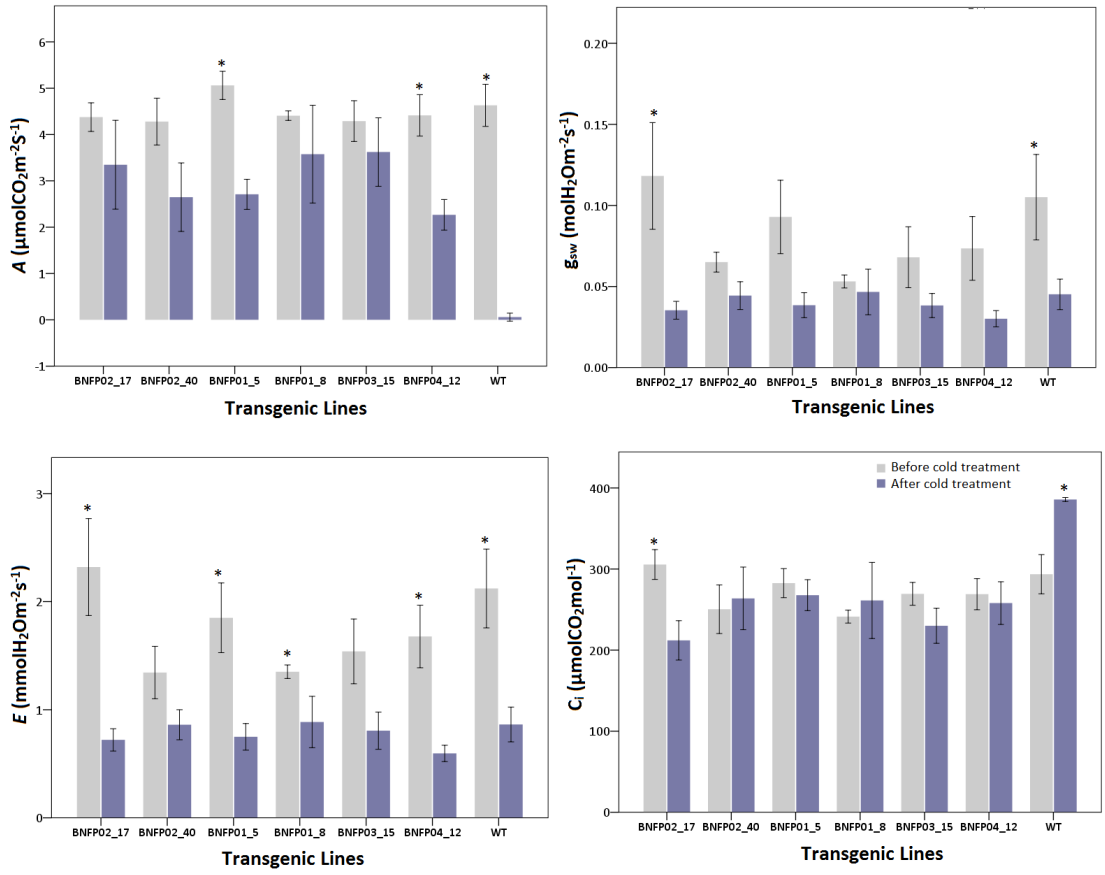


Figure 5.10. Gas exchange parameters — net photosynthetic rate (*A*, µmol CO_2 $m^{-2}S^{-1}$), stomatal conductance (g_{sw}, mol H_2O $m^{-2}s^{-1}$), transpiration rate (*E*, mmol H_2O $m^{-2}s^{-1}$), and intercellular CO_2 concentration (C_i, µmol CO_2 mol^{-1}) measured before and after freezing treatment of 6 T_3 lines of rapid-cycling *B. napus* (BNFP) and untransformed control WT. Bars represent standard errors, and asterisks indicate significant difference ($p<.05$) before and after freezing treatment.

Net photosynthetic rate *A* decreased after exposure to freezing stress, and the reduction was significant for BNFP01_5 with mean difference of 2.35, 95% CI [1.3 to 3.4] and BNFP04_12 with mean difference of 2.14, 95% CI [0.78 to 3.5]. Highest decrease was observed in WT at 4.57, 95% CI [3.5 to 5.6]. Significant reduction after freezing stress in stomatal conductance was only observed in BNFP02_17 by 0.08, 95% CI [-.006 to 0.17] and WT by 0.06, 95% CI [-0.004 to 0.12]. With *E,* significant reduction was observed in all T_3 lines except for 2 lines – BNFP02_40 and BNFP03_15. Highest

decrease in E was observed in BNFP02_17 with mean difference of 1.6, 95% [0.37 to 2.82] followed by WT which was reduced by 1.25, CI 95% [0.33 to 2.17]. The response of the T3 lines and WT after freezing treatment varied in terms of $C_{i,}$ wherein means increased in some lines and decreased in others. However, the changes in most lines were not significant. A significant change was only observed in BNFP02_17 in which Ci decreased by 93.6, CI 95% [19.04 to 168.15]; and WT which increased and had a mean difference of -92.2, CI 95% [-148.25 to -36.14].

5.3.2.4 Fluorescence and gas exchange relationships

Pearson's product-moment correlation-based relationships among all photosynthetic measurements in cold-treated plants, are shown in Table 5.4. Among the measurements, correlation was highly significant with *A* and all other measurements within the ACBP6-overexpressing T_2 BNW plants; however, in the T_3 BNFP plants, *A* was not correlated with g_{sw} and *E*. Highest correlation was observed between *E* and g_{sw} for both experiments. Ci was negatively correlated with Y(II) and *A*, for both experiments.

Table 5.4. Pearson's product-moment correlation in ACBP6-overexpressing T_2 lines of *B. napus* 'Westar' and T_3 lines of rapid-cycling *B. napus* based on chlorophyll fluorescence parameters – PSII operating efficiency [Y(II)] and electron transport rate (ETR); and gas exchange parameters – net photosynthetic rate (*A*), stomatal conductance (g_{sw}), transpiration rate (*E*) and intercellular CO_2 concentration (Ci) .

	A	Ci	g_{sw}	*E*	Y(II)	ETR
			T_2 lines			
Ci	-.61**					
g_{sw}	.61**	0.13				
E	.64**	0.1	.98**			
Y(II)	.68**	-.46**	.41**	.44**		
ETR	.65**	-.43**	.39**	.42**	.81**	
WUE	-.50**	0.13	.65**	-.76**	0.09	.51**
			T_3 lines			
Ci	-.81**					
g_{sw}	0	.45*				
E	0.02	.42*	.99**			
Y(II)	.60**	-.59**	-0.15	-0.15		
ETR	.56**	-.54**	-0.13	-0.16	.97**	
WUE	-.46*	.85**	-0.34	-.93**	-0.33	0.32

*** Correlation is significant at the 0.01 level (2-tailed), and * at the 0.05 level (2-tailed).*

The R^2 values of the modelled boundary layer of the relationships of major photosynthetic parameters before and after freezing were at least 90% (Table 5.5). The obtained models explored the photosynthetic performance of ACBP6-overexpressing plants and untransformed controls after cold-treatment as shown in Figures 5.11, 5.12 and 5.13. The boundary line for PSII operating efficiency Y(II) of the plants before freezing in both T_2 and T_3 plants peaked around 0.8, between net photosynthetic rate or CO_2 assimilation rate (*A*) values of 3-6 μmol CO_2 $m^{-2}S^{-1}$ (Figure 5.11). After exposure to freezing temperature, the T_2 boundary value defined by a parabola shifted into an exponential curve with the wild-type or untransformed plants at the lowermost axes in the scatter plot. A proportion of treated ACBP6-overexpressing plants fell inside the boundary of the non-cold treated plants. In the T_3 population, there was a clear separation of values of the cold-treated and untreated plants, with the boundary layer shifting into lower Y(II) and *A*. In terms of *A* and rate of electron transport (ETR), there was an obvious shift of boundary in *A* with the parabola opening in a larger range, covering the minimum value of ETR in the cold treated plants. Despite limited net photosynthesis (*A*) or CO_2 assimilation, Y(II) was able to increase in the ACBP6-overexpressing lines, in comparison to the wild-type plants which were clustered in the lower axes of both parameters.

Table 5.5. Modelled boundary layer equations on relationships of chlorophyll fluorescence and gas-exchange parameters obtained before and after freezing experiments of *B. napus* 'Westar' T_2 and rapid-cycling *B. napus* T_3 lines.

Samples	Treatment	Relationship	Boundary layer equation	R^2
B. napus 'Westar' T_2 lines	24°C	Φ_{PSII}/A	$-0.08782 * A^2 + 0.701*A - 0.5659$	0.95
	-4°C		$0.7289*e^{(0.007772*A)} - 0.5808 *e^{(-23.43*A)}$	0.99
	24°C	A/ETR	$-0.009203* ETR^2 + 0.5628*ETR - 2.769$	0.99
	-4°C		$-0.007904* ETR^2 + 0.3824*ETR - 0.2544$	0.95
	24°C	A/Ci	$-2.559x10^{-07}*e^{(0.04572 *Ci)} + 5.156*e^{(0.0004021*Ci)}$	0.98
	-4°C		$-0.0003824*Ci^2 + 0.1977 *Ci - 20.05$	0.98
	24°C	Ci/g_{sw}	$329.8*e^{(0.0003257*Ci)} - 414.7*e^{(-0.04084*Ci)}$	0.99
	-4°C		$352*e^{(-0.0001371*Ci)} - 505.1*e^{(-0.05267 *Ci)}$	0.99
	24°C	A/g_{sw}	$-0.0003868*g_{sw}^2 + 0.07463*g_{sw} +1.889$	0.96
	-4°C		$-0.00101 *g_{sw}^2 + 0.1469*g_{sw} - 0.4544$	0.98
	24°C	ETR/g_{sw}	$-0.003656*g_{sw}^2 + 0.5317 *g_{sw} + 26.87$	0.97
	-4°C		$-0.007219*g_{sw}^2 + 1.1*g_{sw} + 0.6599$	0.99
Rapid-cycling *B. napus* T3 lines	24°C	Φ_{PSII}/A	$-0.1147* A^2 + 1.031*A - 1.501$	0.94
	-4°C		$-0.05708 * A^2 + 0.3148 *A + 0.1299$	0.98
	24°C	A/ETR	$-0.0368* ETR^2 + 2.06*ETR - 21.95$	0.90
	-4°C		*Cubic spline interpolant (piecewise polynomial)*	1
	24°C	A/Ci	$-0.0001383 *Ci^2 + 0.06917 *Ci - 2.686$	0.99
	-4°C		$-0.0001005 *Ci^2 + 0.02906 *Ci + 3.872$	0.99
	24°C	Ci/g_{sw}	$-9909*g_{sw}^{(-1.218 + 363.5)}$	0.99
	-4°C		$-0.1073 *g_{sw}^2 + 0.07481*g_{sw} +31.76$	0.99
	24°C	A/g_{sw}	$-0.0002851 *g_{sw}^2 + 12.03 *g_{sw} +1.053$	0.98
	-4°C		$-0.006865 *g_{sw}^2 + 0.6821 *g_{sw} - 10.93$	0.99
	24°C	ETR/g_{sw}	$78.6*e^{(-0.007547 * gsw)} + 3.535 *e^{(-0.0342* gsw)}$	0.96
	-4°C		$-0.04016 *g_{sw}^2 + 0.6821 *g_{sw} - 40.43$	0.92

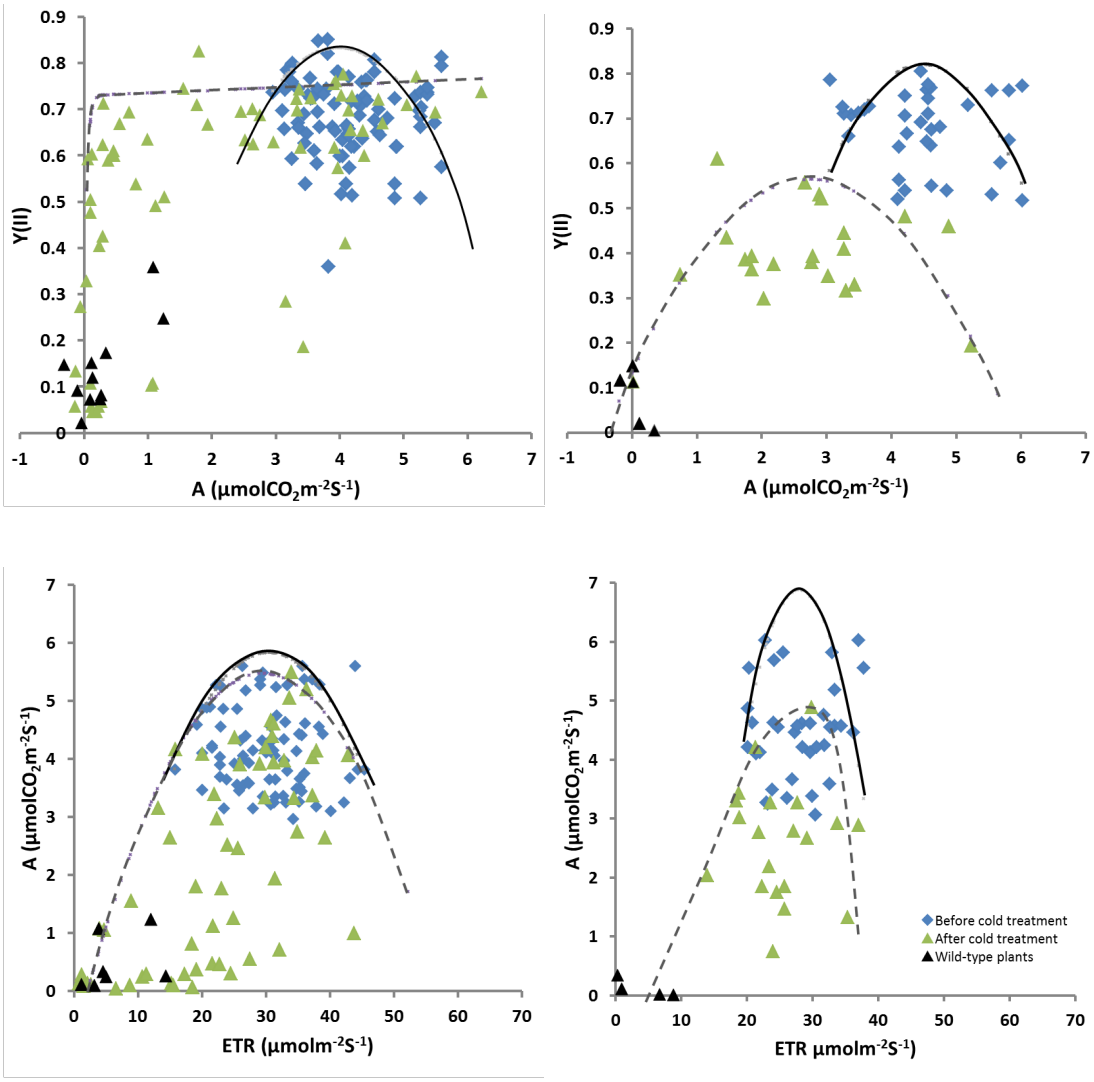


Figure 5.11. Relationships between main fluorescence and gas exchange-based parameters: PSII operating efficiency Y(II), net photosynthesis (*A*) and rate of electron transport (ETR) measured in leaves after freezing treatment of BNW T_2 lines (left) and BNFP T_3 lines (right). The solid black lines are fitted boundary layer models of the untreated plants and the dashed lines are fitted boundary layer models after cold treatment.

Because of the observed increase in intercellular CO_2 concentration (Ci) after treatment, the net photosynthesis or the assimilation rate of CO_2 (*A*) was examined against Ci, and Ci as a function of stomatal conductance (g_{sw}). The boundary layer for the untreated plants for both T_2 and T_3 expressed a logarithmic curve where a saturation point was reached at around 350 µmol CO_2 mol^{-1} and *A* values spread around 3-6 µmol$CO_2$$m^{-2}S^{-1}$ (Figure 5.12). After freezing, *A* and Ci showed a predominantly inverse relationship. In both T_2 and T_3, a proportion of the plants showed very minimal *A* despite high Ci. In T_2, some treated plants fell inside the boundary layer of the non-treated plants, while majority of cold-treated plants fell in the lower boundary line of the parabola. All cold-treated untransformed (wild-type) plants showed the lowest *A*, yet had very high Ci.

To determine how Ci was related to stomatal functions, Ci was plotted against stomatal conductance (g_{sw}). The boundary layer for the untreated plants showed a logarithmic curve of Ci plateauing around 300 µmol CO_2 mol^{-1} and g_{sw} of 80 mol H_2O $m^{-2}s^{-1}$, for both T2 and T3. The T_2 boundary for the cold-treated plants was slightly higher than the untreated plants, both expressing a logarithmic pattern. The boundary layer for the T_3 plants showed a narrow parabola, with g_{sw} limited below 80 mol$H_2Om^{-2}s^{-1}$. A very distinct clustering of plants that can't be fitted inside the modelled boundary was observed in T_2 after freezing. These plants had very high Ci (300-400 µmol$CO_2$$mol^{-1}$) in a very narrow range of g_{sw} (around 10-50 mol H_2O $m^{-2}s^{-1}$). The untransformed plants fell under this range. Even with low stomatal conductance g_{sw}, there was a high intercellular CO_2 concentration in the substomatal layer observed after cold-treatment. Similarly the wild-type plants in the T_3 experiment extended beyond the modelled boundary, displaying the same trend of high Ci and low g_{sw}.

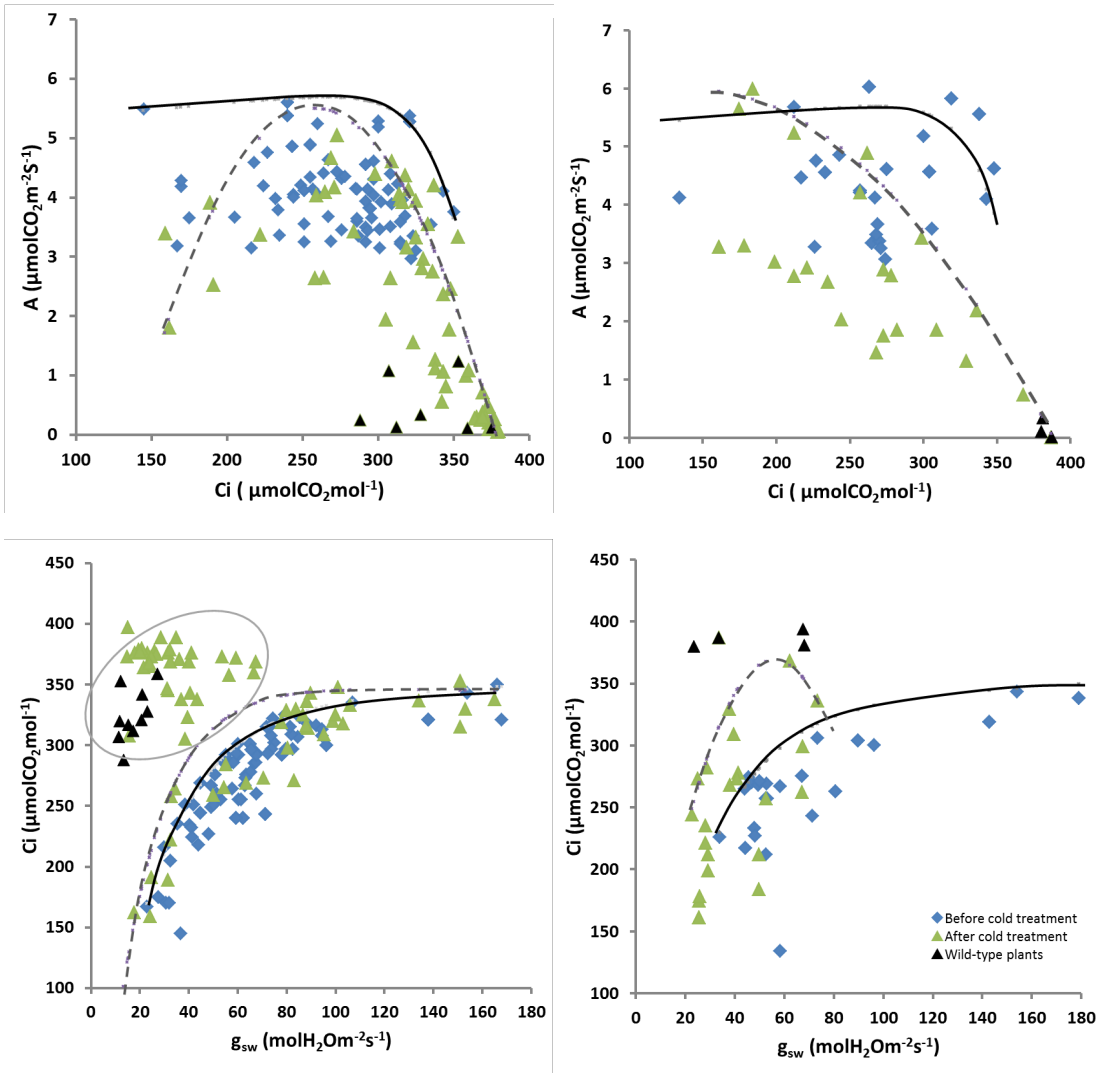


Figure 5.12. Net photosynthetic rate (*A*) as a function of intercellular CO_2 concentration (Ci); and intercellular CO_2 concentration (Ci) as a function of stomatal conductance (g_{sw}) measured in leaves after freezing treatment of BNW T_2 lines (left) and BNFP T_3 lines (right). The solid black lines are fitted boundary layer models of the untreated plants and the dashed lines are fitted boundary layer models after cold treatment.

The relationship of stomatal conductance and the net photosynthesis in terms of CO_2 assimilation (*A)* and PSII operating efficiency Y(II) is shown in Figure 5.13. In T_2, there was a downward shift of boundary of *A* and g_{sw} after treatment, while in T_3, a very apparent limited outer boundary of the cold-treated plants was observed, as shown by the very narrow parabola, in comparison to the wide parabola of the untreated plants. Almost similar trends were observed with ETR and g_{sw}. The untransformed plants had the lowest value of *A* and ETR for a given g_{sw} in T_3, while in T_2, the untransformed plants together with some transformed T_2 plants, had the lowest A and ETR.

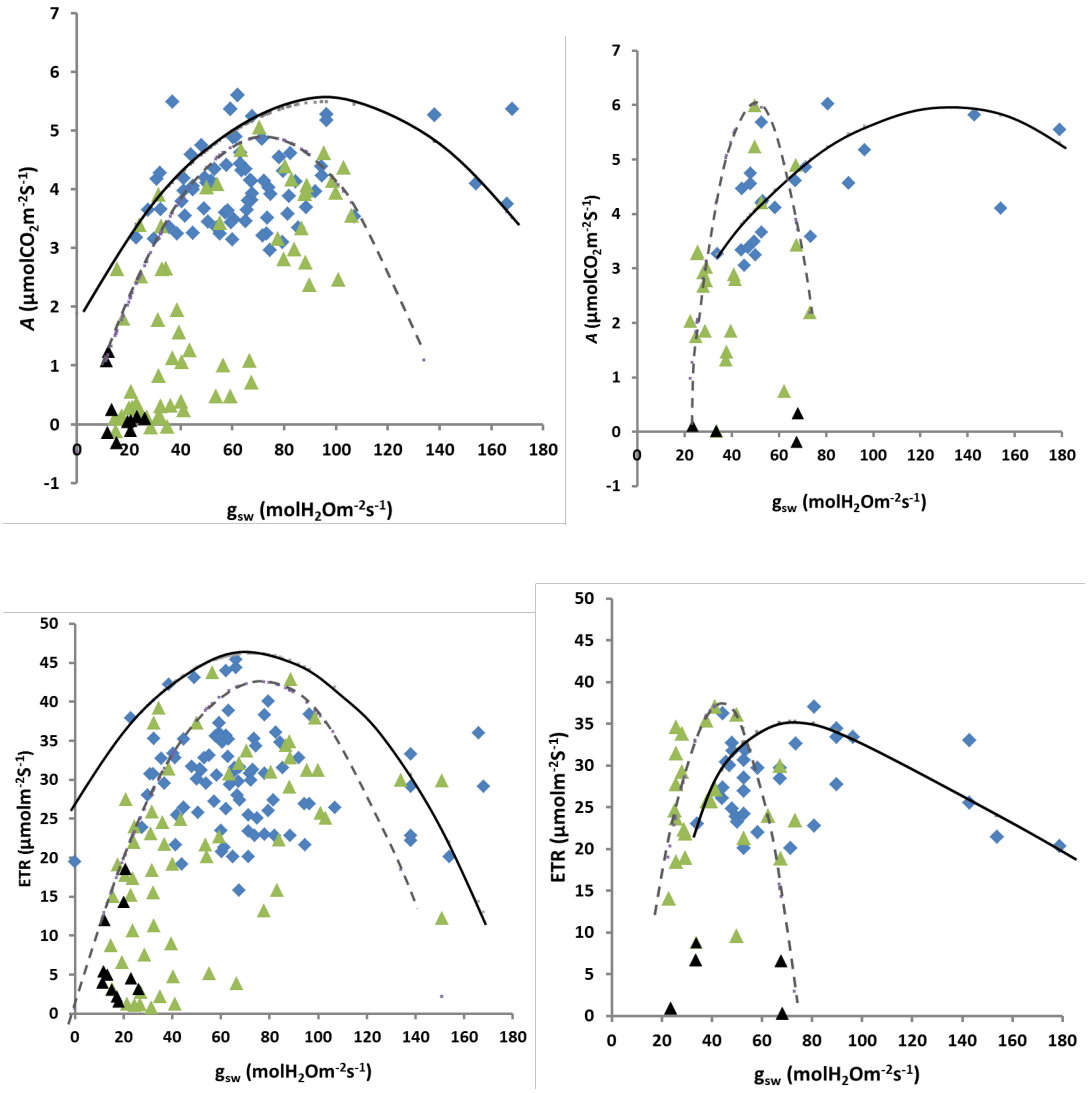


Figure 5.13. Net photosynthetic rate (*A*) and PSII-driven rate of electron transport (ETR) as a function of stomatal conductance (g_{sw}) measured in leaves after freezing treatment of BNW T_2 lines (left) and BNFP T_3 lines (right). The solid black lines were fitted boundary layer models of the untreated plants and the dashed lines were fitted boundary layer models after cold treatment.

5.3.2.5 Image analysis of rapid-cycling *B. napus* seeds exposed to freezing

The number of pods or silique analysed per transgenic plant, ranged from 6-12, as each plant varied in terms of viable pods for analysis. Figure 5.14 shows images of stained seed cotyledons with varying level of fluorescence, and the corresponding percent living tissue computed. A total of 301 pods were analysed from the 6 T_3 lines and 1 untransformed control. The mean living tissue (%) for the T_3 lines and WT analysed is shown in Table 5.6. All details of statistical analysis performed are displayed in Appendix 16.

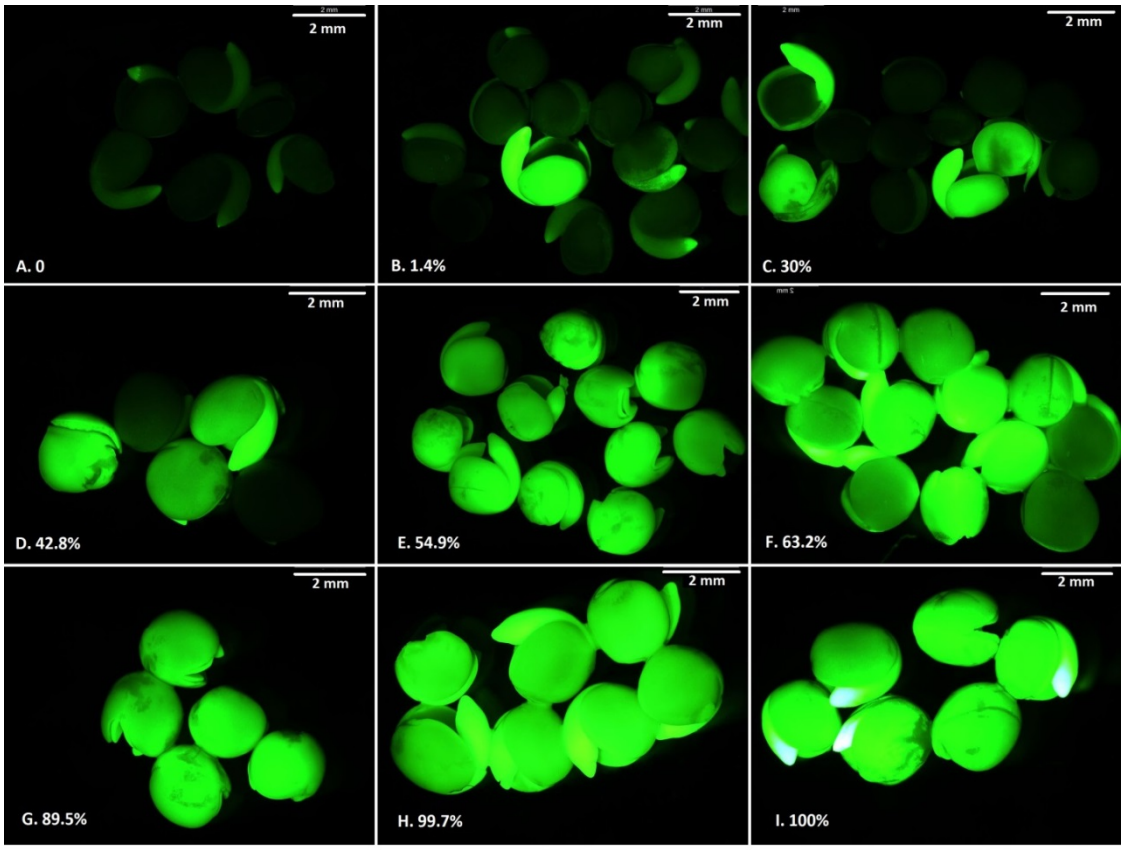


Figure 19. Fluorescein diacetate (FDA)-stained seed cotyledons (photo A to I) of ACBP6-overexpressing rapid-cycling *B. napus* T_3 lines. after exposure to freezing viewed under blue fluorescent light. For each image, the mean living tissue (%) analysed using MATLAB image processing algorithm is indicated.

Table 5.6. Living tissue (%) analysed based on fluorescein diacetate (FDA) - stained cotyledons after freezing treatment of ACBP6-overexpressing rapid-cycling *B. napus* T_3 (BNFP) lines.

Transgenic lines	Living tissue (%)
BNFP01_5	77.32^{c}
BNFP01_8	61.65^{bc}
BNFP02_17	76.65^{c}
BNFP02_40	66.53^{bc}
BNFP03_15	66.69^{bc}
BNFP04_12	47.73^{b}
WT	26.15^{a}

^{abc}*Means for groups which are homogenous have the same letters, based on Tukey's HSD post hoc test.*

The percent living tissue computed based on FDA-stained cotyledons was significantly different for the 6 ACBP6-overexpressing T_3 lines and wild-type control analysed, $F(6,294) = 12.483$, $p < .0005$. Tukey post-hoc analysis revealed that the percent living tissue of embryos of the untransformed control after subjecting to freezing was significantly lower compared to the T_3 transgenic plants, ($p < 0.0005$), except for the T_3 line BNFP04_12 ($p = 0.092$).

A Pearson's product-moment correlation (Table 5.7) was conducted to assess the relationship between seed embryo vitality (percent living tissue) and the vegetative tissue assessment (electrolyte leakage assay EL, PSII operating efficiency Y(II) and net photosynthesis *A*) before and after exposure to the freezing treatment. Among the vegetative measurements, there was a strong positive correlation between Y(II) and *A*, $r = 0.541$; and a strong negative correlation ($r = -0.558$) with electrolyte leakage. Percent

living tissue in the embryo was strongly correlated with Y(II), (*r = 0.614*), and moderate negative correlation with electrolyte leakage values (*r = -0.384*).

Table 5.7. Pearson's correlation for PSII operating efficiency Y(II), living tissue (%), and electrolyte leakage (EL), and seed embryo vitality (Living tissue) of ACBP6-overexpressing rapid-cycling *B. napus* T3 (BNFP) lines.

	Y(II)	Living tissue (%)	EL (log10)
Living tissue (%)	.614**		
EL (log10)	-.558**	-.384*	
A (log10)	.541**	0.329	-.567**

*** Correlation is significant at the 0.01 level (2-tailed).*
** Correlation is significant at the 0.05 level (2-tailed).*

5.4 Discussion

Cultivars of *B. napus* responded differently to freezing at -4°C. Zhongyouza (ZHONGYUOZA-8-30940), a Pol CMS Hybrid from China, and Spectrum (AG-Spectrum) an Australian cultivar with blackleg resistance, showed consistent lower vegetative electrolyte leakage in the two trials. However, there was variation in the level of electrolyte leakage between the two trials, which may have been caused by the inconsistency of the actual temperature within the freezing chamber and duration of freezing between the two trials. The second trial had a lower minimum temperature (-5.29 ± 0.56°C) compared to trial 1 (-4.4 ± 0.33°C). The plants closest to the glass door of the freezer had very minimal electrolyte leakage, thus this space was not ideal for further freezing experiments. The shorter duration of the freezing regime in the first trial may have resulted in less leaf electrolyte leakage. The stepwise freezing regime used in trial 2 produced sufficient damage that also distinguished differences between the cultivars. In addition, plant age may have affected the physiological responses of plants to freezing. The plants in the first trial being older and thus may have had more tolerance compared to the plants in the second trial. Thus in the subsequent freezing experiments, these sources of variations were properly controlled by not using the space close to the door, and reinforcing door insulation with a sheet of 1 cm thick extruded polystyrene foam. The temperature spike during the opening of freezer door when nucleating ice crystals were introduced was not an issue as the temperature increase was very minimal (maximum of 2°C) for a very short period (4-7 minutes), and the temperature stabilised rapidly when the door were closed (Chen et al., 2009).

The two cultivars ‘ZHONGYUOZA-8-30940’ and ‘AG-Spectrum’, that exhibited consistently low freezing damage based on leaf electrolyte leakage, are potentially frost

tolerant. Further assessment of frost tolerance on these cultivars especially at the seed filling stage is recommended, since this stage is the most susceptible to frost damage in the field.

Transgenic *B. napus* over-expressing the *A. thaliana* acyl-coenzyme A-binding protein6 (ACBP6) were tolerant to freezing temperature of -4°C, even without undergoing cold acclimation. In *Arabidopsis*, ACBP6 was shown to facilitate transport of cytosolic acyl-CoA and maintenance of acyl-CoA esters during production of fatty acids in the plastids. ACBP6 were shown to bind phosphatidylcholine, and *ACBP6*-overexpressing plants also showed low levels of phosphatidylcholine and accumulation of phosphatidic acid. These abilities of ACBP6 to regulate phospholipid metabolism enabled ACBP6-overexpressing plants to withstand freezing temperatures (Chen et al., 2008).

The ACBP6-induced tolerance to freezing temperature was evident from three aspects of freezing injury estimation: leaf electrolyte leakage to assess plasma membrane damage, chlorophyll fluorescence to assess damage in the plant's light-harvesting apparatus; and gas exchange to measure assimilation of CO_2 and transpiration. Additionally, the observed tolerance to freezing injury was extended to maturing developing seed cotyledons, based on tissue viability staining.

The degree of injury to the plasma membrane was high for both wild-type plants of Westar (WES) and rapid-cycling *B. napus* (WT). With the ACBP6-overexpressing plants, the Westar lines (BNW) showed generally lower electrolyte leakage compared with the rapid-cycling transgenic lines (BNFP) primarily because of development stage and plant architecture differences between the two genotypes. The Westar plants were in pre-reproductive stage, with thicker and larger leaves; while the fast plants were in mid-reproductive stage with thinner and smaller leaves. In the ACBP6-overexpressing lines, the freezing tolerance was comparable to that of non-acclimated relatively hardy winter-

type rapeseed, which has an LT_{50} of approximately -5.0°C - the temperature wherein 50% of electrolyte leakage was observed (Bauer et al., 1994; Rife & Zeinali, 2003). A similar threshold temperature was also determined in a study in wheat where freezing temperature profiles were based on actual frost events in South Australia (Chen et al., 2009).

The effect of exposure to freezing in the untransformed plants was clearly reflected in fluorescence parameters [Y(II) and ETR] under light source with photosynthetic photon flux density of ~150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and net photosynthesis based on net assimilation of CO_2 by the leaf. There was a clear indication of cold stress injury to the PSII operating efficiency in the wild-type plants, with greatly reduced effective quantum yield of PSII Y(II), and PSII-driven rate of electron transport (ETR). The apparent damage to photosystem II as measured by chlorophyll fluorescence parameters has been used as an indicator of low temperature stress in *Brassica* (Bauer et al., 1994; Rapacz, 1999; Rapacz et al., 2001; Savitch et al., 2005).

ACBP6-overexpressing *B. napus* 'Westar' plants were able to maintain PSII efficiency while the untransformed Westar decreased by 77%. Although significant reduction in Y(II) was evident in ACBP6-overexpressing fast plants (maximum reduction of 35%), PSII-driven rate of electron transport was maintained. In chilling sensitive plants i.e. tomato and cucumber, the chloroplasts were directly affected during chilling stress, disrupting PSII functions, due to damage in membrane proteins (Choluj et al., 1997). PSII is the first protein complex in the light-dependent reactions of photosynthesis, termed as water-to-plastoquinone oxidoreductase, composed of numerous transmembrane proteins involved in chlorophyll-protein light-harvesting complex (Mellis, 1999), which can be directly affected by freezing damage in susceptible plants. Exposure of plants to low

temperature stress has also been shown to increase the susceptibility of plants to photoinhibition, by inhibiting repair of the photosynthetic apparatus, and slow down the electron transport, which resulted in higher proportion of closed PSII centres (Mellis, 1999). On the basis of fluorescence parameters, ACBP6-overexpressing plants were able to retain to a certain degree the integrity of the chlorophyll-protein complex in the chloroplast stroma.

The decrease of net photosynthetic rate or the assimilation of CO_2 (*A*) on all plants, except for 4 ACBP6-overexpressing BNFP T3 lines, was expected as freezing affects the integrity of the chloroplast (Choluj et al., 1997; Shen et al., 1990). Exposure to low temperature primarily inhibits the assimilation of CO_2 in the photosynthetic pathway (Leegood & Edwards, 1996; Ort, 2002) resulting in the reduction of PSII efficiency (Baker & Rosenqvist, 2004). However, an increase in Y(II) during exposure to low temperature was associated with the plants ability to scavenge reactive oxygen species, if coupled with an increase in CO_2 assimilation (Fryer et al., 1998). For both Westar and fast plants there was a continued increase of *A* despite Y(II) plateauing at a range of 0.6 to 0.8 in Westar plants and 0.4 to 0.6 in fast plants. In corn, CO_2 assimilation was greatly reduced as photosynthesis was inhibited after exposure to low temperature. This resulted in the formation of reactive oxygen species, as electron flux to O_2 increased (Fryer et al., 1998).

In the wild-type controls for both experiments, ETR and *A* were both limited at a minimum, while the ACBP6-overexpressing plants showed maintenance of ETR despite limited *A*. Exposure to low temperature decreases the rate of electron transport as CO_2 fixation is also reduced (Rizza et al., 2001). In tomato, light-saturated rate of CO_2

assimilation and PSII linear rate of electron transport decreased after exposure to chilling temperature in the dark (Flexas et al., 1999).

A contrasting trend occurred for both Westar and rapid-cycling plants with regards to the intercellular CO_2 concentration (Ci) against net photosynthesis (*A*) and stomatal conductance (g_{sw}). After freezing, there was high variability in Ci over a range of g_{sw} in the T_2 population. For Westar and rapid-cycling plants; *A* was concentrated to a certain range of Ci and also showed an inverse relationship in the most frost damaged plants. The untransformed plants displayed a high Ci over a narrow range of g_{sw}, while within the ACBP6-overexpressing plants, an increasing trend was observable of Ci over a wide range of g_{sw}. The T_3 transformed plants were limited around 50 $molm^{-2}s^{-1}$, with increasing trend over this narrow range, while the untransformed plants displayed a high level of Ci. These results indicated that after freezing treatment, the causal effect of stomatal closure on the rate of photosynthesis did not apply because CO_2 concentration was high even when stomatal conductance was low. This has been observed in Jatropha (Ploshuck et al., 2014) where depletion of photosynthesis is not caused by stomatal closure but by other factors. The low g_{sw} after freezing could have been due to the disruption in the photosynthetic apparatus, i. e. damage in the functions of guard cells (Allen & Ort, 2001). In this situation, closure of stomata may have been caused by a high presence of CO_2 in the stomatal cavity due to loss of Rubisco activity (Allen & Ort, 2001). The loss of Rubisco activity may extend until the next morning after an overnight exposure to chilling stress (Allen & Ort, 2001). In C3 plants, under limiting Ci, net photosynthesis is expected to increase with Ci (Taiz and Zeiger, 1998). In chilling-sensitive species such as corn, soybean and tomato, exposure to low temperature overnight depressed the photosynthesis machinery and greatly affected the plant's

photosynthetic performance the following day especially under a highly radiant sky (Ort, 2002). Low temperature induces changes in multiple metabolic pathways, associated with circadian regulated processes, such as sucrose phosphate synthase and nitrate reductase (Ort, 2002).

In *B. napus* 'Westar', the overexpression of proteins involved in abiotic stress response such as the CBF/DREB1-like transcription factors, imparted increased photochemical efficiency and photosynthetic capacity, which were observed both in non-acclimated and acclimated plants. Leaf electrolyte leakage (LE_{50}) of the transgenic plants over-expressing the transcription factors BNCBF5 and BNCBF17, reached -5.3 and -10.2°C, while the untransformed control was only 3.7°C. Over-expression of these 2 cold-related transcription factors increased net photosynthesis (CO_2 assimilation rate, *A*), photochemical efficiency of PSII and linear electron transport rate (Savitch et al., 2005). Indeed in the same manner, ACBP6-overexpressing plants of Westar and rapid-cycling plants acquired tolerance to freezing temperature. This observation was however limited to non-acclimated plants. Further investigation on cold acclimation is recommended to investigate how ACBP6 would alter the photosynthetic machinery of *B. napus* based on chlorophyll fluorescence and gas exchange relationships, to tolerate freezing temperature. Rapacz (2001) showed that in *B. napus* cold-acclimation significantly enhanced freezing tolerance (Rapacz, 2001). Membrane stability was maintained even in freezing temperature when plants were cold acclimated (Mahajan & Tuteja, 2005). Cold acclimation is important for plants to survive frost events during winter. During cold acclimation, plants undergo changes that enable them to survive frost events and make them function well during the entire cold winter season. For example, the winter-type oilseed rape has the natural capacity to form a rosette or compact plant morphology and

the ability to increase its photosynthetic rate ensuring storage of ample energy for winter survival (Rapacz, 1999).

Cold acclimation significantly increases frost tolerance in spring form of oilseed rape to a level comparable to that of the winter types (Rapacz, 1999). Maximum increase in cold tolerance of both winter and spring types of oilseed rape can be achieved after 3 days of cold acclimation at 5°C (Rife and Zeinali, 2003). In a gene mapping study in *Brassica* for frost tolerance, genomic regions associated with acclimated freezing tolerance and acclimation ability were discovered in *B. rapa* (Teutonico et al., 1995). Recent studies on frost and cold tolerance were already largely focused on the genes directly controlling this plant response. The cold-inducible nature of acyl-coenzyme A-Binding protein (ACBP6) which enhances freezing tolerance was elucidated in *Arabidopsis* by Chen et al. (2008). Overexpression of ACBP6 in *Arabidopsis* conferred freezing tolerance and the effect was enhanced after cold acclimation, independent of the action of the more well-known cold-responsive *(COR*) genes.

Viability staining in developing seed embryos of rapid-cycling *B. napus* showed significantly higher proportion of living tissues in *ACBP6*-transformed plants compared with the untransformed plants. This indicated that *ACBP6*-induced tolerance to freezing extended into the plant's reproductive tissues. In *Arabidopsis*, the overexpression of ACBP6 imparted freezing tolerance on the flowers, and the expression was coupled with induction of *COLD-RESPONSIVE* (*COR*) genes (Liao et al. 2014). Exposure to freezing temperature affects brassica plants differently based on their growth stages. During seed maturation, storage lipids and proteins are being produced at a high rate especially during the predessication, peaking before the moisture content drops to around 50% (Crouch and Sussex 1981). At the same moisture level, the seeds exhibit a change in response and

sensitivity when exposed to ABA levels and osmotic potential (Finkelstein et al. 1985), indicating that at this stage, a major developmental change had occurred.

Exposure of canola seeds to freezing temperature disrupts the normal development of seeds, especially at this stage. Specifically, it affects the degreening process and promotes synthesis of green pigments (Johnson-Flanagan et al.1990). When examined for lipid content, seeds with about 55% moisture content decreased lipid quantity when exposed to freezing. The elongation and unsaturation pattern of fatty acids were also greatly affected (Johnson-Flanagan et al., 1991). Seeds with more than 80% moisture content exposed to freezing temperature were unable to develop and germinate (Johnson-Flanagan & Thiagarajah, 1990). After a freezing event, there was a high rate of seed desiccation (Johnson-Flanagan et al., 1991).

In summary, non-acclimated *B. napus* plants overexpressing ACBP6 showed enhanced freezing tolerance as determined by leaf electrolyte leakage, chlorophyll fluorescence and gas exchange measurements. The ACBP6-overexpressing plants showed minimal reduction of efficiency of PSII and maintenance of PSII-driven electron transport. The tolerance to freezing was further observed in the plants' capacity for CO_2 assimilation, but unlike in other abiotic stresses such as drought (Flexas et al. 2002), CO_2 assimilation was not limited by closure of stomata as indicated by a high internal concentration (C_i). Damage in the function of guard cells and membrane injury in the mesophyll layer could be attributed to the accumulation of CO_2 moleucles in the substomatal layer, which were not assimilated into the Calvin cycle. ACBP6-overexpressing plants showed significantly higher CO_2 assimilation compared with the wild type plants. Furthermore, they also showed less tissue injury in the developing seed embryo. ACBP6-overexpressing plants should also be evaluated with cold acclimation

and investigate how ACBP6 can alter the photosynthetic machinery of the plants in response to freezing stress.

Chapter 6

General Discussion

Frost is a major abiotic stress of canola crops in Australia, especially at the grain-filling stage resulting in poor seed fill and poor oil quality (GRDC, 2009). The objective of this thesis was to develop frost tolerant *B. napus* using the rapid-cycling genotype of *Brassica* and the cultivated variety *B. napus* 'Westar', through *Agrobacterium*-mediated transformation with *Arabidopsis thaliana ACYL-COA-BINDING PROTEIN6* (*ACBP6*). The ACBP6 was previously determined to confer freezing tolerance in *A. thaliana* (Chen et al., 2008). The specific aims of this study were to: (i) establish *in vitro* tissue culture regeneration protocol for rapid-cycling *Brassica napus and B. juncea* (ii) transform these genotypes, and *B. napus* 'Westar' with *Arabidopsis ACBP6* to produce frost tolerant transgenic plants, and (iii) evaluate transformed plants for frost tolerance in the vegetative and reproductive stage.

6.1 *In vitro* regeneration of rapid-cycling *Brassica*

In vitro regeneration of rapid-cycling *B. napus* and rapid-cycling *B. juncea* was established from four-day-old cotyledon explants. Optimum concentration of BAP and NAA for regeneration of explants for rapid-cycling *B. napus* and rapid cycling *B. juncea* was 5 μM of BAP and 2 μM of NAA, whereas for *B. napus 'Westar'*, higher concentration of BAP (20 μM) was required for optimum regeneration. Regeneration frequency obtained for rapid-cycling *B. napus*, *B. juncea* and *B. napus* Westar were 53%, and 76%, respectively. *In vitro* culture of *Brassica* species is highly dependent on the gentoypes (Christey at al., 1997; De Block et al., 1989; Moloney at al., 1989). The type, amount and

combination of plant growth regulators are one of the major aspects of optimisation for different genotypes of *Brassica* (Ono et al., 1994; Loudon et al., 1989). Previous studies on regeneration of rapid-cycling *B. rapa* from cotyledon explants also required similar level of auxin but a much lower cytokinin (Teo et al., 1997). Similarly, with *B. napus* 'Westar', shoot regeneration was influenced by the addition of BAP at concentrations ranging from 5to 20 μM, with very minimal or no NAA (Sharma et al., 1990; Ono et al., 1994; Moloney et al., 1989).

Cotyledon explants from all long duration *Brassica* species have been used to regenerate shoots *in vitro* (Bhuiyan et al., 2009; Cogbill et al., 2010; Hachey et al., 1991; Ono et al., 1994; Tang et al., 2003); with *B. napus* four-day-old cotyledons having the highest regeneration potential and being the most conducive tissue for *Agrobacterium*-mediated transformation (Hachey et al. 1991; Tang et al. 2003; Bhuiyan et al. 2009; Ono et al. 1994; Cogbill et al. 2010). Compared with *B. napus* 'Westar', the cotyledon size of the rapid-cycling genotypes was very small at 2 mm in the widest part of the cotyledon and less than 1 mm diameter; hence, precise excision of the petiole – excluding the apical meristem, was very critical to the success in establishing *in vitro* cultures. Manipulation of tissues in the culture was quite intricate with the rapid-cycling genotypes. The physical trauma in manipulating delicate shoots during shoot clump separation (or wounding of the shoot base and improper separation of shoots) was a major stress factor (Gaj, 2001) that may have contributed to the few situations of non-formation of roots in the *B. napus* fast plants when the elongated shoots were transferred to rooting medium.

The 'fast plant' characteristic of the rapid-cycling brassicas was evident during shoot regeneration in comparison to 'Westar'. The rapid-cycling brassicas started shoot elongation and formation of morphologically distinct leaves at 10-14 days, compared to

20-25 days for Westar. This was also evident in other *in vitro* studies with rapid-cycling *B. rapa* (Teo et al., 1997) and *B. oleracea* (Cheng et al., 2001). Long duration *B. napus* usually produce fully elongated shoots with distinct leaves after 30 days in culture (Moloney et al., 1989; Khehra & Mathias, 1992).

Cotyledon explants from rapid-cycling genotypes of *B. napus* and *B. juncea* exhibited high competency to induction of morphogenesis *in vitro* with the addition of optimum concentration of BAP and NAA. Regeneration of shoots was rapid and did not undergo a distinct intermediary callus phase. These characteristics were ideal for production of transgenic plants through *Agrobacterium*-mediated transformation. To date, this is the first study to report successful direct shoot regeneration of rapid-cycling *B. napus* and *B. juncea* using cotyledon explants.

6.2 *Agrobacterium*-mediated transformation of *Brassica* with the *Arabidopsis ACYL-COA-BINDING PROTEIN6*

Brassica napus 'Westar' and rapid-cycling *B. napus* were successfully transformed with plasmid pAT593, harbouring the *ACBP6* through *Agrobacterium*-mediated transformation; while transformation of rapid-cycling *B. juncea* was unsuccessful. The binary plasmid pAT593 (W. Meng, HKU; unpublished data) contained the CaMV *35S* promoter driving *ACBP6*, a neomycin phosphotransferase II (*NPTII*) encoding kanamycin resistance, and nopaline synthase (nos) terminator. The transformation procedure and *in vitro* regeneration of transgenic plants employed a multi-step process based on the protocol of Bhalla and Singh (2008), with the sequential steps of explant preparation, *Agrobacterium* inoculation, co-cultivation, shoot initiation and outgrowth, transformant selection, and rooting. The concentrations of BAP and NAA for

shoot regeneration of rapid-cycling *B. napus* and *B. napus* 'Westar' were as previously optimised (Section 6.1).

The rapid developmental changes in rapid-cycling *B. napus* accelerated the recovery of transgenic plants within 6-7 weeks, compared to the long duration counterpart of 10 to 14 weeks (Bhalla and Singh, 2008). The rapid formation of shoot initials was immediately observed after co-cultivation; shoot development *in vitro* was faster for the rapid-cycling *B. napus* compared to Westar. Thus, it was essential to closely observe the daily development of tissue growth for the right timing of subculturing into the appropriate tissue culture medium. After 3-4 weeks, separation of individual plantlets was possible for the rapid-cycling plants, hence, recovery of transgenic plants was faster for these genotypes. Over 2 years, T_3 generation of rapid-cycling *B. napus* was attained while Westar transgenic generation only developed to T_2.

Transformation efficiency was 6.1% for rapid-cycling *B. napus* and 4.9% for Westar. A huge proportion of the regenerated well-developed shoots consequentially died during the final selection step of treatment with 50 mg/L kanamycin, and some were unable to establish roots. In the case of rapid-cycling *B. juncea*, no transgenic plants were recovered. The presence of kanamycin during the shoot initiation and shoot outgrowth stages resulted in *B. juncea* explants unable to produce fully-developed shoots with well-formed leaves. Hyperhydricity in rapid-cycling *B. juncea* contributed to poor development under selection pressure with kanamycin, and sensitivity to physical damage during subculture. The inability of shoots to develop under selection pressure was quite common in other *Brassica* transformation studies, wherein the same concentration of kanamycin prevented growth of untransformed shoots but also inhibited shoot maturation and rooting of transformed shoots (Berthomieu & Jouanin, 1992; Radke et al., 1992).

This could have been due to the low expression of NPTII, and inability of the shoots to establish roots, a common occurrence *in vitro* transformed plants (Maheshwari et al., 2011).

PCR and RT-PCR analysis of independent transgenic plants (T_0) of rapid-cycling *B. napus* and 'Westar' confirmed the presence of the transgene *ACBP6* and *NPTII*; while Western blot of leaf tissues confirmed expression of ACBP6 protein in the leaves. A total of 10 independently transformed plants (6 rapid-cycling *B. napus* and 4 *B. napus* 'Westar') that showed consistent positive results for PCR, RT-PCR and Western analysis, and produced highly viable and good amount of seeds, were forwarded to the next generation. PCR analysis for segregation of *ACBP6* DNA revealed that in the T_1 generation, all lines tested were highly likely to contain a single copy of the *ACBP6* transgene as it followed the 3:1 ratio for Mendelian inheritance of a single dominant gene. However, when analysed for *NPTII* DNA and expression of kanamycin resistance, 3 lines deviated from the tested ratio. The inconsistency of the inheritance of the *ACBP6* transgene and the *NPTII* marker gene extended screening and selection period when forwarded to T_1 and T_2 generations. Inconsistent inheritance and integration of the transgenes in both rapid-cycling *B. napus* and 'Westar transgenic parents and progenies were characterized by the integration of only one of either genes in T_0 based on PCR of either *ACBP6* or *NPTII,* non-Mendelian and unstable inheritance of the transgenes in T_1 and T_2 generations, *NPTII* positive plant sensitive to kanamycin, phenotypic variation in kanamycin resistance, and chimerism in the transformed plants. Transgene integration is commonly inherited as a single Mendelian locus regardless of its copy number. However, non-Mendelian inheritance can also be observed due to instability of the transgene during

transmission to the next generation; or because it is poorly expressed (Matzke & Matzke, 1998).

There are many possible reasons why a transgene can become unstable in terms of expression and inheritance. Unstable transmission of the marker genes (i.e. *NPTII*) has been widely observed in *Brassica* and tobacco (Potrykus et al., 1985; Sikdar, 1999). The dosage effect or the variation observed with *NPTII* gene expression, wherein there is a phenotypic variation observed in the kanamycin resistant plants was also observed by Sikdar (1999), while chimerism and weaker expression of resistant phenotype was also observed by De Block et al. (1989) as reflected in the non-consistency of kanamycin and resistance with enzymatic assay results, and Southern blot.

When chimerism occurs, there is a very high chance that the T_1 generation would not reflect the expected gene inheritance; such is the case in the initial transformation experiments. Introduced DNA could have contained deletions during integration into the genome (Potrykus et al., 1985), as reflected in the PCR results of the T_0 plants, wherein some plants were positive only for either *ACBP6* or *NPTII*. The transgene could have been undetected or appear to have been lost in various stages of growth and the integration can be very variable in different generations. This may have been caused by certain sites in the plant genome being unfavorable for stable and heritable transgene integration (Srivastava et al., 1996), such as sites with highly repetitive sequences (Matzke & Matzke, 1998). Gene silencing effects may have also occurred due to methylation. The presence of transposable elements, incompatible sequences, and high GC content are the usual sites for DNA methylation. *Brassica napus,* being a polyploid, is inherently composed of repetitive DNA sequences and abundant transposable elements, making it susceptible for transgene silencing (Matzke & Matzke, 1998).

6.3 Frost tolerance in ACBP6-overexpressing *B. napus*

The freezing regime for frost screening was optimised during freeze screening with different oilseed *B. napus* cultivars. A step-wise decrease of temperature was used with a minimum test temperature of -4°C (actual mean temperature was -5.2°C ± 0.79), approximately similar to the LT_{50} (-5.5°C) of non-acclimated winter type of *B. napus* plants (Rife and Zeinali, 2003). A similar threshold temperature was also identified in a study in wheat where freezing temperature profile was based on actual frost events in South Australia (Chen et al, 2009). The temperature regime used was able to distinguish vegetative stage, cultivar-specific variation response to freezing based on electrolyte leakage assay. Two potentially frost tolerant *B. napus* cultivars which had the least leaf electrolyte leakage in two trials were identified, namely, 'ZHONGYUOZA-8-30940' a Pol CMS Hybrid from China, and 'AG-Spectrum' an Australian cultivar with blackleg resistance. Since this result only measured frost tolerance in the vegetative stage, further characterization at the seed filling stage is recommended, being the most susceptible stage for frost damage in the field. This opens the possibility of further studies to identify the genes involved in frost tolerance in these genotypes and the transfer of tolerance to commercial varieties using non-transgenic technology.

ACBP6-induced freezing tolerance in ACBP6-overexpressing non-acclimated rapid-cycling *B. napus* and *B. napus* 'Westar' was evident based on leaf electrolyte leakage assessing plasma membrane damage, chlorophyll fluorescence to assess damage in the plant's light-harvesting apparatus; and gas exchange to measure assimilation of CO_2 and transpiration. Tolerance to freezing injury was also observed in developing seed embryos, based on image analysis of tissue stained with fluorescein diacetate (FDA) viability stain.

Electrolyte leakage in the ACBP6-overexpressing plants was significantly lower with the least leakage of 37%, compared to the non-transformed plants with leakage up to 96%. The measurement of chlorophyll fluorescence was based on methods of Genty (1989), and estimated the effective photochemical quantum efficiency of photosystem II, Y(II), and the apparent rate of electron transport (ETR). The apparent damage to photosystem II as measured by chlorophyll fluorescence parameters has served as indicator of low temperature stress in *Brassica* (Bauer et al., 1994; Rapacz, 1999; Rapacz et al., 2001; Savitch et al., 2005). The ACBP6-overexpressing plants showed minimal reduction of efficiency of PSII and maintained the PSII-driven electron transport after exposure to -4°C. PSII is the first protein complex in the light-dependent reactions of photosynthesis, and the ACBP*6*-overexpressing plants were able to retain to a certain degree the integrity of the chlorophyll-protein complex in the chloroplast stroma.

The tolerance to freezing was further observed in the ACBP6-overexpressing plants' capacity for CO_2 assimilation. ACBP6-overexpressing plants showed significantly higher CO_2 assimilation compared with the untransformed controls. Exposure to low temperature primarily inhibits the assimilation of CO_2 in the photosynthetic pathway (Leegood & Edwards, 1996; Ort, 2002) resulting in the reduction of PSII efficiency (Baker & Rosenqvist, 2004). However, an increase in photosynthetic yield during exposure to low temperature has been shown to be associated with the plants ability to scavenge reactive oxygen species, if coupled with an increase in CO_2 assimilation (Fryer et al., 1998). This trend was observed for both *Brassica* types wherein there was a continued increase of net photosynthesis (*A*) despite Y(II) plateauing at a range of 0.6 to 0.8 in ACBP6-overexpressing Westar plants and 0.4 to 0.6 in the rapid-cycling plants.

Modelling of the boundary layer of the relationships between chlorophyll fluorescence and gas-exchange parameters showed shifts in the plants' performance after exposure to freezing stress, and revealed a proportion of ACBP6-overexpressing plants performing similarly with non-stressed plants. The gas-exchange measurements recorded very high concentration of intercellular CO_2 concentration (Ci) against net photosynthesis (*A*) and stomatal conductance (g_{sw}). Accounting for the limiting effect of stomatal closure after exposure to stress, Ci normally decreases. Unlike in other abiotic stress situations such as drought (Medrano et al., 2002; Reynolds-Henne et al., 2010) CO_2 assimilation was not limited by closure of stomata as indicated by a high internal CO_2 concentration (Ci), as also observed in tomato (Artuso et al., 2000) and Jatropha (Ploschuk et al., 2014). Damage to the function of guard cells and membrane injury in the mesophyll layer could have been attributed to the accumulation of CO_2 molecules in the substomatal layer, which were not assimilated into the Calvin cycle.

The fluorescence signal emitted by the FDA-stained developing seed embryos of T_3 rapid-cycling *B. napus* after freezing showed significantly higher proportion of live tissues in ACBP6-overexpressing plants compared to the untransformed plants. This indicated that *ACBP6*-induced tolerance to freezing extended into the plant's reproductive tissues. In *Arabidopsis*, the overexpression of ACBP6 imparted freezing tolerance to the flowers, and the expression was coupled with induction of *COLD-RESPONSIVE* (*COR*) genes (Liao et al. 2014). The *ACBP6* derived freezing tolerance in the seeds is a very ideal trait for canola production wherein seeds form the harvest, and the most susceptible to frost.

6.4 Future Directions

The development of recombinant *B. napus* plants over-expressing *Arabidopsis* ACBP6 demonstrated tolerance to freezing temperature, not only in the vegetative stage but also in the developing seed embryos. This shows the potential of the acquired tolerance to freezing injury to be used in the canola industry, where frost damage in the grain-filling stage is a major problem, resulting in poor seed fill and poor oil quality. The expressed tolerance to freezing in the seeds can be best characterized by transcription and lipid profiling analyses.

ACBP6-overexpressing plants should also be evaluated with cold acclimation and investigated as to how ACBP6 can alter the photosynthetic machinery of the plants in response to freezing stress. Rapacz (2001) showed that in *B. napus* cold-acclimation significantly enhanced freezing tolerance while in *A. thaliana*, ACBP6-overexpression displayed enhanced freezing tolerance in the leaves after cold-acclimation and independent of the induction of *COR* genes (Chen et al., 1998). Liao et al (2014) determined that in *A. thaliana* the mechanism of expression of ACBP6 varied in different organs, thus it would have been worthwhile to determine in *B. napus* if the ACBP6-induced freezing tolerance involved induction of *COR* genes. Homologous overexpression of *B. napus BNCBF/DREB1*-like genes which activated *COR* genes increased constitutive freezing tolerance and regulated chloroplast development to increase efficiency of photosynthetic machinery (Savitch et al., 2005). Compounding effect of *COR* genes and enhanced lipid metabolism by ACBP6 would potentially increase freezing tolerance in *B. napus*.

The successful transformation of rapid-cycling *B. napus* provides a platform for a ‘model’ plant which can be an alternative to *A. thaliana,* but with a genetic background

similar to the economically important canola crop. The short life-cycle and small plant habit of rapid-cyling *Brassica napus* makes it very ideal for transformation studies for improved abiotic and biotic stress performance.

With the limited freezing chamber and temperature control used in this study, it is highly recommended that the same trials would be repeated in a specialized frost simulation facility. Alarger sample size and more measurement points including post-freezing survival and performance are highly recommended.

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Appendices

Appendix 1. Analysis of variance (ANOVA) of regeneration response of the three genotypes in different concentration of NAA and BAP.

A. ANOVA for regeneration response in terms of Regeneration Efficiency and Regeneration Frequency among the three genotypes.

	Source	*SS*	*df*	*MS*	*F*	*P*
Regeneration Efficiency	Between Groups	14411.0	2	7205.514	9.014	.000
	Within Groups	81537.1	102	799.383		
	Total	14411.0	2	7205.514	9.014	.000
Regeneration Frequency	Between Groups	6525.9	2	3262.939	9.670	.000
	Within Groups	34416.5	102	337.417		
	Total	40942.4	104			

B. ANOVA for regeneration response of the three genotypes in different media.

	Source	*SS*	*df*	*MS*	*F*	*P*
Regeneration Efficiency	Between Groups	16224.8	6	2704.135	3.324	.005
	Within Groups	79723.3	98	813.503		
	Total	95948.1	104			
Regeneration Frequency	Between Groups	5143.9	6	857.311	2.347	.037
	Within Groups	35798.5	98	365.291		
	Total	40942.4	104			

C. ANOVA results for regeneration response of BNFP, BJFP and Westar in different media.

	Source	*SS*	*df*	*MS*	*F*	*P*
BJFP						
Regeneration Efficiency	Between Groups	12694.039	6	2115.673	18.217	0.000
	Within Groups	3251.852	28	116.138		
	Total	15945.891	34			
Regeneration Frequency	Between Groups	4433.580	6	738.930	11.105	0.000
	Within Groups	1863.210	28	66.543		
	Total	6296.790	34			
BNFP						
Regeneration Efficiency	Between Groups	23386.384	6	3897.731	17.087	0.000
	Within Groups	6387.160	28	228.113		
	Total	29773.545	34			
Regeneration Frequency	Between Groups	6661.093	6	1110.182	14.296	0.000
	Within Groups	2174.321	28	77.654		
	Total	8835.414	34			
Westar						
Regeneration Efficiency	Between Groups	29141.587	6	4856.931	20.370	0.000
	Within Groups	6676.049	28	238.430		
	Total	35817.637	34			
Regeneration Frequency	Between Groups	16113.933	6	2685.655	23.719	0.000
	Within Groups	3170.370	28	113.228		
	Total	19284.303	34			

Appendix 2. Means of regeneration frequency and regeneration efficiency of BNFP, BJFP and Westar, in different combinations of NAA and BAP.

Regeneration Frequency

Treatment	BAP (μM)	NAA (μM)	BNFP			BJFP			Wes		
			Number of plates (*n*)	Total number of explant	*M*	Number of plates	Total number of explant	*M*	Number of plates (*n*)	Total number of explant	*M*
1	13	1	5	49	20.4	5	47	14.9	5	47	51.6
2	5	0.5	5	50	30	5	49	26.7	5	49	18.2
3	10	0.5	5	49	18.2	5	49	14.4	5	49	18.2
4	20	0.5	5	50	8	5	49	8.2	5	49	54.9
5	5	2	5	45	53.3	5	46	43.6	5	46	21.8
6	10	2	5	48	22.9	5	48	18.7	5	50	24.0
7	20	2	5	49	12.4	5	47	10.4	5	47	76.9
8	0	0	5	49	0	5	48	0	5	48	0.0

Regeneration Efficiency

Treatment	BAP (μM)	NAA (μM)	BNFP			BJFP			Wes		
			Number of plates (*n*)	Total number of explant	*M*	Number of plates	Total number of explant	*M*	Number of plates (*n*)	Total number of explant	*M*
1	13	1	5	49	26.4	5	47	16.9	5	47	72.4
2	5	0.5	5	50	38	5	49	32.9	5	49	24.4
3	10	0.5	5	49	16.2	5	49	14.4	5	49	24.7
4	20	0.5	5	50	8	5	49	8.2	5	49	75.3
5	5	2	5	45	91.1	5	46	68	5	46	23.8
6	10	2	5	48	24.9	5	48	20.9	5	50	44.0
7	20	2	5	49	16.4	5	47	12.7	5	47	102.0
8	0	0	5	49	0	5	48	0	5	48	0.0

Appendix 3. One-way analysis of variance of rooted plantlets.

A. One-way analysis of variance of number of rooted BJFP, BNFP and Westar plantlets.

Source	*SS*	*df*	*MS*	*F*	*P*
Between Groups	8.9	2	4.5	18.5	.000
Within Groups	14.6	60	.24		
Total	23.6	62			

B. Tukey's HSD post hoc test

Genotype	N	Mean
BJFP	21	2.05 [a]
BNFP	21	2.67 [b]
Westar	21	2.95 [b]

[ab] *Means for groups in homogenous subsets. Std. Error = 0.152*

C. One-way analysis of variance of number of rooted plantlets derived from regeneration medium with different concentrations of NAA and BAP.

Source	*SS*	*df*	*MS*	*F*	*P*
Between Groups	2.9	6	.481	1.305	.270
Within Groups	20.7	56	.369		
Total	23.6	62			

Appendix 4. Regeneration response of cotyledon explants to varying concentration of *Agrobacterium*.

A. Regeneration of BNFP and BNW explants inoculated with different *Agrobacterium* concentration.

Agrobacterium Concentration (OD=650)	BNFP	No. of regenerating explant*	Observation	BNW	No. of regenerating explant*	Observation
0.05	30	9	Explants expanded, callus and shoot initials formed.	25	3	Explants expanded, callus and shoot initials formed.
	25	11		22	5	
	26	13		29	11	
0.1	25	8	Explants expanded, callus and shoot initials formed.	20	9	Explants expanded, callus and shoot initials formed.
	23	15		19	12	
	25	16		24	13	
0.5	25	4	Non-growing explants have necrotic petiole end; Agrobac contamination	23.0	8.0	Necrosis on petiole end of non-regenerating explants. Agrobac contamination.
	24	2		20.0	5.0	
	23	8		26.0	5.0	

**Regenerating explants = green explants showing shoot initiation at shoot induction medium only.*

B. Mean regenerating explants in different *Agrobacterium* concentration.

Agrobacterium Concentration (OD=650)	*N*	Mean (%) regenerating explant
0.05	6	23.1[a]
0.1	6	32.8[a]
0.5	6	53.9[b]

C. One-way analysis of variance of mean regenerating explants *Agrobacterium* concentration.

Source	*SS*	*df*	*MS*	*F*	*P*
Between Groups	2994.6	2	1497.3	9.3	.002
Within Groups	2424.2	15	161.6		
Total	5418.8	17			

Appendix 5. Gene sequences for *B. napus* acyl-coA-binding protein, *A. thaliana* acyl-CoA-binding protein and neomycin-kanamycin phosphotransferase type II

gi|509264|emb|X77134.1| B.napus mRNA for acyl-CoA binding protein

GATCTGCGCAATTTATCTCAACTTCTGACGTCTAATCATCATGGGTTTGAAGGAGGA
CTTTGAGGAGCACGCTGAGAAAGTCAAGAAGCTCACCGCGAGCCCATCTAACGAG
GACTTGCTCATCCTCTACGGTCTCTACAAGCAAGCCACCGTTGGGCCAGTGACCAC
CAGTCGTCCTGGGATGTTCAGCATGAAGGAAAGAGCCAAGTGGGACGCTTGGAAG
GCCGTTGAAGGGAAATCAACGGACGAAGCCATGAGTGACTACATCACTAAGGTGA
AGCAACTCCTTGAAGCAGAGGCTTCCTCCGCTTCAGCTTGATGGTGATCTATTAGTG
CTCTTATGCAGTAATTCTATCTAAGCTTCAAATAAGTTTAGCATAAGAGCTTGTTCT
TTTACTTCTTG

gi|145336317|ref|NM_102916.3| *Arabidopsis thaliana* acyl-CoA-binding protein 6 mRNA, complete cds

AGGTTTATCATGACGCCCATATATATCTCACGCGTTGTCCTCGTCTTCTCCGTCTTAC
ACTGATTTAATTCTCCTACCAATCTCAACTTCCGACGTCTATTCATCATGGGTTTGA
AGGAGGAATTTGAGGAGCACGCTGAGAAAGTGAATACGCTCACGGAGTTGCCATC
CAACGAGGATTTGCTCATTCTCTACGGACTCTACAAGCAAGCCAAGTTTGGGCCTG
TGGACACCAGTCGTCCTGGAATGTTCAGCATGAAGGAGAGAGCCAAGTGGGATGCT
TGGAAGGCTGTTGAAGGGAAATCATCGGAAGAAGCCATGAATGACTATATCACTA
AGGTCAAGCAACTCTTGGAAGTTGCTGCTTCCAAGGCTTCAACCTGATGAATCAAA
TCCTCATCTGCAGTAACTTTATCTTAAGCATCAAAATAACATTGCATAAGACTTGTT
CTTGCTCTTGTGTTTCTATCATATTTAAGCTATCTACTTTGTGACATGGTGTGATCTC
TTAAAAATGCTTGATATTGGTTAAAACAGAGAATCATGATGCAAACTAAATCCATA
AGTTATTTTTGGTCCGTCCTCGATATGGTCTTAGTTAAAACAGTTGAATTCAAGATG
ATATATTCGTTCTGGTCCGT

gi|62528955|gb|AY909580.1| Synthetic construct neomycin-kanamycin phosphotransferase type II mRNA, complete cds

CTGCAGACCATGATTGAGCAGGATGGACTGCATGCTGGCAGCCCTGCTGCTTGGGT
GGAGAGACTGTTTGGCTACGATTGGGCTCAGCAGACCATTGGCTGTTCTGATGCCG
CTGTGTTCCGGTTGTCTGCACAAGGTCGGCCTGTGTTGTTTGTGAAGACAGATCTGT
CTGGAGCCCTCAACGAACTCCAGGATGAGGCTGCCAGACTGAGCTGGTTGGCCACC
ACAGGAGTGCCTTGTGCTGCTGTGCTGGATGTGGTGACTGAGGCTGGCAGAGACTG
GCTGCTGCTGGGAGAGGTGCCTGGCCAGGACCTGCTGAGCAGCCACCTGGCCCCAG
CTGAGAAAGTCAGCATCATGGCTGATGCCATGAGAAGACTGCACACCCTGGACCCT
GCCACCTGTCCTTTCGACCACCAAGCCAAGCACAGAATTGAGAGAGCCAGAACCAG
AATGGAGGCTGGCCTGGTGGACCAGGATGACCTCGATGAGGAGCACCAGGGCCTG
GCCCCAGCCGAGTTGTTTGCCCGGTTGAAGGCCAGAATGCCAGATGGAGAGGATTT
GGTGGTGACACATGGAGATGCCTGTCTGCCTAACATCATGGTGGAGAATGGCAGAT
TCTCTGGCTTCATTGACTGTGGCCGGCTGGGAGTGGCTGACAGATACCAGGACATT
GCATTGGCCACCAGAGACATTGCAGAGGAGTTGGGAGGAGAGTGGGCTGACAGAT
TCCTGGTGCTGTATGGCATCGCTGCCCCTGACAGCCAGAGAATCGCCTTCTACCGGC
TGCTGGATGAGTTCTTCTGAGAGCTC

Appendix 6. *B. napus* cultivars used for frost tolerance screening.

Line	Source	Characteristics
B. napus 06-6-3737	China	double low quality, chill resistance
B. napus 06-6-3737	China	double low quality, chill resistance
B. napus P30830	China	double low quality, high oil content, high plant
B. napus FAN-023-30851	China	double low quality, low branch location and many branches
B. napus ZHONGSHUANG-4-30872	China	double low quality, tolerance to *Sclerotinia sclerotium*
B. napus ZHONGYOUZA-8-30940	China	Pol CMS Hybrid, high oil content
B. napus FAN-168-30960	China	Shattering resistance
B. napus FAN-189-30970	China	double high quality, low oil content
B. napus YU-178-20045	China	double low quality, long pod and big seed
B. napus 03-P74-4-20047	China	double low quality, strong growth vigor
B. napus 03-P74-6-20048	China	double low quality, very early
B. napus Oscar	Australia	conventional, mid-maturity, wide adaptation
B. napus Lantern	Australia	conventional, mid-maturity, high oil and protein
B. napus Monty	Australia	conventional, very early maturity (older seed used)
B. napus Tranby	Australia	early maturity, short height and TT traits
B. napus AG-Outback	Australia	early-mid maturity, moderate blackleg resistance, moderate oil content, moderate-high yield
B. napus Mystic	Australia	early-mid maturity, moderate blackleg resistance, moderate oil content, moderate yield
B. napus Rainbow	Australia	mid maturity, moderate blackleg resistance, moderate oil content, moderate-high yield
B. napus Surpass400	Australia	early-mid maturity, high blackleg resistance, high oil content, moderate yield
B. napus AG-Spectrum	Australia	mid maturity, blackleg resistance, high oil content, high yield
B. napus AV-Sapphire	Australia	mid maturity, blackleg resistance, high oil content, high yield

Appendix 7. Analysis of variance (ANOVA) on mean electrolyte leakage of different *B. napus* cultivars screened for freezing tolerance (Trial 1).

A. Test for interaction effect between cultivars and replications, based on mean electrolyte leakage.

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	77729.9[a]	58	1340.2	4.5	0	0.7
Intercept	457037.7	1	457037.7	1518.3	0	0.9
Replication	5124.8	3	1708.3	5.7	0	0.1
Cultivar	27567.7	14	1969.1	6.5	0	0.5
Replication*Cultivar	45183.4	41	1102.0	3.7	0	0.6
Error	32509.6	108	301.0			
Total	605425.2	167				
Corrected Total	110239.6	166				

[a] *R Squared = .705 (Adjusted R Squared = .547)*

B. Analysis for simple main effects (Replication).

Replications		*SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
1	Contrast	13358.2	14	954.2	3.17	0	0.3
	Error	32509.6	108	301.0			
2	Contrast	14230.8	14	1016.5	3.38	0	0.3
	Error	32509.6	108	301.0			
3	Contrast	19074.8	14	1362.5	4.53	0	0.4
	Error	32509.6	108	301.0			
4	Contrast	26724.1	13	2055.7	6.83	0	0.5
	Error	32509.6	108	301.0			

C. Analysis for simple main effects (Cultivar).

Cultivar		*SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
06-63737	Contrast	454.19	3	151.39	0.503	0.68	0.014
	Error	32509.62	108	301.02			
P30830	Contrast	3149.24	3	1049.75	3.487	0.02	0.09
	Error	32509.62	108	301.02			
30851	Contrast	5356.29	3	1785.43	5.931	0.00	0.14
	Error	32509.62	108	301.02			
Zhongshuang	Contrast	3245.68	3	1081.89	3.594	0.02	0.09
	Error	32509.62	108	301.02			
Zhongyouza	Contrast	107.87	3	35.96	0.12	0.95	0.0
	Error	32509.62	108	301.01			
FAN168	Contrast	6429.22	3	2143.07	7.12	0	0.165
	Error	32509.62	108	301.01			
FAN189	Contrast	10758.73	3	3586.24	11.91	0	0.25
	Error	32509.62	108	301.01			
20045	Contrast	5952.60	3	1984.20	6.59	0	0.16
	Error	32509.62	108	301.01			
20047	Contrast	5925.16	3	1975.05	6.56	0	0.15
	Error	32509.62	108	301.01			
20048	Contrast	3811.69	3	1270.57	4.22	0.007	0.10
	Error	32509.62	108	301.01			
Oscar	Contrast	1426.68	3	475.56	1.58	0.19	0.04
	Error	32509.62	108	301.01			
Monty	Contrast	28.10	2	14.05	0.05	0.95	0.00
	Error	32509.62	108	301.01			
Mystic	Contrast	1334.08	3	444.69	1.48	0.22	0.04
	Error	32509.62	108	301.01			
Spectrum	Contrast	271.36	3	90.45	0.3	0.82	0.01
	Error	32509.62	108	301.01			
Sapphire	Contrast	1485.5	3	495.17	1.64	0.18	0.04
	Error	32509.62	108	301.01			

D. Tukey HSD (honest significance difference) test for replications in Trial 1.

Replication	N	Mean electrolyte leakage
1	43	52.70[ab]
2	44	55.55[ab]
3	45	61.14[b]
4	35	46.63[a]

[ab]*Means for groups in homogeneous subsets are displayed.*

E.

F. Tukey HSD (honest significance difference) test for cultivars in Trial 1.

Cultivar	N	Mean electrolyte leakage
Spectrum	11	26.92[a]
Zhongyouza	11	28.09[ab]
20047	12	45.77[ab]
30851	11	46.32[ab]
FAN168	12	51.03[abc]
20045	10	51.79[abc]
Oscar	11	53.55[bcd]
20048	11	59.88[cd]
Sapphire	12	60.58[cd]
P30830	12	61.52[cd]
Mystic	12	62.14[cd]
FAN189	12	62.47[cd]
Monty	9	65.56[cd]
Zhongshuang	9	68.98[cd]
06-63737	12	73.363[d]

[ab]*Means for groups in homogeneous subsets are displayed.*

Appendix 8. Analysis of variance (ANOVA) on mean electrolyte leakage of different *B. napus* cultivars screened for freezing tolerance (Trial 2).

A. Test for interaction effect between cultivars and replications, based on mean electrolyte leakage.

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	166944.760[a]	162	1030.52	375.26	.000	.99
Intercept	1314324.936	1	1314324.94	478611.62	.000	1.00
Replication	27093.098	8	3386.64	1233.24	.000	.98
Cultivar	20527.247	20	1026.36	373.75	.000	.98
Replication*Cultivar	117599.818	134	877.61	319.58	.000	.99
Error	447.618	163	2.75			
Total	1504499.368	326				
Corrected Total	167392.377	325				

[a]*R Squared = .997 (Adjusted R Squared = .995)*

B. Analysis for simple main effects (Replication).

Replication		*SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
1	Contrast	6196.24	17	364.48	132.73	.000	.93
	Error	447.62	163	2.75			
2	Contrast	9017.96	16	563.62	205.24	.000	.95
	Error	447.62	163	2.75			
3	Contrast	15761.72	14	1125.84	409.97	.000	.97
	Error	447.62	163	2.75			
4	Contrast	6799.84	17	399.99	145.66	.000	.94
	Error	447.62	163	2.75			
5	Contrast	11270.68	18	626.15	228.01	.000	.96
	Error	447.62	163	2.75			
6	Contrast	30098.30	16	1881.15	685.02	.000	.98
	Error	447.62	163	2.75			
7	Contrast	23909.20	18	1328.29	483.69	.000	.98
	Error	447.62	163	2.75			
8	Contrast	14864.22	20	743.21	270.64	.000	.97
	Error	447.62	163	2.75			
9	Contrast	20208.75	18	1122.71	408.83	.000	.98
	Error	447.62	163	2.75			

C. Analysis for simple main effects (Cultivar).

Cultivar		*SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Oscar	Contrast	1693.23	4	423.31	154.15	.00	.79
	Error	447.62	163	2.75			
20045	Contrast	8350.04	7	1192.86	434.38	.00	.95
	Error	447.62	163	2.75			
P3737	Contrast	4632.00	6	772.00	281.12	.00	.91
	Error	447.62	163	2.75			
30851	Contrast	10182.11	7	1454.59	529.69	.00	.96
	Error	447.62	163	2.75			
Zhongshuang	Contrast	4299.94	7	614.28	223.69	.00	.91
	Error	447.62	163	2.75			
Surplus	Contrast	13384.74	7	1912.11	696.29	.00	.97
	Error	447.62	163	2.75			
Monty	Contrast	799.72	7	114.25	41.60	.00	.64
	Error	447.62	163	2.75			
Mystic	Contrast	1749.77	7	249.97	91.03	.00	.80
	Error	447.62	163	2.75			
Fan 168	Contrast	2783.25	5	556.65	202.70	.00	.86
	Error	447.62	163	2.75			
FAN189	Contrast	2142.09	6	357.01	130.01	.00	.83
	Error	447.62	163	2.75			
Lantern	Contrast	5418.62	8	677.33	246.65	.00	.92
	Error	447.62	163	2.75			
Zhangyouza	Contrast	16984.95	8	2123.12	773.13	.00	.97
	Error	447.62	163	2.75			
20048	Contrast	16583.85	8	2072.98	754.88	.00	.97
	Error	447.62	163	2.75			
20047	Contrast	653.40	6	108.90	39.66	.00	.59
	Error	447.62	163	2.75			
Sapphire	Contrast	1913.01	7	273.29	99.52	.00	.81
	Error	447.62	163	2.75			
Tranby	Contrast	5185.06	7	740.72	269.73	.00	.92
	Error	447.62	163	2.75			
Westar	Contrast	389.62	6	64.94	23.65	.00	.47
	Error	447.62	163	2.75			
Spectrum	Contrast	8909.81	6	1484.97	540.75	.00	.95
	Error	447.62	163	2.75			
Rainbow	Contrast	5059.70	7	722.81	263.21	.00	.92
	Error	447.62	163	2.75			
P30830	Contrast	19168.38	8	2396.05	872.52	.00	.98
	Error	447.62	163	2.75			
Outback	Contrast	14409.63	8	1801.20	655.91	.00	.97
	Error	447.62	163	2.75			

D. Tukey HSD (honest significance difference) test for replications in Trial 2.

Replicate	N	Mean electrolyte leakage
1	36	71.60[a]
2	34	65.26[b]
3	30	49.69[c]
4	36	81.95[d]
5	38	72.60[e]
6	34	57.39[f]
7	38	58.75[g]
8	42	62.66[g]
9	38	54.38[h]

E. Tukey HSD (honest significance difference) test for Cultivars in Trial 2.

Cultivar	N	Mean electrolyte leakage
20048	18	49.60[a]
0778ectrum	14	51.89[b]
Zhangyouza	18	54.67[c]
P30830	18	54.75[c]
P3737	14	56.53[cd]
30851	16	56.65[cd]
Outback	18	58.14[d]
Zhongshuang	16	62.42[e]
Lantern	18	62.80[ef]
Surplus	16	62.90[ef]
Mystic	16	64.97[fg]
20047	14	66.62[g]
Fan 168	12	66.86[g]
20045	16	69.16[h]
Westar	14	69.43[h]
Tranby	16	72.35[i]
Sapphire	16	73.36[ij]
Rainbow	16	74.44[ij]
Monty	16	74.57[j]
Oscar	10	75.12[j]
FAN189	14	77.83[k]

Appendix 9. Analysis of variance (ANOVA) on the freezing response measurements of ACBP6-transformed *B. napus* 'Westar' and untransformed control.

A. Two-way analysis of variance of electrolyte leakage (%).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	67623.72[a]	31	2181.41	3.99	.00	.72
Intercept	237397.77	1	237397.77	434.16	.00	.90
Line	23262.76	7	3323.25	6.08	.00	.47
Block	18603.71	3	6201.24	11.34	.00	.41
Line * Block	19470.56	21	927.17	1.69	.07	.43
Error	26246.26	48	546.79			
Total	344424.62	80				
Corrected Total	93869.98	79				

[a]*R Squared = .720 (Adjusted R Squared = .540)*

B. Two-way analysis of variance of net photosynthetic rate (*A*, µmol CO_2 $m^{-2}S^{-1}$).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	169.65[a]	30	5.65	3.38	.00	.68
Intercept	218.08	1	218.08	130.34	.00	.73
Line	37.12	7	5.30	3.17	.01	.32
Block	78.07	3	26.02	15.55	.00	.49
Line * Block	35.51	20	1.78	1.06	.42	.31
Error	78.64	47	1.67			
Total	533.68	78				
Corrected Total	248.28	77				

[a.] *R Squared = .683 (Adjusted R Squared = .481)*

C. Two-way analysis of variance of stomatal conductance (g_{sw}, mol H_2O $m^{-2}s^{-1}$).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	.07[a]	30	.00	2.49	.00	.61
Intercept	.17	1	.17	190.27	.00	.80
Line	.02	7	.00	3.26	.01	.33
Block	.02	3	.01	9.05	.00	.36
Line * Block	.02	20	.00	.92	.56	.28
Error	.04	47	.00			
Total	.33	78				
Corrected Total	.11	77				

[a]*R Squared = .614 (Adjusted R Squared = .368)*

D. Two-way analysis of variance of intercellular CO_2 concentration.

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	76647.88[a]	30	2554.93	.96	.55	.3
Intercept	7542580.87	1	7542580.87	2818.21	.00	.98
Line	10188.58	7	1455.51	.55	.79	.07
Block	18726.47	3	6242.16	2.33	.08	.13
Line * Block	45625.26	20	2281.26	.85	.64	.27
Error	125789.50	47	2676.37			
Total	8566460.00	78				
Corrected Total	202437.38	77				

[a]*R Squared = .379 (Adjusted R Squared = -.018)*

E. Two-way analysis of variance of transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	20.95[a]	30	.69	2.53	.00	.62
Intercept	80.92	1	80.92	293.16	.00	.86
Line	7.56	7	1.08	3.91	.00	.37
Block	6.32	3	2.11	7.64	.00	.33
Line * Block	5.51	20	.27	.99	.48	.29
Error	12.97	47	.28			
Total	131.47	78				
Corrected Total	33.92	77				

[a]*R Squared = .618 (Adjusted R Squared = .373)*

F. Two-way analysis of variance of effective photochemical quantum yield of PSII, Y(II).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	3.04[a]	31	.098	2.202	.01	.59
Intercept	15.56	1	15.56	349.82	.00	.88
Line	1.35	7	.19	4.32	.00	.39
Block	.782	3	.26	5.86	.00	.27
Line * Block	.63	21	.03	.68	.83	.24
Error	2.05	46	.04			
Total	22.59	78				
Corrected Total	5.08	77				

[a]*R Squared = .597 (Adjusted R Squared = .326)*

G. Two-way analysis of variance of electron transport rate (ETR, μmol $m^{-2}S^{-1}$).

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	8812.52[a]	31	284.27	3.47	.00	.70
Intercept	25320.90	1	25320.90	309.10	.00	.87
Line	2529.55	7	361.36	4.41	.00	.40
Block	3065.45	3	1021.81	12.47	.00	.45
Line * Block	2701.62	21	128.65	1.57	.10	.42
Error	3768.19	46	81.92			
Total	40710.68	78				
Corrected Total	12580.72	77				

[a]R Squared = .700 (Adjusted R Squared = .499)

H. Two-way analysis of variance of F_0 – minimal initial fluorescence yield

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*PartialEta Squared*
Corrected Model	530778.70[a]	31	17121.89	1.78	.04	.54
Intercept	16365067.53	1	16365067.53	1698.86	.00	.97
Line	245951.60	7	35135.94	3.65	.00	.36
Block	103145.69	3	34381.89	3.57	.02	.19
Line * Block	232277.98	21	11060.86	1.15	.34	.34
Error	443116.25	46	9632.96			
Total	18588481.22	78				
Corrected Total	973894.96	77				

aR Squared = .545 (Adjusted R Squared = .238

I. Two-way analysis of variance of Fmax – maximal fluorescence yield.

Source	*Type III SS*	*df*	*MS*	*F*	*P*	*Partial Eta Squared*
Corrected Model	19130938.73[a]	31	617127.06	3.14	.00	.68
Intercept	100324908.86	1	100324908.86	510.22	.00	.92
Line	7140075.45	7	1020010.78	5.18	.00	.44
Block	5905971.93	3	1968657.31	10.01	.00	.39
Line * Block	4875553.14	21	232169.19	1.18	.31	.35
Error	9044973.35	46	196629.86			
Total	139785034.84	78				
Corrected Total	28175912.08	77				

aR Squared = .679 (Adjusted R Squared = .463)

Appendix 10. Means of measurements on chlorophyll fluorescence and gas-exchange measurements taken on *ACBP6*-transformed BNW T_2 and Westar before (A) and after freezing or cold treatment (B).

A. Measurements before cold treatment

	A	g_{sw}	C_i	*E*	VpdL	PAR1	Y(II)	ETR	F_0	F_{max}	PAR2
BnW01	3.87	0.06	271.60	1.36	2.27	140.00	0.68	27.98	463.45	1596.45	98.36
BnW02	4.51	0.07	252.91	1.43	2.28	142.82	0.65	26.66	447.09	1264.36	87.73
BnW03	4.51	0.10	300.73	1.94	2.15	143.00	0.63	27.86	548.27	1512.27	107.27
BnW04	4.10	0.06	259.17	1.31	2.25	132.83	0.69	34.13	602.58	1672.17	125.33
BnW05	4.33	0.07	271.67	1.45	2.29	138.17	0.70	31.94	481.83	1491.00	107.42
BnW06	4.03	0.06	275.75	1.40	2.27	127.00	0.70	32.73	572.42	1821.17	110.25
BnW07	4.01	0.06	266.67	1.30	2.28	135.33	0.74	33.37	494.75	1777.42	105.33
Westar	4.02	0.08	290.82	1.61	2.24	136.00	0.66	27.04	494.83	1312.75	97.92
Total	4.17	0.07	273.45	1.47	2.26	136.73	0.68	30.30	514.02	1559.12	105.18

B. Measurements after cold treatment

Line	EL	A	g_{sw}	C_i	*E*	VpdL	PAR1	Y(II)	ETR	F_0	F_{max}	PAR2
BnW01	47.53	1.96	0.06	329.60	1.21	2.23	103.10	0.54	18.01	473.50	1349.57	101.98
BnW02	44.25	3.19	0.06	297.43	1.26	2.17	127.00	0.55	22.97	479.11	1302.44	97.30
BnW03	36.80	2.35	0.05	302.50	1.13	2.27	116.25	0.62	27.71	549.46	1528.92	109.50
BnW04	37.15	2.41	0.07	337.20	1.49	2.22	108.40	0.52	20.82	506.39	1380.09	95.07
BnW05	56.95	1.89	0.06	330.82	1.26	2.27	105.64	0.48	20.24	466.88	1174.30	89.06
BnW06	48.75	2.54	0.07	338.70	1.45	2.22	114.30	0.52	21.19	489.07	1422.10	87.97
BnW07	71.84	1.39	0.04	335.27	0.80	2.35	110.36	0.49	17.82	503.42	1128.90	85.27
Westar	93.96	0.27	0.02	332.55	0.48	2.28	110.45	0.14	6.61	364.18	455.11	45.53
Total	55.96	1.91	0.05	327.46	1.12	2.26	111.13	0.47	18.99	475.21	1196.20	87.69

EL - Electrolyte leakage (%), A- Net photosynthetic rate ($\mu molCO_2 m^{-2} S^{-1}$), g_{sw} - Stomatal conductance ($molH_2Om^{-2}s^{-1}$), E - Transpiration rate ($mmolH_2Om^{-2}s^{-1}$), C_i - Intercellular CO_2 concentration ($\mu molCO_2 mol^{-1}$), Y(II) – Effective photochemical quantum yield of $PSII_I$, ETR - Electron transport rate ($\mu molm^{-2}S^{-1}$), VpdL – leaf to air vapour pressure deficit, kPa; PAR1- photosynthetically active radiation using Li-Cor 6400 measurement; F0-initial fluorescence Fv'/Fm'; Fmax – maximal fluorescence Fv'/Fm'; PAR2 - photosynthetically active radiation measured with MINI-PAMII

Appendix 11. Analysis of variance (ANOVA) on chlorophyll fluorescence and gas-exchange measurements of untreated (24°C) and cold-treated (-4°C) *ACBP6*-transformed *B. napus* 'Westar' and untransformed control.

A. ANOVA of measurement means on combined untreated and treated T2 samples and control.

	Source	*SS*	*df*	*MS*	*F*	*P*
A	Between Groups	271.701	15	18.113	11.722	.000
	Within Groups	236.423	153	1.545		
	Total	508.125	168			
g_{sw}	Between Groups	.047	15	.003	3.213	.000
	Within Groups	.150	153	.001		
	Total	.198	168			
Ci	Between Groups	156437.026	15	10429.135	4.928	.000
	Within Groups	323808.737	153	2116.397		
	Total	480245.763	168			
E	Between Groups	17.290	15	1.153	4.052	.000
	Within Groups	43.529	153	.285		
	Total	60.819	168			
Y(II)	Between Groups	3.480	15	.232	8.607	.000
	Within Groups	4.177	155	.027		
	Total	7.657	170			
ETR	Between Groups	8774.562	15	584.971	6.428	.000
	Within Groups	14104.640	155	90.998		
	Total	22879.202	170			
F0	Between Groups	509800.356	15	33986.690	2.009	.018
	Within Groups	2622356.715	155	16918.430		
	Total	3132157.071	170			
Fmax	Between Groups	17044872.195	15	1136324.813	4.953	.000
	Within Groups	35563268.938	155	229440.445		
	Total	52608141.133	170			
Par	Between Groups	49564.615	15	3304.308	3.142	.000
	Within Groups	163014.780	155	1051.708		
	Total	212579.395	170			
VpdL	Between Groups	.365	15	.024	.729	.752
	Within Groups	5.176	155	.033		
	Total	5.541	170			
PARi	Between Groups	33274.172	15	2218.278	5.104	.000
	Within Groups	67362.355	155	434.596		
	Total	100636.526	170			

B. Post-hoc analysis (Tukey's HSD) of chlorophyll fluorescence and gas exchange parameters on combined untreated and treated T_2 samples

Samples	N	Y(II)	ETR	A	gsw	E	Ci
A_BNW01	11	0.68bc	27.98bc	3.87cde	0.06abc	1.36bc	271.6abcd
A_BNW02	11	0.65bc	26.66bc	4.51e	0.07bc	1.43bc	252.91a
A_BNW03	11	0.63bc	27.86bc	4.51e	0.09c	1.94c	300.73abcd
A_BNW04	12	0.69bc	34.13c	4.10cde	0.06abc	1.31bc	259.17ab
A_BNW05	12	0.7bc	31.94bc	4.33de	0.06bc	1.45bc	271.67abcd
A_BNW06	12	0.7bc	32.73c	4.03cfe	0.06abc	1.39bc	275.75abcd
A_BNW07	12	0.74c	33.37c	4.01cde	0.06abc	1.30bc	266.67abc
A_WES	12	0.66bc	27.04bc	4.02cde	0.08bc	1.61bc	290.82abc
B_BNW01	10	0.54bc	18.01bc	1.96ab	0.06abc	1.21abc	329.6bcd
B_BNW02	9	0.55bc	22.97bc	3.19bcde	0.06abc	1.26abc	297.43abcd
B_BNW03	8	0.62bc	27.71bc	2.34bc	0.05abc	1.13abc	302.5abcd
B_BNW04	9	0.52bc	20.82abc	2.41bc	0.07bc	1.49bc	337.2d
B_BNW05	11	0.48b	20.24abc	1.89ab	0.06abc	1.26abc	330.82cd
B_BNW06	10	0.52bc	21.19bc	2.54bcd	0.06bc	1.45bc	338.7d
B_BNW07	10	0.49b	17.82ab	1.38ab	0.04ab	0.80ab	335.27cd
B_WES	11	0.14a	6.61a	0.27a	0.017a	0.47a	332.55cd

Homogenous means are shown as subsets with similar letter. Sample labels starting with A were untreated set, while samples with B were treated.

Appendix 12. Analysis of variance (ANOVA) on chlorophyll fluorescence and gas-exchange measurements of untreated (24°C) means of *ACBP6*-transformed *B. napus* 'Westar' and untransformed control.

	Source	*SS*	*df*	*MS*	*F*	*P*
A	Between Groups	4.68	7	0.67	1.37	0.23
	Within Groups	40.39	83	0.49		
	Total	45.07	90			
g_{sw}	Between Groups	0.01	7	0.00	2.09	0.05
	Within Groups	0.06	83	0.00		
	Total	0.08	90			
C_i	Between Groups	19280.15	7	2754.31	1.68	0.12
	Within Groups	136006.38	83	1638.63		
	Total	155286.53	90			
E	Between Groups	3.49	7	0.50	2.28	0.04
	Within Groups	18.16	83	0.22		
	Total	21.64	90			
VpdL	Between Groups	0.16	7	0.02	2.06	0.06
	Within Groups	0.97	85	0.01		
	Total	1.14	92			
PAR1	Between Groups	2330.64	7	332.95	0.75	0.63
	Within Groups	37961.64	85	446.61		
	Total	40292.28	92			
Y(II)	Between Groups	0.10	7	0.01	2.00	0.06
	Within Groups	0.61	85	0.01		
	Total	0.71	92			
ETR	Between Groups	789.84	7	112.83	2.35	0.03
	Within Groups	4079.73	85	48.00		
	Total	4869.57	92			
F_0	Between Groups	246654.72	7	35236.39	1.62	0.14
	Within Groups	1847719.23	85	21737.87		
	Total	2094373.96	92			
F_{max}	Between Groups	3328447.74	7	475492.53	2.60	0.02
	Within Groups	15516411.95	85	182546.02		
	Total	18844859.70	92			
PAR2	Between Groups	9785.57	7	1397.94	2.23	0.04
	Within Groups	53320.33	85	627.30		
	Total	63105.89	92			

Appendix 13. Analysis of variance (ANOVA) on the freezing response measurements of ACBP6-transformed rapid-cycling *B. napus* and untransformed control.

	Source	*SS*	*df*	*MS*	*F*	*P*
EL	Between Groups	6107.5	6	1017.9	8.2	0
	Within Groups	2876.6	23	125.07		
	Total	8984.1	29			
A	Between Groups	41.6	6	6.9	3.9	0.0
	Within Groups	37.3	21	1.8		
	Total	78.9	27			
g_{sw}	Between Groups	0.0	6	0	0.4	0.9
	Within Groups	0.0	22	0		
	Total	0.0	28			
Ci	Between Groups	87522.9	6	14587.2	4.7	0.0
	Within Groups	64436.8	21	3068.4		
	Total	151959.8	27			
E	Between Groups	0.3	6	0.0	0.5	0.8
	Within Groups	2.1	23	0.1		
	Total	2.4	29			
VpdL	Between Groups	0.0	6	0.0	0.7	0.7
	Within Groups	0.2	23	0.01		
	Total	0.3	29			
PAR1	Between Groups	78193.0	6	13032.2	15.0	0
	Within Groups	19629.7	23	853.5		
	Total	97822.7	29			
PhY	Between Groups	0.6	6	0.1	11.3	0
	Within Groups	0.2	23	0.0		
	Total	0.7	29			
ETR	Between Groups	2224.1	6	370.7	10.0	0
	Within Groups	849.9	23	36.9		
	Total	3074.1	29			
F0	Between Groups	535660.2	6	89276.7	2.4	0.06
	Within Groups	863641.2	23	37549.6		
	Total	1399301.4	29			
Fmax	Between Groups	3223440.9	6	537240.2	4.7	0.00
	Within Groups	2589242.8	23	112575.8		
	Total	5812683.8	29			
Par2	Between Groups	1988.9	6	331.5	1.5	0.2
	Within Groups	5058.5	23	219.9		
	Total	7047.4	29			

Appendix 14. Analysis of variance (ANOVA) on chlorophyll fluorescence and gas-exchange measurements of untreated (24°C) means of ACBP6-transformed rapid-cycling *B. napus* and untransformed control.

	Source	*SS*	*df*	*MS*	*F*	*P*
A	Between Groups	2.28	6	0.38	0.52	0.79
	Within Groups	21.22	29	0.73		
	Total	23.50	35			
g_{sw}	Between Groups	0.02	6	0.00	1.38	0.26
	Within Groups	0.06	29	0.00		
	Total	0.08	35			
C_i	Between Groups	16591.42	6	2765.24	1.43	0.24
	Within Groups	56047.33	29	1932.67		
	Total	72638.75	35			
E	Between Groups	4.38	6	0.73	1.56	0.19
	Within Groups	13.54	29	0.47		
	Total	17.92	35			
VpdL	Between Groups	1.70	6	0.28	0.90	0.51
	Within Groups	9.17	29	0.32		
	Total	10.87	35			
PAR1	Between Groups	9436.94	6	1572.82	1.33	0.28
	Within Groups	34374.03	29	1185.31		
	Total	43810.97	35			
Y(II)	Between Groups	0.04	6	0.01	0.85	0.54
	Within Groups	0.21	29	0.01		
	Total	0.25	35			
ETR	Between Groups	170.84	6	28.47	1.10	0.39
	Within Groups	750.57	29	25.88		
	Total	921.42	35			
F0	Between Groups	437053.59	6	72842.26	2.21	0.07
	Within Groups	956781.63	29	32992.47		
	Total	1393835.22	35			
Fmax	Between Groups	97686.41	6	16281.07	0.45	0.84
	Within Groups	1043417.23	29	35979.90		
	Total	1141103.64	35			
PAR2	Between Groups	1020.35	6	170.06	1.53	0.20
	Within Groups	3230.40	29	111.39		
	Total	4250.75	35			

Appendix 15. Means of measurements on chlorophyll fluorescence and gas-exchange measurements taken on ACBP6-transformed BNFP T3 and untransformed control before cold treatment (A) and after cold treatment (B).

A. Measurements before cold treatment

Samples	*A*	g_{sw}	C_i	*E*	VpdL	PAR1	Y(II)	ETR	F_o	F_{max}	PAR2
BNFP02_17	4.38	0.12	305.60	2.32	3.10	108.60	0.65	26.82	690.40	2130.40	89.40
BNFP02_40	4.28	0.07	250.40	1.34	3.14	90.40	0.70	30.08	694.00	2087.40	99.60
BNFP01_5	5.06	0.09	282.60	1.85	2.78	128.00	0.68	29.70	674.80	2081.20	103.60
BNFP01_8	4.41	0.05	241.33	1.35	3.25	90.17	0.66	27.33	768.83	2070.83	101.00
BNFP03_15	4.29	0.07	269.40	1.54	2.76	136.20	0.72	28.54	516.60	2090.60	94.60
BNFP04_12	4.41	0.07	269.00	1.68	2.91	105.20	0.73	29.80	616.80	2215.20	100.20
WT	4.63	0.11	293.60	2.12	2.62	108.40	0.63	23.36	901.20	2183.60	89.20

B. Measurements after cold treatment

Samples	EL	Samples	*A*	g_{sw}	C_i	*E*	VpdL	PAR1	Y(II)	ETR	F_o	F_{max}	PAR2
BNFP02_17	59.51	3.35	0.04	212.00	0.72	2.06	171.25	217.50	0.50	28.65	553.00	1173.00	136.00
BNFP02_40	65.41	2.65	0.04	263.80	0.86	2.00	174.80	191.40	0.44	27.15	857.83	1561.83	146.40
BNFP01_5	56.07	2.71	0.04	267.75	0.75	2.00	37.19	350.04	0.39	24.28	572.00	945.60	146.80
BNFP01_8	62.83	3.58	0.05	261.23	0.89	1.95	176.00	223.33	0.47	31.67	881.67	1639.00	157.67
BNFP03_15	50.73	3.62	0.04	230.00	0.81	2.01	130.21	239.50	0.34	18.45	553.75	866.25	127.75
BNFP04_12	72.58	2.27	0.03	258.00	0.60	2.06	173.50	223.50	0.42	25.78	536.75	939.50	144.00
WT	95.42	0.06	0.05	385.80	0.86	1.96	179.40	216.20	0.08	4.67	628.70	694.00	138.30

EL - Electrolyte leakage (%), A- Net photosynthetic rate ($\mu molCO_2 m^{-2} S^{-1}$), g_{sw} - Stomatal conductance ($molH_2Om^{-2}s^{-1}$), E - Transpiration rate ($mmolH_2Om^{-2}s^{-1}$), C_i - Intercellular CO_2 concentration ($\mu molCO_2 mol^{-1}$), FV'/FM' - Maximum quantum Fv'/Fm' of PSII Φ_{PSII}, ETR - Electron transport rate ($\mu molm^{-2}S^{-1}$), VpdL – leaf to air vapour pressure deficit, kPa; PAR1- photosynthetically active radiation using Li-Cor 6400 measurement; F0-initial fluorescence Fv'/Fm'; Fmax – maximal fluorescence Fv'/Fm'; PAR2 - photosynthetically active radiation measured with MINI-PAMII

Appendix 16. Tissue vitality analysis of seed embryos of ACBP6-overexpressing T_3 lines of rapid-cycling *B. napus*.

A. Descriptive statistics of T_3 *Brassica napus* Fast Plants analysed for percent living tissue after freezing stress, based on FDA staining.

Percent Living Tissue				95% Confidence Interval for Mean				
T_3 Line	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Min	Max
BNFP02_17	34	76.65	23.18	3.98	68.56	84.74	13.63	100
BNFP02_40	76	66.53	29.04	3.33	59.89	73.17	2.02	100
BNFP01_5	40	77.32	32.63	5.16	66.88	87.75	0	100
BNFP02_8	59	61.64	31.47	4.09	53.44	69.85	0	100
BNFP03_15	28	66.69	34.52	6.52	53.31	80.08	0.02	100
BNFP04_12	31	47.73	31.58	5.67	36.14	59.31	0	98.55
WT	33	26.14	26.94	4.69	16.59	35.69	0	68.71
Total	301	61.80	33.31	1.92	58.02	65.58	0	100

B. ANOVA table for percent living tissue of the T_3 lines and control.

	Source	*SS*	*df*	*MS*	*F*	*p*
Percent living tissue	Between Groups	67587.03	6	11264.51	12.483	0
	Within Groups	265295.9	294	902.367		
	Total	332882.9	300			

C. Tukey's HSD test showing a pairwise comparison of the untransformed BNFP against the ACBP6-trasnformed T_3 BNFP lines.

Lines		Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
WT	BNFP017	-50.501648*	7.340617	0	-72.9985	-28.0048
	A40	-40.386732*	6.262404	0	-59.5792	-21.1943
	A5	-51.170369*	7.06425	0	-72.8203	-29.5205
	A8	-35.499901*	6.52984	0	-55.512	-15.4878
	C15	-40.546775*	7.71828	0	-64.2011	-16.8925
	D12	-21.583354	7.513526	0.092	-44.6102	1.44346

END