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Prospects for Transgenic and Molecular Breeding for Cold Tolerance in Canola (*Brassica napus* L.)

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1. Introduction

Oilseed rape has become a major crop in North America, with cropland dedicated to rapeseed production increasing from 4,391,660 ha in 2001 to 7,103,725 ha in 2010 in both U.S.A. and Canada (Canola Connection, 2011; National Agricultural Statistics Service, 2011). Most of these are cultivated in spring in the Canadian Prairie Provinces and the northern Great Plains of the USA.

Canola is cultivated both during winter and spring seasons in the United States and this exposes the crop to winter kill, frost, and high temperatures, during the reproductive period. The temperatures during winter and spring are known to influence all the crucial steps of the reproductive cycle including gametogenesis, pollination, fertilization and embryogenesis (Angadi, 2000). Winter rapeseed has been successfully grown in the Pacific Northwest, southern Great Plains, Midwest, and southeast regions of the USA. The hardiest cultivars will routinely survive winters in the north east of USA but survival is inconsistent further south (Rife *et al.,* 2001). Winter-grown canola (*Brassica napus* L.) production is limited mostly by frost and winter-kill in the southern canola-growing regions of the United States (Singh *et al.,* 2008). For instance, the late freeze in 2007 resulted in significant damage to most of the winter canola cultivars at the National Winter Canola Variety Trials in Alabama, U.S. (Cebert and Rufina, 2007). Winter hardiness and freezing tolerance are a major concern for improving production consistency in many regions of the canola growing countries.

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Introduction and cultivation of new crops in a given environment require management practices and trait selection that enable optimum performance of the crop. Canola is an important oilseed crop and its cultivation is expanding, particularly in the western world because of its importance as both an oilseed and a bio-diesel crop.

1.1 Cold tolerance

The ability of rapeseed plants to tolerate very low temperatures depends essentially on their development and the degree of hardening it has achieved. Unhardened plants can survive -4°C, while fully-hardened spring-type rapeseed can survive much lower temperatures (-10° to -12°C). Hardened winter rapeseed can survive short periods of exposure to temperatures between 15° and -20°C. Unhardening happens fairly fast after the plants initiate active growth (Sovero, 1993). The plants are typically best adapted to survive the winter in rosette stage with 6 to 8 leaves. Small plants are usually not as capable of surviving over-wintering, while plants with more leaves often start the stem elongation prematurely, exposing the meristem tissue to cold, making it more susceptible to damage. The hardening requirements of rapeseed have not been fully characterized. Winter type canola tend to harden faster, achieve higher degree of cold tolerance and unharden slower than spring types, but it is likely that variable hardening requirements could also be found within both types. Some differences in cold hardiness have been observed among both winter and spring types, however, it is unclear whether these are due to differences in ultimate achievable cold hardiness or differences in hardening requirements. The absence of snow cover during the coldest period of the winter decreases the plants' chances to survive. Ice formation on the soil surface can damage the crown area of the plants and reduce survival rate (Sovero, 1993).

1.2 Vernalization requirement

Most winter rapeseed cultivars will require three weeks of near-freezing temperatures in the field to get fully vernalized and start rapid generative growth. In controlled environments, eight weeks at 4^0C temperature is sufficient for full vernalization. In spring planting, winter rape will typically start slow generative growth after the prolonged rosette stage, and some cultivars may start blooming towards the end of the growing season. Differences in this respect are sometimes useful in distinguishing between morphologically similar cultivars. Different vernalization requirements are apparent among winter rape cultivars. A high vernalization requirement does not necessarily result in good winter hardiness, as many of the winter type cultivars from extreme maritime environments, such as Japan, require a long vernalization period yet have little tolerance for low temperatures (Sovero, 1993).

Some spring type cultivars do not exhibit any vernalization response at all, but in some cases the generative development can be accelerated with brief chilling treatment. In spring planting, only a few cool nights are usually needed. Vernalization response in spring types also tends to disappear in a long day environment. In spite of the variability in vernalization requirements within both types, the differences between the two types i.e. winter and spring canola are fairly clear with no overlap in the initiation of blooming in either spring or fall planting (Sovero, 1993).

1.3 Cold stress symptoms and its after effects

Cold stress symptoms can arise only after a cold temperature event; however, mild symptoms of herbicide injury may often be confused with symptoms caused by cold stress temperatures or nutrient-deficient soil (Figure 1 to 3). Recovery from cold stress will be rapid as temperatures increase. Nutrient stress symptoms are unlikely to occur at the cotyledon stage as nutrient demands at this stage are generally low (Boyles. 2011).

Fig. 1 shows that since the 1st and 2nd leaves are of normal size, the purpling observed is not herbicide injury. The purpling is as a result of anthocynin production caused by cold temperatures. Purpling may be towards the base, on the leaf margins or may cover entire young leaves of the plant. This symptom will diminish as temperatures increase.

Fig. 2 exhibits cupping caused by cold temperatures and symptoms quickly diminish as temperatures increases.

Fig. 3 indicates that cupping was caused by a low level herbicide residue. Variation in herbicide carryover means uninjured (red arrow) and injured yellow arrow and plants may be found in close proximity. Cold stress generally causes more uniform damage.

Fig. 1. Purpling of leaves due to low temperature.

Fig. 2. Cupping of leaves due to low temperature.

Fig. 3. Leaves damaged by herbicide carryover.

1.4 Methods of measuring cold stress

Field sites often exhibit either complete survival or complete winter-kill. Because of this variability, laboratory procedures to measure freezing tolerance have been developed including plant tissue water content (Brule-Babel and Fowler, 1988), ion leakage from plant cells after a freezing stress (Teutonico *et al.,* 1993), and changes in luminescence (Brzostowicz and Barcikowska, 1987). Further, meristem regrowth after plants are subjected to freezing temperatures is commonly used (Andrews and Morrison, 1992).

Laboratory freezing tolerance procedures have allowed investigators to gather information on cold tolerance that would otherwise be unobtainable in the field. Freezing tolerance of plant tissue is evaluated by measuring whether the tissues are alive or dead after subjecting the tissue to a range of freezing temperatures. The extent of damage caused by the freezing can be evaluated by placing plant tissue in distilled water and measuring the electrical conductivity of the resultant solution (Madakadze *et al.*, 2003; Murray *et al.,* 1989). An increased rate of electrolyte loss is interpreted as evidence and the extent corresponds to damage. The electrolyte leakage (EL) method (Oakton CON 510TDS electrical conductivity meter: Eutech Instruments, Singapore) is based on objective measurements, which utilizes small quantities of tissue, and is relatively cheap. However, it takes more time than the chlorophyll fluorescence method (OS1-FL portable pulse-modulated fluorometer: Opti-Sciences, Tynggsboro, MA).

Freezing on the functionality of the photosynthetic apparatus can be used to assess the cold tolerance of plant genotypes (Chengci *et al.,* 2005). The photosynthetic apparatus function can be evaluated by measuring the ratio of chlorophyll variable fluorescence (Fv) over the maximum fluorescence value (Fm) (F_v/F_m), which indicates the efficiency of the excitation capture by open photosystem II reaction centers (Frachebound *et al*., 1999; Rizza *et al.,* 2001). A significant reversible decrease in F_v/F_m was found in all genotypes of oat (*Avena sativa* L.) during acclimation to low, nonfreezing temperatures, and F_v/F_m measurement was found to be highly correlated with field-evaluated frost damage (Rizza *et al*., 2001). Measurement of F_v/F_m is rapid and noninvasive.

2. Effect of cold stress on plant performance

2.1 Cold stresses reduce plant productivity

To make early spring seeding feasible, suitable canola cultivars must be selected. The suitable cultivars must have quick germination emergence, and establishment at low temperatures, and seedlings must be tolerant to early spring freezing and thawing events. Low temperatures reduce both the final percentage as well as the rate of germination, which leads to delayed and reduced seedling emergence of canola (Zheng et al., 1994). Early spring frosts are more problematic with fall-seeded canola, which emerges earlier than canola seeded in early spring (Willenborg *et al.,* 2004). Fall seeding of canola does present some challenges in the Canadian Prairie Provinces and the northern Great Plains of the USA. Fall seeding frequently entails seeding into hard, cold soil, which ultimately results in poor soil-to-seed contact (Kirkland and Johnson, 2000).

Mild winter climate in Northern Alabama, USA is conducive for optimum productivity of winter canola (Cebert and Rufina, 2007). However, the region is also vulnerable to late frost

during mid to late spring (late March to Mid April). In April, 2007, twenty winter canola genotypes at varying stages of flowering and early pod-filling (Figures 4-9) were exposed to three incidences of naturally occurring severe spring frosts that reduced most of the yield components (Cebert and Rufina, 2007).

Fig. 4-9 show the extent of damage resulting from cold stress after freeze from April 5 to 9, 2007 in North Alabama, U.S.A.

Fig. 4. Canola field at flowering after spring 2007 hard freeze in Northern Alabama.

Fig. 5. Early maturity breeding line (lower right) completely destroyed by late spring frost.

Fig. 6. Several cultivars did not suffer much damage. Cultivar, Kadore produced the highest yield despite the freeze followed by an exceptional drought period.

Fig. 7. Primary yield loss due to dropping off of fertilized flowers after late spring frost.

Fig. 8. Destruction of photosynthetic green tissues due to late spring frost.

Fig. 9. Destruction of developing pods due to late spring frost.

Early seeded canola may encounter suboptimal soil temperatures for seed germination and seedling establishment in the northern Great Plains (Chengci *et al.*, 2005). The optimum

germination temperature for canola was reported to be 5°C (Morrison *et al*., 1989) and 0.4 to 1.2°C (Vigil *et al*., 1997). Soil temperature in April in most canola production areas of the northern Great Plains is usually lower than the previously given optimum temperatures (Zheng *et al*., 1994). Spring canola seeded into suboptimal soil temperatures had lower emergence and stand establishment rates due to the seed rotting in the cold soils (Blackshaw, 1991; Livingston and de Jong, 1990). A significant reduction in canola germination was found at temperatures less than 10°C (Nykiforuk and Johnson-Flanagan, 1994), and it took as long as 18 days for 50% emergence at 5°C (Blackshaw, 1991). Vigil *et al.* (1997) reported that 65 to 81 growing degree days (GDD) are required for spring canola seedlings to emerge. Differences in GDD have also been found among the species and seed lots (Nykiforuk and Johnson-Flanagan, 1994)

One can determine an optimal seeding date in the northern Great Plains or other regions with similar climate and soil conditions as central Montana (Chengci *et al*., 2005). First, days from seeding to emergence can be predicted from long-term weather data based on the base temperature for germination (T_b) and growing degree days for 50% emergence. Second, based on the cold tolerance information, one can decide which cultivar to plant and the risk of frost damage for a given early seeding date. Third, using the information on days to 50 % flowering in combination with long-term weather data, the maximum daily temperatures at flowering stage, optimum seeding date can be forecasted; thus, the potential impact of maximum temperature on seed yield can be estimated for a given seeding date.

2.2 Plant responses to cold stress

Plants acclimatize to survive metabolic lesions because of intracellular ice formation, as well as to survive the dehydrative effects of frost (Kacperska, 1984). Fowler *et al.,* (1996) found that after the vernalization requirement was met in wheat (*Triticum aestivum* L.) and rye (*Secale cereale* L.), cold acclimation declined. Laroche *et al.,* (1992) did not observe this reduction in cold acclimation in rapeseed but they estimated cell survival on excised leaves to determine freezing tolerance and not crown meristem survival.

The relationship between vernalization requirements and cold tolerance is not clear as different observations have been reported. While Markowski and Rapacz (1994) found little relationship between these traits by comparing vernalization requirements and frost resistance of winter rape lines derived from doubled haploid, Rapacz and Markowski (1999) found a significant correlation between vernalization requirement and both frost resistance and field survival when looking at older, high erucic acid cultivars. Long vernalization requirements are expected to delay a plant from entering the reproductive growth phase, a cold sensitive plant growth stage (Fowler et al., 1996). Rife and Zeinali (2003) found that rapeseed plants may withstand cold temperatures under field conditions more effectively prior to vernalization saturation than after the vernalization requirement has been met.

Under field conditions, rapeseed plants often survive cold events in December and January only to be killed by less severe cold events in February and March (Rife and Zeinali, 2003). One theory to explain this is that after vernalization saturation takes place, rapeseed plants do not have the same ability to recover after a warming event as unvernalized plants. This has been documented in winter cereals. Fowler et al., (1996) found reductions in lethal temperature 50's (LT_{50}s) of 5°C or more for many cultivars between Day 49 and 84 of

acclimation at 4°C. This study suggested that this may not be the case in three rapeseed cultivars: A112, Ceres, and Plainsman. However, the spike of increased cold tolerance was more pronounced in unvernalized seedlings. Under the variable temperature conditions present during Great Plains winters, this phenomenon could have a substantial impact on winter survival before vernalization saturation and selecting genotypes with increased vernalization requirements could have a positive effect on winter survival.

Early seeding between late March and mid April has been proposed as the key to achieve a good and stable canola seed yield in central Montana, USA (Chengci *et al*., 2005). This study indicated that the optimal seeding rate for early spring seeded canola is 32 to 65 seeds m^{-2}. Although early spring seeded canola is expected to encounter cold soil temperatures and frequent frosts, canola can germinate at less than 4°C and requires 42 to 81 growing degree days (GDD) for 50% flowering for emergence. Further studies are needed to test the threshold temperature and duration of canola genotypes to cold stress. Several genotypes were found to have favorable characteristics for the semi-arid region in the northern Great Plains, such as low T_b, fast emergence, relatively cold tolerant, early flowering, and good seed yield and oil content. Further, Chengci *et al*., (2005) observed that a high F_v/F_m and low EL reading after a snowstorm event indicated less effect on Photosynthetic System II and cell membranes by cold stress. A large recovery of F_v/F_m and slow leakage rate (longer time to reach 50% total leakage) also indicate a frost tolerance of the plants. There were variances observed among the cultivars in F_v/F_m, $\Delta(F_v/F_m)$, EL, and hours to 50% total leakage (HTL_{50}). However, as the authors noted, there was no evidence of cell membrane damage by the snowstorm, EL readings ranged from 12.7 to 20.4 μS cm^{-1}, and the time to reach 50% of the total leakage (≈600 μS cm^{-1} after autoclaving) ranged from 212 to 276 h. Several cultivars had greater F_v/F_m readings and less $\Delta(F_v/F_m)$ than others. Neither EL nor the F_v/F_m readings were correlated with seed yield. However, the positive correlations between $\Delta(F_v/F_m)$ and biomass indicated that cultivars having faster biomass growth may be sensitive to cold stress. Results in this study also indicated that canola did not suffer severe frost damage by the snowstorm on 12 May 2004. Canola seedlings at the early seeding dates encountered several snowstorms in May and early June over 3 yr from 2002 to 2004, but no severe frost damage to the seedlings was observed. Canola was also found to have the ability to withstand subzero temperatures in other studies after reclamation. Kirkland and Johnson (2000) found fall-seeded canola survived eight consecutive nights of frost with the temperature dropping as low as −8°C in 1994. Further, Johnson *et al.* (1995) observed that canola seedlings were able to tolerate temperatures of −6°C without significant reductions in plant stands in North Dakota

2.3 Effect of cold stress during flowering and pod formation

Farmers in central Alberta experienced a record string of killing frosts for their Canola plants in late May to early June, 2000. Seedling canola was severely injured by these frosts and significant reseeding occurred. The surviving density was on average 32 to 43 plants/m^2 and about 11/m^2 in the worst areas. Continuing frosts hampered the recovery of the canola seedling but eventually new growth appeared after 10 days. The canola stand had a slow recovery for a few weeks following damage, but initially looked quite poor. By flowering, the stand began to fill in but differences in maturity were evident between areas that suffered different amounts of stand thinning. The stand continued to improve through flowering and pod fill. The crop matured in September with the thin areas maturing later

than the better areas by one to two weeks. Plant counts at harvest averaged 43 plants/m^2. The thin crop that was questioned in spring eventually yielded 2,128 kg/ha gross with 2 to 3% green. Although this crop probably would have yielded higher if frost had not occurred, the yield was satisfactory and equivalent or better than reseeded canola. Early spring germinated stands can suffer damage due to subsequent heavy spring frosts. Polymer-coated seeds show promise to reduce the untimely germination but carry extra costs (Canola Council of Canada, 2011). However, Christian *et al.,* (2004)) observed that by decreasing osmotic potential and temperature, germination significantly reduced on both coated and uncoated canola seeds. Polymer-coated seeds exhibited delayed germination even in the absence of moisture stress, an effect that was magnified at more negative water potentials and at a lower temperature. Median germination time of polymer-coated seed was significantly higher than for uncoated control seed throughout all temperature and osmotic potential treatments.

Several types of polymer seed coats have recently been developed with the intent to extend the fall planting period (Zaychuk and Enders, 2001). The polymers are specifically designed to prevent germination of canola seeds until spring by absorbing water into the polymer coat matrix but preventing the passage of sufficient amounts of water to the seed coat to begin germination. After water entry into the polymer coat matrix, freezing is required to create microfractures in the polymer coat that act as water channels for imbibition. Polymer seed coats have been shown to decrease imbibition (Chachalis and Smith, 2001) and final germination percentages (Valdes and Bradford, 1987). This may also be a problem with the polymer coats developed specifically for fall seeding canola as Gan et al., (2001) observed reduced canola emergence during a dry spring following a dry fall. Lower yields were also observed in polymer coat treatments.

To make early spring seeding feasible, suitable canola cultivars must be selected. The suitable cultivars must have quick germination, emergence, and establishment at low temperatures, and seedlings must be tolerant to early spring freezing and thawing events. Freezing on the functionality of the photosynthetic apparatus can be used to assess the cold tolerance of plant genotypes. The photosynthetic apparatus function can be evaluated by measuring the F_v/F_m, which indicates the efficiency of the excitation capture by open Photosystem II reaction centers (Rizza et al., 2001). Rizza *et al* (2001) observed a significant reversible decrease in F_v/F_m in all genotypes of oat (*Avena sativa* L.) during acclimation to low, nonfreezing temperatures, and F_v/F_m measurement was found to be highly correlated with field-evaluated frost damage.

The late freeze of April 5-9, 2007, in Northern Alabama, resulted in significant economic damage to most of the winter canola cultivars that were evaluated for the National Winter Canola Variety Trials at the Hazel Green, Alabama location (Cebert and Rufina, 2007). Five consecutive nights of low temperatures: 0.6, 1.1, -5.6, -5.6, and 0.6^{o}C with chilling hours of 8, 16, 18, 24, and 17occurred from April 5-9, 2007, respectively. These dates correspond to days 192, 193, 194, 195, and 196 after planting, when all cultivars were beyond 50% blooming (Figures 4-9). Damage on the primary stem was highest in early blooming cultivars (Figure 5). Cultivars which were in full bloom between days 178-183 (March 22-27) suffered a complete loss. Seeds per pod and days to 50% blooming were the two factors other than the extent of freeze damage which influenced seed yield (Table 1). In Alabama

Cultivar	Estimated freeze damage (%)	Estimated yield (Kg ha-1)
Baros	80.0a†	374.6i
Virginia	65.0ab	685.7hi
Viking	60.0abc	838.9fghi
Hybrista	56.7abc	782.8ghi
Abilene	55.0abcd	851fghi
Taurus	53.3abcd	902.7fghi
Trabant	53.3abcd	893.6fghi
Baldur	40.0bcde	1195.6defgh
Rasmus	35.0bcdef	972.2fgh
Falstaff	28.3cdef	1332.5defg
Summer	26.7cdef	924.7fghi
Ceres	21.7def	1399.2cdef
Wichita	21.7def	1721.2bcd
Satori	16.7ef	1392.3cdef
Jetton	16.7ef	1061.8efgh
Kalif	16.7ef	2094.6ab
Ovation	10.0ef	1578bcde
Kronos	8.3ef	1624.8bcd
Kadore	1.7f	2552.0a
Plainsman	1.7f	1900.6bc

†Means with the same letter are not significantly different at a = 0.05

Table 1. Estimated freeze damage and yield (Kg ha-1) of canola cultivars following late spring frost damage and exceptional drought conditions in National Winter Canola Variety Trial, Alabama A&M University, USA 2007.

A&M University, U.S. experimental plots, Cebert and Rufina (2007) reported an inverse response among cultivars between freeze damage and seed yield. Cultivars Kadore, Kalif and Plainsman with the highest seed yields were also the last ones to reach 50% blooming, approximately five days before the freeze.

3. Breeding for crop improvement

3.1 Genetics

As described by Nakashima and Yamaguchi-Shinozaki (2006), the basic characteristics of plants forced them to survive in environments with variable environmental stresses such as cold stress and osmotic stress, which includes drought and high salinity. Plants, therefore exhibit an increase in freezing tolerance in response to low, non-freezing temperatures. This concept of acclimating to stressful environmental conditions has been widely investigated,

especially with the use of model plant *Arabidopsis thaliana* (Yamaguchi-Shinozaki and Shinozaki, 1994; Jaglo-Ottosen *et al.,* 1998; Lee *et al.,* 2001; Taji *et al.,* 2002; Zhang *et al.,* 2004b; Carvallo *et al.,* 2011).

However, contribution towards the improvement of *Brassica* species, which eventually led to the development of canola, is the creation of the Triangle of U (Figure 10) by Nagaharu, U (1935). The interpretation of three common diploid species (*B. campestris*, *B. nigra* and *B. oleracea*) being the origin of the current tetraploid *Brassica* species continues to be the foundation for contemporary development and enhancement for both vegetables and oilseed crops within the genus, indicative in improvement of *B. rapa* by Ofori *et al.,* (2008). An extensive assortment of loci for various physiological and morphological traits in the different *Brassicica* species have been determined and identified through both classical and molecular methods. Through the use of embryo rescue techniques, interspecific and intergeneric hybridization hindrances such as male-sterility and self-incompatibility were overcome (Nishi *et al.,* 1959; Quazi, 1988; Mohaptra and Bajaj, 1987). As delineated by Harberd (1969), the tedious procedures for embryo rescue in *Brassica* species resulted in the exchange of genes to produce cultivars with improved resistance for pests, variation for seed coat color, changes in fatty-acids composition and tolerance to herbicide. Sacristan and Gerdemann (1986), successfully transferred blackleg resistance to *B. napus* from *B. juncea* using embryo culture, while similar techniques were successfully employed to transfer both aphid-resistance and triazine herbicide-resistance among and between the various *Brassica* species (Kott *et al.,* 1988).

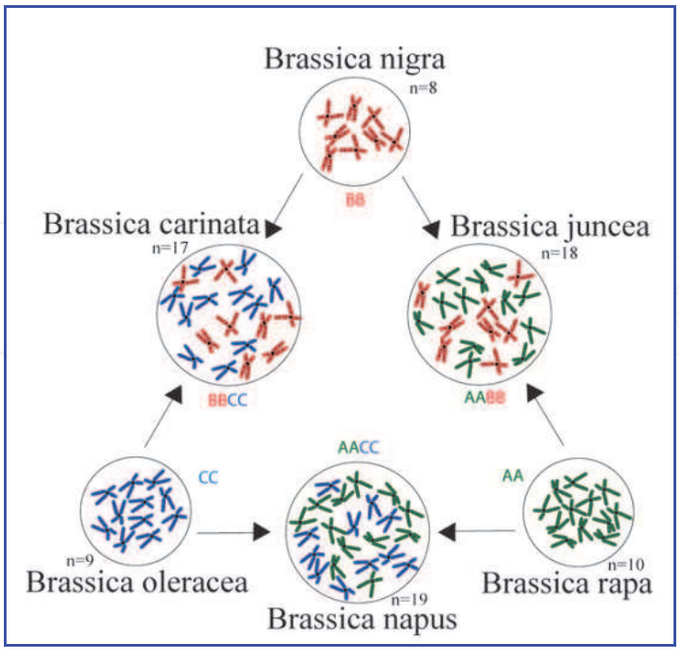

Fig. 10. The Triangle of U, by Woo Jang-choon (Nagaharu U, 1935).

Microspore culture and protoplast fusion are other protocols which have been employed successfully in the development of improved *Brassica* cultivars. According to results obtained by Ryschka *et al.,* (2007), hybrid formation between haploid at the level of protoplasts fusion obtained from different species has the potential to combine divergent genomes which may not be possible otherwise. Traditional non-molecular systems provided the foundation to identify Quantitative Trait Loci (QTLs) for early genomic mapping. These systems of hybridization coupled with current biotechnology have energized the search for novel procedures to create distinctive desirable cultivars.

3.2 Breeding for cold tolerance

Current molecular tools such as SSRs (Single Sequence Repeats), SNPs (Single Nucleotide Polymorphism) and ESTs (Expressed Sequence Tags) are being used to identify genes of economic significance including cold tolerance in *Brassica* species as reported in studies by Ofori *et al.,* (2008) and Thomashow (1999). Specific genes that respond to cold and osmotic stress in plants have been elucidated by Zhang *et al.,* (2004a), and Shinozaki *et al.,* (2003) and summarized by Lata and Prasad (2011). Results from these findings along with molecular makers have enabled the creation of high density genetic and physical maps of new genes that will allow the enhancement of genetic variation for desired traits such as response to cold stress. The most recent of such results is the release of the *Brassica rapa* genome-sequence by the "*Brassica* Genome Sequencing Project Consortium" (2011). Initiatives to identify stress related genes have provided significant results in understanding functional genomics of abiotic stress. The Genome Canada/Genome Prairie project studied a range of genomics and proteomics technologies to determine how plants respond to various environmental stresses at the gene level, particularly to cold. A general insight among the findings is the discernment that cold tolerance genes are induced under conditions other than low temperatures but also due to dehydration, high salt and other abiotic stress.

Many studies (Sangwan *et al.,* 2001; Zhu *et al.,* 2009; Chinnusamy *et al.,* 2010; Chen *et al.,* 2011) have reported significant findings that provide novel sources of cold tolerance genes which can be introgressed into new hybrids and open pollinated cultivars. New data are being generated with genomics tools to exploit the use of genetic maps derive from various *Brassica* species and those from *Arabidopsis*. The accumulation of ESTs and SNPs is producing significant information on genome polymorphism and sequence data for all stress related characteristics in *Brassica* species. Kreps *et al.,* (2002) used transcriptome changes for *Arabidopsis* to identify genes in response to stress treatment including cold. Their findings showed approximately 30% of the transcriptones demonstrated sensitivity to regulation to common stress, with most being unambiguous in response for specific stimuli. Genes identified as circadian controlled were also found to be associated in response to cold stress stimuli. Similar findings were also reported by Trischuk *et al.,* (2006) and Nakashima and Yamaguchi-Shinozaki (2006) who further indicated that cold acclimation, while primarily influenced by temperature, is also moderated by factors such as light intensity, day length, cultural practices, and other abiotic stresses such as drought, dehydration and salinity. Dehydration responsive element binding (DREBs) have been identified as important plant transcription factors (TFs) in regulating expression of many stress-inducible genes. These DREBs elements have been identified in many plant species as indicated in Table 2 in their response to cold stress (Lata & Prasad, 2011).

DREB TFs	Species	Accession no.	Stress response	References
DREB1A	*Arabidopsis thaliana*	AB007787	**Cold**	Liu *et al.*, 1998
DREB2C	*Arabidopsis thaliana*	At2g40340	Salt, Mannitol, **Cold**	Lee *et al.*, 2010
CBF1	*Arabidopsis thaliana*	U77378	**Cold**	Gilmour *et al.*, 1998
CBF2	*Arabidopsis thaliana*	AF074601	**Cold**	Gilmour *et al.*, 1998
CBF3	*Arabidopsis thaliana*	AF074602	**Cold**	Gilmour *et al.*, 1998
OsDREB1A	*Oryza sativa*	AF300970	**Cold**, Salt, Wounding	Dubouzet *et al.*, 2003
OsDREB1B	*Oryza sativa*	AF300972	**Cold**	Dubouzet *et al.*, 2003
OsDREB1C	*Oryza sativa*	AP001168	Drought, Salt, **Cold**, ABA, Wound	Dubouzet *et al.*, 2003
OsDREB2A	*Oryza sativa*	AF300971	Drought, Salt, faintly to **Cold**, ABA	Dubouzet *et al.*, 2003
OsDREB1F	*Oryza sativa*		Drought, Salt, **Cold**, ABA	Wang *et al.*, 2008
OsDREB2B	*Oryza sativa*		Heat, **Cold**	Matsukura *et al.*,2010
OsDREBL	*Oryza sativa*	AF494422	**Cold**	Chen *et al.*, 2003
TaDREB1	*Triticum aestivum*	AAL01124	**Cold**, Dehydration, ABA	Shen *et al.*, 2003
WCBF2	*Triticum aestivum*		**Cold**, Drought	Kume *et al.*, 2005
WDREB2	*Triticum aestivum*	BAD97369	Drought, Salt, **Cold**, ABA	Egawa *et al.*, 2006
HvDREB1	*Hordeum vulgare*	DQ012941	Drought, Salt, **Cold**	Xu *et al.*, 2009
ZmDREB2A	*Zea mays*	AB218832	Drought, Salt, **Cold**, Heat	Qin *et al.*, 2007
PgDREB2A	*Pennisetum glaucum*	AAV90624	Drought, Salt, **Cold**	Agarwal *et al.*, 2007
GmDREBa	*Glycine max*	AY542886	**Cold**, Drought, Salt	Li *et al.*, 2005
GmDREBb	*Glycine max*	AY296651	**Cold**, Drought, Salt	Li *et al.*, 2005
PpDBF1	*Physcomitrella patens*	ABA43697	Drought, Salt, **Cold**, ABA	Liu *et al.*, 2007
PNDREB1	*Arachis hypogea*	FM955398	Drought, **Cold**	Mei *et al.*, 2009
DvDREB2A	*Dendrathema*	EF633987	Drought, Heat, ABA, **Cold**	Liu *et al.*, 2008
DmDREBa	*Dendronthema3moriforlium*	EF490996	**Cold**, ABA	Yang *et al.*, 2009
DmDREBb	*Dendronthema3moriforlium*	EF487535	**Cold**, ABA	Yang *et al.*, 2009
PeDREB2	*Populus euphratica*	EF137176	Drought, Salt, **Cold**	Chen *et al.*, 2009

*Adapted from: Lata and Prasad (2011).

Table 2. Transcription factors identified as regulators in the expression abiotic stress including cold tolerance*

3.3 Limitations of classical breeding

Brassica species having characteristics such as sporophytic pollen self-incompatibility, male sterility along with restoration for cytoplasmic male sterility offered overwhelming possibilities for genetic modification. The production of haploids and doubled haploids using microspores has accelerated the production of homozygous lines in the *Brassica* species (Cardoza and Stewart, 2004). Somatic cell fusion has facilitated the development of interspecific and intergeneric hybrids in the sexually incompatible species of *Brassica*. Those characteristics have been further exploited by combining traditional non-molecular protocols with modern biotechnology including molecular markers in marker-assisted selection and breeding, and transformation technology to introduce desired genes into elite cultivars.

However, classical breeding which relies largely on homologous recombination between chromosomes to generate genetic diversity (Garcia *et al*., 1998), limits the extent of further improvement as it does not allow the precise understanding of genomic composition. The continuation of current technology in identifying selectable markers to aid in the assortment of segregating population is a major contribution that will extend the use of classical breeding. However, the use of plant transformation which allows the isolation and insertion of single genes into elite cultivars could accelerate crop improvement once the technology becomes routine.

4. Molecular breeding for crop improvement

4.1 Genomic approach

Functional genomics strategies to understand a plant's response to abiotic stress and to exploit this knowledge to improve crop yield, quality & quantity under adverse environmental conditions is a priority focus for northern temperate climates. Genomic approaches are likely to have particular value for *Brassica* crop improvement because they have the potential to identify transcriptional, biochemical, and genetic pathways that contribute to agronomic properties. Examples include revealing transcriptional pathways that are correlated with oil quality and disease resistance (e.g. specific resistance genes and downstream transcriptional pathways). The application of such knowledge to *Brassica* crop improvement program is likely to take the form of improved cultural practices and precise molecular breeding. Approaches such as marker-assisted selection and transgenesis will facilitate transfer of genes for desirable traits into elite or classic cultivars of *Brassica*, with the goal of improving agronomic performance while preserving traditional quality traits.

A complete genome sequence provides unlimited information in the sequenced organism as well as in the related taxa (Yang *et al.,* 2005). Korea *Brassica* Genome Project (KBGP) in conjunction with Multinational *Brassica* Genome Project (MBGP) have sequenced chromosome 1 (cytogenetically oriented chromosome #1) of *Brassica rapa*. They selected 48 seed BACs on chromosome 1 using EST genetic markers and FISH analyses. They also reported that the comparative genome analyses of the EST sequences and sequenced BAC clones from *Brassica* chromosome 1 revealed homeologous partner regions on the *Arabidopsis* genome and a syntenic comparative map between *Brassica* chromosome 1 and *Arabidopsis* chromosomes. In-depth sequence analyses of five homeologous BAC clones and an *Arabidopsis* chromosomal region revealed overall co-linearity, with 82% sequence similarity.

The data indicated that the *Brassica* genome has undergone triplication and subsequent gene losses after the divergence of *Arabidopsis* and *Brassica*.

Cheung *et al*. (2009) analyzed homoeologous regions of *Brassica* genomes at the sequence level. These represented segments of the *Brassica* A genome as found in *Brassica rapa* and *Brassica napus* and the corresponding segments of the *Brassica* C genome as found in *Brassica oleracea* and *B. napus*. Analysis of synonymous base substitution rates within modeled genes revealed a relatively broad range of times (0.12 to 1.37 million years ago) since the divergence of orthologous genome segments as represented in *B. napus* and the diploid species. Similar and consistent ranges were also identified for single nucleotide polymorphism and insertion-deletion variation. Genes conserved across the *Brassica* genomes and the homoeologous segments of the genome of *Arabidopsis thaliana* showed almost perfect collinearity. Numerous examples of apparent transduplication of gene fragments, as previously reported in *B. oleracea*, were also observed in *B. rapa* and *B. napus*, indicating that this phenomenon is widespread in *Brassica* species. They also concluded that the majority of the regions studied, the C genome segments were expanded in size relative to their A genome counterparts. Further, they observed considerable variation, even between the different versions of the same *Brassica* genome, for gene fragments and annotated putative genes suggesting that the concept of the pan-genome might be particularly appropriate when considering *Brassica* genomes. Thus characterization of complete *Brassica* genome and comparative genome analyses with *Arabidopsis* using genomics approach will increase the knowledge of the biological mechanisms of the *Brassica* species that will allow targeted approaches to reduce the number and impact, which could enable a sustainable and environmentally-sound, farming policy.

4.1.1 Molecular markers

Molecular markers have been used extensively to analyze genetic diversity and to create linkage maps in *Brassica* crops. Most importantly, they have been used widely to map agronomically important genes in *Brassica* genomes and to assist canola breeding and selection procedures. According to Snowdon and Friedt (2004), the major challenges that face *Brassica* geneticists and breeders are (i) the alignment of existing genetic and physical maps in order to agree on a consensus map with a standardized nomenclature, and (ii) to compile and integrate relevant phenological, morphological, and agronomic information with allelic information for *Brassica* oilseed germplasm. Therefore, association mapping can be used to exploit available genetic resources outside the narrow rapeseed gene pool. Molecular markers linked to agronomically important traits have been reported, and most of them are now integrated into oilseed breeding programmes. It is important to note that genome research and marker assisted applications in *Brassica* began to flourish in the late 1980s (Snowdon and Freidt, 2004), when the first restriction fragment length polymorphism (RFLP) linkage maps for *B. oleracea* (Slocum *et al.,* 1990), *B. rapa* (Song *et al*., 1991), and *B. napus* (Landry *et al.,* 1991) were developed. The development of PCR techniques over the last two decades has lead to the rise of new marker technologies, and this has enabled the generation of high-density molecular maps through the amplification of highly polymorphic anonymous PCR fragments. Some of the DNA-based marker systems that have been used in *Brassica* are random amplified polymorphic DNA markers (RAPD; Williams et al., 1990),

amplified fragment length polymorphisms (AFLP; Vos *et al.*, 1995), and inter-simple sequence repeats (ISSR; Zietkiewicz *et al.*, 1994).

PCR-based markers that meet the requirements and capacity of rapeseed breeders has emerged due to the ability to convert anonymous PCR markers that are closely linked to loci controlling traits of interest into sequence characterized amplified region (SCAR) or sequence tagged site (STS). Progress has also been made with the development of simple sequence repeat markers, also termed microsatellites (SSR; Grist *et al.*, 1993). These markers are highly polymorphic, robust, and relatively inexpensive. They are valuable to use for map alignment among different crosses simply because of their co-dominant nature. The number of publicly available *Brassica* SSR primers is increasing as a result of publicly funded international initiatives (www.brassica.info/ssr/SSRinfo.htm). Another important marker system is the single-nucleotide polymorphisms (SNPs), which results from single-base substitutions in the DNA sequence. These are the most abundant form of DNA polymorphism in most organisms, and they offer an opportunity to develop extremely fine genetic maps. This is because they can be used to uncover allelic variation directly within the expressed sequences, and to develop haplotypes based on gametic phase disequilibrium for analysis of quantitative traits.

The application of all these marker techniques towards developing maps for cold tolerance in *Brassica* crops will greatly improve the production of canola in temperate regions. Collaborative research with many research groups to improve stress tolerance in canola by utilizing these marker systems should be encouraged through funding. The results obtained through these collaborative studies can contribute to the sustainable oil and food production in canola.

4.1.2 Gene expression during cold stress in *Brassica* species

Exposure of cold-hardy species to low, non-freezing temperatures induces genetic, morphological and physiological changes in plants, which results in the development of cold hardiness and the acquisition of freezing tolerance (Savitch *et al.*, 2005). The ability of plants to acquire freezing tolerance from cold acclimation has been shown to involve the reprogramming of gene expression networks (Seki *et al.*, 2001, Fowler and Thomashow, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2002). Photosynthetic cold acclimation has been reported to be an essential component of the development of cold hardiness and freezing tolerance and requires the complex interaction of low temperature, light and chloroplast redox poise (Gray *et al.*, 1997, Wanner and Junttila, 1999).

The long-term cold acclimation has also been shown to be associated with morphological changes, such as compact dwarf morphology, increased leaf thickness caused by increased mesophyll cell size, increased specific leaf weight, marked decrease of leaf water content and an increase in cytoplasmic volume relative to vacuole volume (Stefanowska *et al.*, 1999; Strand *et al.*, 1999; Stefanowska *et al.*, 2002). In fact, it has been suggested that such structural changes might be necessary to account for the increase in stromal and cytosolic enzymes and metabolites in cold-acclimated leaves (Strand *et al.* 1999).

Several genes associated with cold hardiness have been studied in various crops. But in *Brassica napus,* limited report exists. Savitch *et al.* (2005) studied the effects of over expression

of two *Brassica* CBF/ DREB1-like transcription factors (BNCBF5 and 17) in *Brassica napus* cv. Westar. They reported that in addition to developing constitutive freezing tolerance and constitutively accumulating COR gene mRNAs, BNCBF5 and 17 over expressing plants also accumulate moderate transcript levels of genes involved in photosynthesis and chloroplast development as identified by microarray and Northern analyses. These include GLK1 and GLK2-like transcription factors involved in chloroplast photosynthetic development, chloroplast stroma cyclophilin ROC4 (AtCYP20–3), β-amylase and triose-P/Pi translocator. In parallel with these changes, increases in photosynthetic efficiency and capacity, pigment pool sizes, increased capacities of the Calvin cycle enzymes, and enzymes of starch and sucrose biosynthesis, as well as glycolysis and oxaloacetate/malate exchange were seen, suggesting that BNCBF over expression has partially mimicked cold-induced photosynthetic acclimation constitutively. Taken together, they suggested that BNCBF/DREB1 over expression in *Brassica* not only resulted in increased constitutive freezing tolerance but also partially regulated chloroplast development to increase photochemical efficiency and photosynthetic capacity.

The role of hsp90 in adaptation to cold temperature stress has also been studied. Characterization of the expression of hsp90 genes of *Brassica napus* using northern blot analysis and immunoblotting have shown that the hsp90 mRNA and protein are present in all *B. napus* tissues examined, albeit at different levels (Krishan *et al.,* 1995). High levels of hsp90 mRNA and protein were found in young and rapidly dividing tissues such as shoot apices and flower buds, suggesting that hsp90 may have an important role in plant growth and development. A significant increase in hsp90 mRNA levels was detected in seedlings exposed to 5°C. The transcript levels reached a maximum within 1 d of cold treatment and remained elevated for the entire duration of cold treatment. The levels of hsp90 mRNA rapidly decreased to the level found in control plants upon return to 20°C. The cold-induced accumulation of hsp90 mRNA closely resembles the expression of two previously identified cold-regulated genes of *B. napus*. Further, determining the cellular localization of the above genes and proteins during cold acclimation and identifying the proteins associated with them will provide more clues to the cellular basis of cold tolerance.

4.1.3 Microarray based monitoring of gene expression during cold stress

Genome wide transcription analysis in response to stresses is essential to providing the basis of effective engineering strategies to improve stress tolerance in *Brassica* crop plants (Lee et al., 2008). In order to perform transcriptome analysis in *Brassica rapa*, Lee et al. (2008) constructed a *B. rapa* oligo microarray, KBGP-24K, using sequence information from approximately 24,000 unigenes and analyzed cold (4 degrees C), salt (250 mM NaCl), and drought (air-dry) treated *B. rapa* plants. Among the *B. rapa* unigenes represented on the microarray, 417 (1.7%), 202 (0.8%), and 738 (3.1%) were identified as responsive genes that were differently expressed 5-fold or more at least once during a 48-h treatment with cold, salt, and drought, respectively. These results were confirmed by RT-PCR analysis. In the abiotic stress responsive genes identified, they found 56 transcription factor genes and 60 commonly responsive genes. The authors suggested that various transcriptional regulatory mechanisms and common signaling pathway are working together under the abiotic stresses in *B. rapa*. In conclusion, they reported that their new developed 24K oligo

microarray will be a useful tool for transcriptome profiling and this work will provide valuable insight in the response to abiotic stress in *B. rapa* and other *Brassica* species.

4.1.4 Micro RNA could modify regulator gene expression during cold in *Brassica* species

MicroRNAs (miRNAs) are short ribonucleic acid (RNA) molecules (on average only 22 nucleotides long) found in all eukaryotic cells except fungi, algae, and marine plants. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts (mRNAs), usually resulting in translational repression or target degradation and gene silencing. Several miRNAs have been identified that regulate complex process. Kutter et al. (2007) demonstrated that the density and development of stomatal complexes on the epidermis of Arabidopisis thaliana leaves depends on the microRNA-mediated regulation of Agamous-like16 (AGL16), which is a member of the MADS box protein family. AGL16 mRNA is targeted for sequence-specific degradation by miR824, a recently evolved microRNA conserved in the Brassicaceae and encoded at a single genetic locus. They reported that expression of a miR824-resistant AGL16 mRNA, but not the wild type AGL16 mRNA, in transgenic plants increased the incidence of stomata in higher order complexes. By contrast, reduced expression of AGL16 mRNA in the agl16-1 deficiency mutant and in transgenic lines over expressing miR824 decreased the incidence of stomata in higher order complexes. Non overlapping patters of AGL16 mRNA and miR824 localization led to the proposal that the miR824/ AGL16 pathway functions in the satellite meristemoid linage of stomatal development. Since *Brassica* and *Arabidopsis*, derived from same line and diverged around 12 to 20 million years ago, similar conserved miRNA families can be identified in *Brassica* species which help in plant development. Hence, microRNAs so identified will play an essential function in regulating gene expression both in mutlicellular plants and animals.

4.2 Proteomic approach

In the past few decades, considerable efforts have been directed at identifying cold-regulated protein in plant species. Various biochemical responses of plants to low, freezing temperatures have been widely documented. They involve changes in protein content and enzyme activities, metabolic modifications and changes in lipid composition and membrane structure. It is also recognized that *Brassica* species are of value for investigating important key areas for resistance, especially cold resistance, and the great advances have been made in terms of cold-induced genes and cytological mechanisms in the shape of cold resistance (Jiang *et al*., 1996; Diaz *et al*., 1997; Mieczyslaw, 1999; Rapacz, 2002). Cold acclimation is a complex adaptive process by which plants increase their tolerance to extracellular freezing. This process is induced by exposure of the plant to low but nonfreezing temperatures and is accompanied by a variety of biochemical and structural changes in plant cells (Thomashow, 2001). Proteins encoded by cold-regulated genes have an interesting feature in common; they are hydrophilic and remain soluble upon boiling (Gilmour *et al.,* 2000). It is postulated that proteins that display these distinctive properties may have roles in cryoprotection (Thomashow, 2001). Other cold-regulated gene products show similarities to antifreeze proteins or to drought-induced late-embryogenesis-abundant proteins (Gilmour *et al.,* 2000)

and responsive to ABA proteins which suggests a possible role in preventing ice formation and imparting tolerance to dehydration stress, respectively.

4.2.1 Protein expression during cold stress in *Brassica* species

B. napus, a member of the Cruciferae family, cold acclimates just like *Arabidopsis thaliana.* Jaglo *et al.* (2001) showed that *B. napus* has a cold-response pathway related to the CBF cold-response pathway of *Arabidopsis*. cDNA clones encoding two different CBF-like proteins were identified by screening *B. napus* cDNA libraries using PCR-generated probes. The *B. napus* CBF-like proteins were 92% identical in amino acid sequence to each other and approximately 76% identical in sequence to *Arabidopsis* CBF1. An alignment of the *B. napus* proteins with *Arabidopsis* CBF1 indicated that the sequence identity extended throughout the protein, but was greatest in the AP2/EREBP DNA-binding domain includes an alignment of one *B. napus* CBF protein against *Arabidopsis* CBF1). A sequence for a third *B. napus* CBF polypeptide has been deposited by others (accession no. AF084185; N. Zhou, G. Wu, Y.-P. Gao, R.W. Wilen, and L.V. Gusta). Transcripts encoding *B. napus* CBF-like proteins were found to accumulate rapidly (within 30 min) upon exposure of plants to low temperature. They reasoned that if *B. napus* had a similar CBF-like cold-response pathway, then expression of the *Arabidopsis CBF* genes in transgenic *B. napus* might also activate expression of *Bn115* and other cold-regulated genes containing the CRT/DRE-related regulatory elements and increase plant freezing tolerance. Constitutive expression of *Arabidopsis* CBF1, CBF2, and CBF3 in transgenic *B. napus* caused the accumulation of transcripts for *Bn115* and *Bn28* without a low temperature stimulus; *Bn28* encodes an ortholog of the CRT/DRE-regulated cold-responsive gene *COR6.6* (Hajela *et al.,* 1990). Electrolyte leakage experiments indicated that expression of the *Arabidopsis CBF* genes in *B. napus* resulted in an increase in freezing tolerance. The experiments presented above indicated that *B. napus* encodes a CBF cold-response pathway related to that found in *Arabidopsis*.

There is emerging evidence that certain novel hydrophilic and late embryogenesis abundant (LEA) polypeptides participate in the stabilization of membranes against freeze-induced injury. These hydrophilic and late embryogenesis abundant polypeptides are predicted to contain regions capable of forming amphipathic α-helices which are shown to have strong effect on intrinsic curvature of monolayers and their propensity to form hexagonal II phase. They are said to defer their formation at lower temperatures (Epand *et al.,* 1995). An additional hypothesis suggests that the extensive water binding capacity of these hydrophilic proteins might provide a protective environment in the proximity of membranes during freezing and result in membrane stabilization. In addition, there is evidence that protein denaturation occurs in plants at low temperature (Guy *et al.,* 1998) which could potentially result in cellular damage. In these cases, the enhancement of antioxidative mechanisms (Aroca *et al*., 2003), increased levels of sugars in the apoplastic space (Livingston and Henson, 1998), and the induction of genes encoding molecular chaperones (Guy *et al*., 1998), respectively, could have protective effects.

Recently (Chen *et al.,* 2011) studied a gene encoding novel cold-regulated protein with molecular mass of 25 KDa that was isolated from *B. napus* cDNA library using microarray analysis, and is consequently designated as BnCOR25. The data presented in this study revealed that BnCOR25 transcripts were significantly accumulated in roots after cold

treatment. Sumoylation/desumoylation of proteins has been shown to have a pivotal role in cold acclimation (Miura *et al*., 2007). Sumoylation is a post-translational protein modification where small ubiquitin-related modifier (SUMO) proteins are conjugated to protein substrates in a process dependent on SUMO E3 ligases, whereas desumoylation is the removal of SUMO proteins from their target proteins by SUMO proteases. It might protect target proteins from proteasomal degradation because sumoylation prevents ubiquitination (Ulrich, 2005). SIZ1, an *Arabidopsis* SUMO E3 ligase is shown to be required for the accumulation of SUMO conjugates during cold stress.

Transgenic attempts with many structural genes have also been made with a moderate degree of success. The overexpression of genes encoding LEA proteins can improve the stress tolerance of transgenic plants. Expression of the citrus gene encoding a LEA protein, CuCOR19 increased the cold tolerance of transgenic tobacco (Hara *et al.,* 2003). Likewise, the freezing tolerance of *Arabidopsis* was increased by the ectopic expression of the wheat gene WCS19 (Dong *et al.,* 2002), the *Arabidopsis* gene COR15A (Artus *et al.,* 1996), and the co-expression of the genes RAB18 and COR47, and XERO2 and ERD10 (Puhakainen *et al*., 2004). The freezing tolerance of strawberry leaves was enhanced by expression of the wheat dehydrin gene WCOR410 (Houde *et al.,* 2004). On the other hand, the expression of two cold-induced LEA proteins from spinach (Kaye *et al.,* 1998) and three desiccation-induced LEA proteins from *C. plantagineum* (Iturriaga *et al.,* 1992) in tobacco did not induce any significant changes in the freezing or drought tolerance of the respective transgenic plants. This may indicate either that not all LEA proteins make a significant contribution to plant stress tolerance, or that they need a particular background to function in, as suggested for transgenic strawberry plants (Houde *et al.,* 2004).

5. Using transgenic approaches to develop cold tolerance

Biotechnology offers new strategies that can be used to develop transgenic canola plants with improved tolerance to cold stress. Rapid advancement in recombinant DNA technology and development of precise and efficient gene transfer protocols have resulted in transformation and regeneration of transgenic lines in canola and other plant species (Wani *et al.,* 2008; Gosal *et al.,* 2009; Wani *et al.,* 2011). Genes that respond to freezing stress have been isolated and characterized, and studies suggest that they contribute to chilling tolerance and cold acclimation (Knight *et al*., 1999; Hsieh *et al*., 2002). Therefore, the transgenic approach should be pursued actively in canola breeding to improve cold tolerance. Efforts have been made to generate transgenic lines, which have shown improved tolerance to cold stress (Savitch, *et al.,* 2005).

Transgenic technology has the ability to improve cold stress in plants by introducing or down-regulating genes that regulate specific trait (Kumar, 2006). In the last few years, several efforts have been made to identify and characterize cold-responsive (*COR*) genes. Homologous components of the *Arabidopsis CBF* cold response pathway have also been found in many plants (Yamagachi-Shinozaki and Shinozaki, 2006). Some of these putative orthologs have been analyzed, and functionally tested. Most of the studies indicate that the expressions of *CBFs* and *CORs* in response to cold stress are similar in many plant species. Thus, they involve rapid cold induced expression of the *CBFs* followed by expression of *CBF*-targeted genes that increase freezing tolerance. It is important to note that as we aspire

to engineer cold stress in Canola, the contitutive expression of *Arabidopsis CBF* genes in other plants resulted in increasing freezing tolerance (Yamagachi-Shinozaki and Shinozaki, 2006). There are also other structural genes that have been used to engineer cold tolerance in some plants with amoderate degree of success. An example is tobacco that was engineered by over-expressing chloroplast glycerol-3-phosphate acyltransferase (*GPAT*) gene from squash and *Arabidopsis* (Murata *et al*., 1992). The transgenic tobacco showed enhanced cold tolerance and an increase in the number of unsaturated fatty acids present in the plant cell wall. In another study, Pennycooke *et al.,* (2003) down-regulated α-Gal (α –Galactosidase) in petunia, and this resulted in transgenic plants with an increased freezing tolerance. This suggested that transformation with α-Gal is another way in which freezing tolerance of plants can be genetically improved. The genes encoding *LEA* proteins can also improve tolerance to cold stress if they are overexpressed in other plants. Citrus gene encoding a *LEA* protein, *CuCOR19* was over-expressed in tobacco and an increased cold tolerance of transgenic tobacco was achieved (Hara *et al.,* 2003). The expression of the wheat dehydrin gene WCOR410, in strawberry leaves also enhanced freezing tolerance (Houde *et al*., 2004). In a separate study, Kim *et al*., (2007) engineered tobacco with ring zinc finger protein (RDCPt) from hot pepper and their results indicated that the expression of this gene improved cold tolerance in transgenic plants when compared to wild type. In another study Su *et al.,* (2010), determined that *MYBS3* was critical in cold adaptation in rice and it enhanced cold tolerance. Their report indicated that transgenic rice constitutively overexpressing *MYBS3* tolerated 4°C for at least 1 week and there were no interferences with the yield. All these studies demonstrate that the relationships among different pathways regulated by cold acclimation are complex. Therefore, it is important to understand the mechanism regulating cold-regulated genes in order to engineer cold tolerant canola. In developing transgenic canola, one can study the genes aforementioned with the aim of over-expressing or down-regulating them in Canola plants. The advent of molecular genetics and biotechnology offers a possibility to genetically engineer Canola to be more tolerant to cold. The technology has been modified to significantly improve breeding efficiency, thus resulting in rapid and accurate incorporation of cold tolerant genes into Canola plants.

5.1 ABA-independent gene regulation to cold stress

Environmental stresses induce the expression of many genes that can be classified into two groups. The first group corresponds to proteins involved in transduction pathways, such as transcription factors, whereas the second group includes effector proteins like the enzymes of osmolyte biosynthesis. Many studies have been focused on transcription factors involved in gene expression regulation. For each signal (salt, drought, and cold), several pathways can be distinguished depending on ABA dependent and ABA independent. ABA-independent expression of stress-responsive genes can occur through dehydration-responsive element (DRE)/C-repeat (CRT) cis-acting elements. The binding factors CBF/DREB1 (CRT-binding factor/DRE-binding factor 1) and DREB2 mediate gene expression in response to cold and drought/salinity, respectively). Interestingly, the CBF4 protein seems to mediate drought response unlike the other CBFs (Haake *et al*., 2002). A particular feature of CBF proteins is their early and transient cold induction, which precedes

the expression of cold-responsive genes. This requires the involvement of a constitutively expressed CBF-transcriptional inducer, which would be activated by cold treatment.

Four orthologues of the *Arabidopsis* CBF/DREB transcriptional activator genes were identified from the winter *Brassica napus*, cv. Jet neuf (Gao *et al.,* 2002). All four BNCBF clones encode a putative DRE/CRT (LTRE)-binding protein with an AP2 DNA-binding domain, a putative nuclear localization signal and a possible acidic activation domain. Deduced amino acid sequences suggested that BNCBFs 5, 7, and 16 are very similar to the *Arabidopsis* CBFI whereas BNCBF17 is different in that it contains two extra regions of 16 and 21 amino acids in the acidic domain. Transcripts hybridizing specifically to BNCBF17 and to one or more of the other BNCBFs accumulated in leaves within 30 min of cold exposure of the *Brassica* seedlings and preceded transcript accumulation of the cold-inducible BN28 gene, a *Brassica* orthologue of the cor6.6 or KIN gene from *Arabidopsis*. Cold-induced accumulation of BNCBF17 mRNA was rapid but was short-lived compared to transcripts hybridizing to BNCBF5/7/16. Transcripts hybridizing to one or more of BNCBF5/7/16 accumulated at low levels after the plants were subjected to prolonged exposure to salt stress. BNCBF17 was not responsive to salt stress. BNCBF transcript accumulation was similar in both spring and winter *Brassica* but the persistence of the transcripts in the cold were generally shorter in the spring than in the winter type. BNCBF5 and 17 proteins bind in vitro to the LTRE domains of the cold-inducible BN115 (cor15a orthologue) or BN28 promoters. Differential binding preferences, however, to LTREs between BNI 15 and BN28 were observed. Mutation of the core CCGAC sequence of the LTRE indicated that BNCBF17 had a lower sequence binding specificity than BNCBF5. Furthermore, experiments indicated that the LTREs were able to drive BNCBF5 and 17 trans-activation of the Lac-Z reporter gene in yeast. We conclude that the BNCBFs reported here could function as trans-acting factors in low-temperature responses in *Brassica*, controlling the expression of cold-induced genes through an ABA-independent pathway. Chen *et al.* (2011) indicated that BnCOR25 protein expression was up-regulated by cold, dehydration, and exogenous ABA treatment, suggesting this gene may be activated via ABA-dependent signal pathway. In this study, the data revealed that BnCOR25 was localized on cell periphery, at which the BnCOR25 protein may bind to cell membrane. In addition, BnCOR25 protein also displays a number of dehyrins' features. Thus, BnCOR25 protein may play a role in cold resistance in a similar manner. It can be concluded, however, that DRE/CRT element, Bn CBFs and Bn COR25 are capable of mediating the cold induced expression of genes in *Brassica napus* via an ABA-independent pathway.

6. Future research

The development of genetically engineered cold tolerant Canola plants by introducing or overexpressing selected genes is a viable option for producing an elite plant. It is also the only option if genes of interest originate from different species or distant relatives. However, the increasing availability of data related to large-scale genome homology between *Arabidopsis* and *Brassica* species means that canola is well-positioned to be among the first major crop species to benefit from continuous progress in plant biotechnology and molecular marker technologies. The sequencing of *B. napus* genome and the emergence of detailed physical maps are of great importance towards engineering cold tolerant canola. The availability of complete gene sequences for *Brassica* can enhance advances in detecting

polymorphisms for many agronomically important candidate genes for cold tolerance. It is also possible to elucidate the genetic control of cold tolerance through allele-trait association studies. This can be implemented by combining SNP haplotype data for *Brassica* candidate genes with pedigree and quantitative trait information. DNA-chip and high-throughput SNP genotyping technologies will accelerate our understanding of cold tolerance in canola through molecular genetics.

7. Conclusions

Cold is an environmental factor that adversely affects the productivity and oil quality and limits the geographical distribution and growing season of canola. Although significant progress has been made to elucidate the genetic mechanisms underlying cold tolerance in Canola, our current understanding is limited to single and shallow temperature stress. In cold regions such as Canada, canola plants are subjected to intense levels of cold stresses, and hence, the response of canola to a combination of different cold temperatures deserves more attention. Newly developed varieties offer an opportunity to test the effects of multiple cold temperatures, and to perform extensive field studies under diverse environments to assess their tolerance. Discoveries in cold stress response in model species such as *Arabidopsis thaliana* can be adapted to canola to identify candidate genes, a key element in molecular plant breeding in canola.

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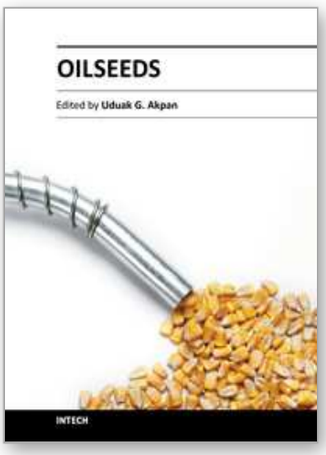

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