A MOLECULAR GENETIC STUDY OF WINTER HARDINESS IN CANOLA (*Brassica napus* L.)

By

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ABSTRACT

A MOLECULAR GENETIC STUDY OF WINTER HARDINESS IN CANOLA (*Brassica napus* L.)

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Cold acclimation increases freezing tolerance of plants and is involved in changes of gene expression. This study was conducted to determine how canola (*Brassica napus* L.) responds to cold acclimation to increase freezing tolerance both in controlled environments and under field conditions. The responses of two cold-regulated genes, BN28 and BN115, from *B. napus* to cold acclimation were also examined to see whether there was a correlation between the degree of gene expression, freezing tolerance, and eventually to winter survival both in controlled environment chambers and in the field.

Cold acclimation increased freezing tolerance of both winter and spring cultivars, and the difference in freezing tolerance among cultivars was due to their ability to acclimate. Freezing tolerance of cultivars was highly correlated with winter survival. Southern blot analysis showed no difference in copy number of the genes among cultivars tested. Though time-course increase in levels of

transcript in one of the genes was highly correlated with the time-course increase in freezing tolerance, no correlation existed at the cultivar level. However, this does not rule out the possible roles of these cold-regulated genes in freezing tolerance of canola.

Planting date is the most important factor which allows canola to withstand freezing stress. Other agronomic practices, such as application of plant growth regulators or nitrogen and phosphorus fertilization did not affect freezing tolerance and winter survival of canola.

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I remember a conversation between me and Dr. Lawrence O. Copeland once I had done something that he did not want. I asked him if he was angry. His answer was "Sometimes parents get angry with their children but that does not mean they do not love them." In an academic sense, Dr. Copeland was my father and I was his child. Thus, I would like to express my sincere gratitude and appreciation for his guidance, advice, encouragement, and financial support during my graduate program. Another nice person to whom my sincere thanks are extended is Dr. Mike Thomashow who offered me the opportunity to study molecular genetics of winter hardiness by providing me with precious laboratory space, equipment and expensive chemicals, as well as helpful guidance and insight to my research.

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LITERATURE REVIEW

INTRODUCTION

All oilseed rape belongs to the mustard family, Brassicaceae, and includes three species of the genus, *Brassica, B. napus, B. campestris, and B. juncea,* known as rape, turnip rape and leaf mustard, respectively (Downey et al., 1975; Downey, 1983; Shahidi, 1990). The name canola was coined by Canadians in 1979 to apply to rapeseed cultivars with low erucic acid and low glucosinolate. Such cultivars may be further described as 'double low' or 'double zero' rapeseed. Consequently, the term canola can be used to describe any rapeseed cultivars with low erucic acid content (2 % or less) in the oil and no more than 30 micromoles per gram of glucosinolate in the defatted meal (Sahhidi, 1968).

Canola production is affected by cold temperature in various ways. The cultivars of winter rapeseed and canola evaluated in the United States have not consistently survived the winter in the northern United States (Hang and Gilland 1982; Kephart et al. 1988; Auld et al. 1989; Smith et

al.,1989; Copeland et al.,1990). Only in areas in which the climate is moderated by proximity to large bodies of water has consistent winter survival of canola been attained (Mahler and Auld 1987, 1988, 1989). Canola production in most of the northern United States has relied on spring cultivars that have lower productivity than winter cultivars because of high summer temperatures and pest problems. Because of lack of cultivars with sufficient freezing tolerance, the safe area for winter *B. napus* production is limited to latitudes below 33°N, though cultivars adapted to latitudes above 33°N have been reported (Mahler and Auld 1987, 1988, 1989; Fribroug et al. 1989). However, in southern areas, winter cultivars are often not exposed to sufficient cool temperature to satisfy stringent vernalization requirements. This eventually delays flowering and results in heat stress during the seed filling stage.

FREEZING TOLERANCE, WINTER SURVIVAL, AND THE EXPRESSION OF COLD-REGULATED GENES

Winter Hardiness and Screening Methods

Winter hardiness of cereal grains and perennial forage crops is of great concern to agronomists in cold northern temperate regions of the world because winter injury severely limits their production area. Consequently, winter hardiness has been the subject of considerable investigation and the development of cultivars with improved winter hardiness is of primary concern. However, the introduction of new cultivars that possess increased winter hardiness has not always been successful (Grafius, 1974).

Winter hardiness implies avoidance of or tolerance to the cumulative effects of winter that a plant encounters, including freezing, heaving, smothering, desiccation, and disease infestation. Climate, soil, plant, and cultural practices interact to determine the degree of injury to a crop following the rigors of winter (Steponkus, 1978). Tolerance and avoidance mechanisms may reside at either the whole plant, tissue, or cellular level. Thus, the undertaking to increase winter hardiness is indeed an ambitious objective, and one that may be insurmountable if approached in its entirety.

A common approach to screen winter hardiness is to expose plants to the rigors of winter in a field situation, although such tests suffer from a lack of control over the severity and consistency of stress imposed on plants (Hill and Salmon, 1927). Consequently, Levitt (1980) indicated that winter conditions that result in differential survival occur only once every ten years. Thus, it is often necessary to grow materials for many years and in several locations to achieve the comparisons desired (Andrews, 1960).

Because of the limitations inherent in screening for winter hardiness under field conditions, there has been a continuous search for rapid and efficient methods for predicting freezing tolerance, and a number of tests have been developed. These usually involve controlled freezing followed by observation of plant recovery. However, this is often a long process that is destructive and requires a large number of plants (Bruce-Babel and Fowler, 1989). Plant tissue water content also has been used to screen for freezing tolerance in winter cereals. Water content of fully acclimated plants was correlated with field evaluation, but was not able to detect small difference between cultivars without excessive replication (Brule-Babel and Fowler, 1989). Recently, the method of choice for evaluating freezing tolerance is to measure the leakage of ions from plant cells after freezing stress by observing the loss in the integrity of cellular membranes (Palta et al., 1977; Steffen et al., 1989). This

method has been used to study freezing injury and recovery in onion bulbs (Arora and Palta, 1991), potato leaves (Palta and Li, 1980), alfalfa cultivars (Sulc et al., 1991), *Arabidopsis* (Gilmour et. al, 1988) and rapeseed (Teutonico et al., 1993).

Though some of the results from artificial freezing tests have been promising, ongoing breeding programs for winter hardiness still rely heavily on field evaluations, largely because breeders are not convinced about alternative methods.

Freezing Injury and Cold Acclimation

Of all the factors related to winter hardiness, direct freezing injury has been cited as the principal cause of winter killing (Quisenberry, 1938). At normal freezing rates under natural conditions, ice nucleation occurs initially outside the cell because the cell has a lower freezing point than the extracellular water. The resultant lowering of extracellular vapor pressure causes more water to leave the cell to form extracellular ice, resulting in protoplasmic dehydration and mechanical collapse of membranes (Levitt, 1980). During very rapid cooling, equilibrium freezing can not occur and ice nucleation occurs inside the cell. This intracellular freezing is usually lethal to the cold-acclimated cell, since the presence of intracellular ice disrupts the cellular membrane. However, freezing rates sufficiently rapid to cause intracellular freezing are not

commonly encountered in the natural environment (Singh and de la Roche, 1988).

Though the plasma membrane is considered to be the primary site of freezing injury (Siminovitch and Scarth 1938; Levitt and Siminovitch, 1940; Palta et al., 1977; Steponkus and Weiser, 1978; Singh 1979a), evidence that photosynthetic capacity can be significantly impaired after a freeze-thaw cycle has indicated that intercellular membranes may be at least as sensitive to extracellular freezing as the plasma membrane (Steffen and Palta, 1987).

In recent years, ultrastructural and biophysical alteration of the plasma membrane during lethal extracellular freezing has been elucidated. Irreversible conversion of the planar membrane bilayer to tightly appressed multilamellar vesicles has been observed in association with lethal extracellular freezing (Gordon and Steponkus, 1984; Pearce and Willison, 1985, Singh et. al, 1987). There is general agreement that under conditions of extracellular ice formation, decrease in temperature or the presence of ice crystals *per se* is not responsible for freezing damage (Heber and Santarius, 1973), and that the process of cellular dehydration is the most disruptive and injurious component of freezing injury.

The most dramatic effect of cold acclimation, although involved in a variety of physical and biochemical changes, is increased freezing tolerance after a period of exposure to low

nonfreezing temperature (Levitt, 1980; Sakai and Larcher, 1987). Biochemical changes include alteration in lipid composition, increased sugar and soluble protein content, and the appearance of new isozyme forms (Levitt, 1980; Sakai and Larcher, 1987; Steponkus and Lynch, 1989). However, it is uncertain whether these biochemical changes are associated with increased freezing tolerance, or whether they are manifestations of metabolic adjustments in response to cold temperature. Some may contribute to the overall fitness of the plant for low temperature survival, while others have specific roles in bringing about increased freezing tolerance. Indeed, it has been demonstrated that changes in membrane lipid composition are directly related to the freezing tolerance of plant cells (Steponkus et al., 1988; Steponkus and Lynch, 1989). In addition, there is evidence that proline and many simple sugars (Santarius, 1973; Strauss and Hauser, 1986; Carpenter and Crowe, 1988), as well as certain soluble polypeptides from spinach (Volger and Heber, 1975) have cryoprotective effects *in vitro*, though whether these molecules contribute significantly to freezing tolerance *in vivo* remains to be determined.

There are several reports of the effect of cold acclimation on the physiology of *Brassica* plants, but these studies do not indicate whether any of these changes are required for freezing tolerance. For example, exposure of *B. napus* to low temperature induced accumulation of reducing

sugars in leaves (Krause et al., 1982), altered the phospholipase-D (Sikorska and Kacperska, 1982), induced seed pigment synthesis (Johnson-Flanagan et al., 1991), inhibited seed photosynthesis (Hodgins et al., 1989), and altered seed chlorophylase and peroxidase activities (Johnson-Flanagan, 1989).

The cold acclimation of a given species is dependent on two factors; genetic capacity of the species to acclimate in order to withstand freezing temperature, and the conditioning or expression of this heritable capacity. Cold acclimation may be influenced by radiation, temperature, photoperiod, precipitation, and developmental stage of plants, with different optimum conditions for different species and cultivars (Steponkus, 1978). Of all the environmental factors influencing cold acclimation, temperature is most important (Olien, 1967; Paulsen, 1968). Low, nonfreezing temperatures are conducive to an increase in hardiness in the fall, and warm temperatures are responsible for its decrease in the spring. Generally, it is thought that most plants will acclimate as temperatures are gradually lowered below 10°C (Alden and Hermann, 1971). However, during acclimation the progressive decline in temperature from relatively high temperatures in early fall, followed by low, nonfreezing temperature in late fall and early winter, followed by freezing temperature in winter is extremely important in the acclimation process. The importance of the progression of

temperature can be supported by the studies of Pomeroy and Andrews (1976). They found that high levels of hardiness can be induced within only 4-6 days in wheat if the hardening temperatures are preceded by warm temperatures, whereas 6-8 weeks at 2-4°C were required for maximum hardiness without preceding warm temperatures (Andrews et al., 1974).

Molecular Genetics of Cold Acclimation

Although the physiological and biochemical changes that occur during cold acclimation could be brought about by preexisting structural and enzymatic changes, it is also possible, as first proposed by Weiser (1970), that cold acclimation involves changes in gene expression. Direct evidence was obtained recently to show that low temperature induced accumulation of specific mRNAs. The appearance of novel transcripts during cold acclimation has been observed in alfalfa (Mohapatra et al., 1989), wheat (Lin et al., 1990; Houde et al., 1991), barley (Cativelli and Bartels, 1990; Dunn et al., 1990), *Arabidopsis* (Hajela et al., 1990; Kurkela and Frank, 1990; Nordin et al., 1991; Gilmour et al., 1992), rice (Binh and Oono, 1992), and *Brassica* (Orr et al., 1992; Weretilnyk et al., 1993).

DNA sequences corresponding to these cold-regulated (*cor*) transcripts have also been isolated and characterized by differential screening of cDNA libraries constructed from these species. These mRNAs appear rapidly upon exposure of

the plants to low temperatures, and deduced amino acid sequences of the products of the some of these cold-regulated genes have been identified. A number of cold-induced transcripts hybridize to the transcripts of *rab* (responsive to ABA) genes (Hahn and Walbot, 1989) or encode polypeptides containing amino acid sequence motifs found in *rab* motifs (Skriver and Mundy, 1990; Gilmour et al., 1992; Houde et al., 1992), suggesting that they may play a role in the tolerance to the cellular desiccation stress that accompanies extracellular freezing. Other cold-induced transcripts encode polypeptides containing stretches rich in alanine and glycine (Kurkela and Franck, 1990; Orr et al., 1992), suggesting that they may play an antinucleating role, although their functions have not been elucidated. The product of one cold-regulated gene has been reported to show cryoprotective properties *in vitro* (Lin and Thomashow, 1992).

In *B. napus*, two cold-regulated cDNA clones were isolated and characterized (Orr et al. 1992; Weretilnyk, 1993). BN28 is homologous to *cor*6.6 of *Arabidopsis* (Gilmour et al., 1992) and encodes abundant mRNA of 0.45 kilobases, induced by the exposure of the whole plant to 2°C, with the transcript appearing within 6 hours of exposure (Orr et al., 1992). The transcript disappears within 20 hours of the return of plant to room temperature. ABA also induced the expression of this gene but heat shock did not. The product of *cor*6.6 from *Arabidopsis* is a 6.6 kDa polypeptide (Gilmour

et al., 1992) and has amino acid homology with KIN1 of *Arabidopsis*, which again shares a sequence similarity with antifreeze protein B from winter flounder. The functions of the products of BN28, *cor6.6* and *kin1* are not yet known. However, based on regions of deduced amino acid homology to flounder antifreeze protein, these genes may possess antinucleation properties (Kurkela and Franck, 1990).

BN115 is another cold-regulated gene isolated from *B. napus* (Weretinyk et al., 1993) which has sequence homology with *cor*15 of *Arabidopsis* (Lin and Thomashow, 1992). This gene encodes abundant mRNA of 0.8 kilobases induced by cold temperature, with the transcript appearing within 1 day of exposure. The transcript disappears within 20 hours of the return of the plant to room temperature. In contrast to BN28, ABA did not induce the expression of this gene, but dehydration leads to a slight increase in transcript at room temperature. Northern blot analysis showed that levels of transcript increased within 24 hours of exposure to low temperature, peaked after 3 days, and remain at elevated level for the duration of the cold treatment. BN115 reveals a single open reading frame that potentially encodes a protein of 14.8 kDa which is matched to that of COR15 (14.7 kDa) of *Arabidopsis* (Lin and Thomashow, 1992). Immunological studies have indicated that this COR15 is processed in plants to a polypeptide of about 9 kDa, and that this polypeptide is located in the soluble fraction of the chloroplast (Thomashow

et al., 1993). The amino terminal sequence of COR15 closely resembles transit peptide target protein to the stromal compartment of the chloroplast. A search of GenBank and EMBL database did not reveal any proteins with significant similarity to COR15.

Mohapatra et al. (1989) have addressed the question of whether cold-regulated gene expression correlates with the degree of freezing tolerance attained during cold acclimation in alfalfa. Three cold-regulated cDNA clones were examined in 4 alfalfa species with different freezing tolerances. The correlation coefficient between the levels of expression of one of the three genes and the degree of freezing tolerance attained by the cultivars was 0.99(***). The other two genes also had a very high positive correlation, while no correlation existed between the levels of freezing tolerance of the cultivars and either the expression of the control genes in the acclimated or nonacclimated plants. Likewise, Houde et al. (1992) found a positive correlation between the level of expression of the wheat cold-regulated gene *Wcs120* and freezing tolerance. Thus, linkage between expression of at least some of the cold-regulated genes and the levels of freezing tolerance implies that the cloned cold-regulated genes might be used to aid in breeding for increased freezing tolerance of crop plants (Thomashow et al., 1990). However, two cases exist in barley in which the levels of *cor* gene expression were found not to correlate with freezing

tolerance; Levels of neither *BLT4* (Dunn et al., 1991) nor *HVA1* (Sutton et al., 1992) were correlated with freezing tolerance.

EFFECT OF PLANTING DATE ON FREEZING TOLERANCE, WINTER SURVIVAL, AND EXPRESSION OF COLD-REGULATED GENES UNDER FIELD CONDITIONS

Effect of Planting Date on Crop Growth and Agronomic Performance, Including Winter Survival.

Planting date is of critical importance to the production of canola and directly impacts subsequent seed yield, winter survival, insect damage, seed oil quality and disease severity (Hang and Gilland, 1982; Auld et al., 1983; Murray et al., 1984; Kephalt et al., 1988; Fribourg et al., 1989; Raymond et al., 1990; Thomas et al., 1990). The optimum planting date differs across production zones. Good cold tolerance in winter cultivars requires that the seedling has six to eight leaves and good crown development prior to onset of severe winter condition (Karow and Pumphrey, 1986; Auld et al., 1989). Winter canola is usually seeded approximately six weeks before the first anticipated hard frost. Canola emerges best when soil temperature is above 10°C (Kephart et al., 1988). In northern parts of the United States, most winter canola is seeded in late summer, following the harvest of an early-maturing cereal crop.

In general, the earlier canola is planted, the better it performs. Seeding in early rather than late August generally

improves winter survival and seed yield wherever winter canola is grown (Noderstagaard, 1972; Huber, 1973; Kondratowicz and Paprocki, 1974; Daniel et al., 1980). There are, however, many exceptions to this rule. Late planting in winter canola is often unavoidable because of double cropping, soil water availability, and other environmental factors (Thomas et al., 1990). Planting too early creates pest and weed problems in winter canola while planting too late may result in very small plants which are more vulnerable to freezing damage (Thomas, 1984).

A significant cultivar x planting date interaction for both seed yield and winter survival has been reported in canola in which seed yield from late plantings was closely associated with winter survival during years in which the plants were exposed to a minimum temperature of -30 °C (Auld et al., 1983).

Several studies have shown that delayed planting affects not only the yield of rapeseed, but also the quality of oil and protein produced (Kondra, 1977; Degenhardt and Kondra, 1981; Christensen, 1984). Delay in planting date reduces the oil content (Scott et al., 1974; Kondra, 1977) and increases protein content of the meal (DePauw et al., 1978). These changes are probably due to delayed late spring growth of plants planted in late fall and subsequent exposure of the plant to increased temperatures during oil formation (Canvin et al., 1965). However, another report indicated that oil

content and plant growth were not sensitive to planting date (Auld et al., 1983).

Development and Maintenance of Freezing Tolerance in the Field

The development of freezing tolerance in plants during the fall (acclimation) and its loss during spring (deacclimation) roughly follows a cyclical pattern (Smith and Nelson, 1985). In studies by Ruelke and Smith (1956) plants began to develop freezing tolerance in early to mid-September as measured by electrical conductance. The development of tolerance continued through autumn until late November, and reached a maximum shortly after permanent freezing of the soil surface after which weekly air temperature remained below freezing. A high level of freezing tolerance was maintained from early December to mid-February, when snow cover provided protection from freezing. Tolerance began to decrease in mid-February with the onset of warmer temperatures and reduced snow cover. Thereafter, it began to drop rapidly after the snow had disappeared and the soil surface thawed in late March (Suneson and Peltier, 1938; Ruelke and Smith, 1956). Winter kill usually occurs during late winter and early spring, when the snow cover has disappeared and plants are exposed to extreme temperature fluctuations above and below freezing. By this time the plants have lost some freezing tolerance in response to warmer temperature and may be unable to reharden satisfactorily, or temperature drop is so rapid that they do

not have time to reharden (Smith and Nelson, 1985).

Environmental Factors Affecting Cold Acclimation of Plants

Cold hardiness of plants is known to be favored by (1) short day length, (2) low temperature, (3) alternation of temperature between warm (20°C) during the day and cold (0-5°C) during night, and (4) adequate radiation for good photosynthetic activity (Tysdal, 1933; Shin et al., 1967). Temperature appeared to be of primary importance, but metabolic processes associated with cold hardiness were also influenced by photoperiod.

Low above-freezing temperatures are conducive to acclimation in the fall, and warm temperatures for deacclimation in spring. Generally, most plants appear to acclimate as temperatures are gradually lowered below 10°C (Alden and Hermann, 1971). Although there appears to be a certain optimal temperature for cold acclimation for a given species (Hodgson, 1964; Olin, 1967), the progressive decline from relatively high temperature in early fall, to low above-freezing temperatures in late fall and early winter, followed by freezing temperature in winter is extremely important in cold acclimation (Steponkus, 1978). Each stage has a distinct role in the overall process of acclimation in respect to succeeding stages and the influence of other environmental factors (Weiser, 1978). The importance of the progression of temperatures is supported by reports by Pomeroy and Andrews (1976). They found that high levels of hardiness could be

induced in only 4-6 days in wheat when hardening temperatures were preceded by warm temperatures, whereas 6-8 weeks at hardening temperature were required for maximum hardiness (Andrews et al., 1974).

Two controversial points have been discussed by many researchers on the role of light in cold acclimation. Dexter (1933a, b) concluded that light only served to provide photosynthate, and in plants such as alfalfa, which have large reserves, cold hardiness could be increased in the dark by cold treatment alone. The suggestion that light *per se* is not a requirement for the acclimation process is supported by reports that the light requirement can be replaced by incubation in sugar solution (Tumanov and Trunova,1963; Steponkus and Lanphear,1967). A second area of apparent conflict in the literature in regard to the role of light in acclimation relates to its function in a photoperiodic role in addition to its photosynthetic role. Short photoperiod generally stimulates acclimation, partly because of the annual decline in photoperiod that accompanies natural acclimation, but also because of reports that some species fail to acclimate under long photoperiods (Hodgson and Bulba, 1956). However, other reports (Turnova, 1965; Paulsen, 1968) indicate that short photoperiods are not required for acclimation of cereal grains, but are required for acclimation of perennial such as alfalfa.

Stress-Induced Gene Expression under Field Condition

Although many studies have demonstrated the expression of stress-induced genes under laboratory conditions (Mundy and Chua, 1988; Close et. al, 1989; Bartel et al., 1990, Hajela et al., 1990; Kukela and Frank, 1990; Skriver and Mundy, 1990; Bray, 1991; Pla et al., 1991; Lang and Palba, 1992), few investigators have examined the expression of such genes under field conditions. Kimpel and Key (1985) showed that mRNA encoding heat shock proteins (HSP) accumulated in leaf tissues of soybean experiencing high temperature in the field. Similar studies were conducted with other crop species by Burke et al. (1985). Both field experiments and growth chamber studies using conditions designed to simulate a day of high-temperature stress showed evidence that HSPs are expressed under natural conditions (Chen et al., 1990; DeRocher et al., 1991). Lorraine et al.(1993) examined the expression of HSPs at the whole plant level in alfalfa under field conditions. The fact that some of the HSPs are expressed in the seed and flowering parts in the absence of heat shock suggests a more widespread occurrence of HSPs in optimal growth environments and their potential role in plant development.

Expression of cold-regulated genes like *cor* genes from *Arabidopsis* (Hajela et al., 1990; Kurkela and Frank, 1990; Nordin et al., 1991, Gilmour et al., 1992) and BN genes from *Brassica napus* (Orr et al.,1992; Weretilnyk, 1993) have not been examined under field conditions.

PLANT GROWTH REGULATORS AND WINTER HARDINESS

Synthetic Plant Growth Regulators and Winter Hardiness

The main plant growth regulators (PGRs) that have increased winter hardiness are CCC (chloromequat chloride, an antigibberellin) and ethephon (2-chloroethyl phosphonic acid, an ethylene-producer). Foliar sprays of CCC have been reported to increase the freezing tolerance of winter wheat (Gusta et al., 1982), potato (Chen and Li, 1976), winter rape (Marth, 1965), and alfalfa (Paquin et al., 1976). Ethephon increased freezing tolerance of sweet cherry (Proebsting and Mills, 1976), apple (Raese, 1977), apple and pear (Ketchie and Murren, 1976), and peach (Durner and Gianfagna, 1988).

However, new PGRs, which are mainly antigibberellins, appear promising. Mefluidide and triazoles (triadimefon, tridimenol, paclobutrazol, uniconazol, and tetcyclacis) have been shown to increase tolerance to chilling (Whitaker and Wang, 1987), heat (Pennypacker et al., 1982), drought (Fletcher and Nath, 1984), and freezing (Paquin et al., 1976; Fletcher and Hofstra, 1985), as well as to cause a transient increase in endogenous ABA concentration in treated plants (Wilkinson, 1982; Asare-Boaham et al., 1986). Triazole compounds are thought to inhibit the biosynthetic pathway of

sterols and GAs (Grossmann, 1990).

Exogenous application of ABA to whole plants, parts of plants or plant protoplast have increased frost tolerance in the absence of cold treatment (Irving, 1969; Rikin et al., 1975; Waldman et al., 1975; Chen et al., 1979; Orr et al., 1986; Reaney and Gusta, 1987; Lang et al., 1989; Mohapatra et al., 1989). A number of researchers have also demonstrated that exposure to low temperature increased ABA concentration in plants. There are two principal explanations for the increase of endogenous ABA during cold acclimation. First, low temperatures *per se* could cause an increase in ABA level (Daie and Campbell, 1981). Second, the accumulation of ABA could be the result of induced water stress in plant (Eze et al., 1983). The increase in endogenous ABA level following low temperature exposure suggests a certain role for ABA in cold hardiness. However, this conflicts with results obtained on the effect of exogenous ABA on cold acclimation. Gusta et al. (1988) found that ABA inhibited growth but had little or no effect on enhanced freezing resistance of winter wheat crowns if applied as a foliar spray or added to the nutrient solution.

Some of the early interest in the use of PGRs on oilseed rape occurred as a result of experiments in Poland (Kacperska-Palacz et al., 1969; Kacperska-Palacz and Wcislinska, 1972; Kacperska-Palacz and Egierszdorff, 1972) which suggested that CCC improved cold hardiness of rape in controlled environment

chambers. The role of ABA in cold acclimation was studied in cell suspension cultures of *Brassica napus* (Orr et al., 1986). ABA could promote freezing tolerance in the absence of low temperatures. Recently, the effects of several plant growth regulators on winter hardiness of canola under field conditions were studied in Canada (Morrison and Andrews, 1993). Some of the triazole PGRs reduced plant size by limiting cell expansion and increasing cell numbers. While cold hardiness and freezing tolerance were increased by certain growth regulators, these effects were not consistent over time and did not result in increased winter survival. This suggests that natural cold hardening may have eclipsed the PGR-induced hardening.

Chemistry and Functions of Ethephon and Mepiquat Chloride, and Their Possible Roles in Winter Hardiness

Terpal-C is a mixture of mepiquat chloride and ethephon, while Cerone contains ethephon. Mepiquat chloride belongs to a group of plant growth retardants known to inhibit GA biosynthesis (Dennis et al., 1965; Lang, 1970). This group of compounds includes AMO-1618, CCC (chlormequat chloride), Phosphon D (chloroponium), and ancymydol, as well as mepiquat chloride. Of all the compounds in this group, only CCC and mepiquat chloride have found large-scale application in agriculture.

Ethephon belongs to the group of compounds which, when applied to plant materials, release ethylene as they

decompose. This group includes ethephon (2-chloroethyl phosphonic acid), silaid (2-chloroethyl methylbis phenylmethoxy silane), alsol (2-chloroethyl tris 2-methoxyethoxy silane) and ACC (1-aminocyclopropane-1-carboxylic acid). Though ethephon has many effects on plant growth and development, the stunting of ethylene-treated plants is now the basis of one of its most important uses, that is, the prevention of lodging in small grains and other crops (Reid, 1985). Ethephon not only reduces stem length, but also stiffens the straw. Ethylene is also known to be released when the plant is exposed to stress, e.g., the stresses resulting from wounding and pathogen attack. Low temperature stress can be reduced by ethephon treatment in some fruit trees by delaying the bloom in the spring and increasing intrinsic winter hardiness of the buds (Durner and Gianfagna, 1988).

The possible mechanisms of these PGRs in winter hardiness have been illustrated by several studies in winter cereals: 1) Some PGRs prevent the dehardening of plants in mid-winter. The ultimate level of freezing tolerance in November was not increased by application of the chemicals. However, they did reduce the loss of freezing tolerance in mid-winter, which is considered to be the most critical period (Gusta et al., 1990). 2) Certain PGRs promote regrowth of roots in the spring, even if most of the root system is winter-killed (Robertson et al., 1992). 3) Some PGRs reduce

the elongation of the subcrown internodes, thereby avoiding exposure to freezing air temperatures (Anderson et al., 1985). 4) Some PGRs stimulate photosynthesis, resulting in increased starch accumulation and enhanced winter hardiness (Astakhova et al., 1988). 5) Certain PGRs inhibit degradation of endogenous ABA, and thus, indirectly affect cold hardiness (Gusta et al., 1990).

All of these effects are inconsistent from year to year (Robertson et al., 1992). The reason for this inconsistency is not known, but probable reasons are lack of uptake, improper growth stage when applied, temperature at the time of application, and severity of winter. A period of relatively warm temperatures is generally required following application to allow the PGRs to result in the desired chemical change.

THE GOALS OF THE THESIS

The available data suggest that the changes in gene expression that occur during cold acclimation may contribute to the increased freezing tolerance of cold acclimated plants. The correlation between the levels of expression of the cold-regulated genes and the degree of freezing tolerance among cultivars or species has been examined in several plant species with several different cold-regulated genes. However, the results are controversial, depending on the plant species and the genes examined.

The primary goal of this research was to examine expression of *cor* genes in a variety of cultivars that differ in freezing tolerance, under both laboratory and field conditions. A relationship between *cor* gene expression and freezing tolerance and, eventually, winter hardiness would provide us not only clues as to the functions of the genes, but also molecular markers for selecting winter hardiness.

In addition to these genetic studies, effects of several agronomic practices such as planting date, seeding rate, nitrogen and phosphorus, and application of chemicals on winter hardiness of canola were also studied.

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CHAPTER I

CORRELATIONS BETWEEN WINTER SURVIVAL, FREEZING TOLERANCE, AND THE EXPRESSION OF COLD-REGULATED GENES, BN28 AND BN115, IN CANOLA (*Brassica napus* L.)

ABSTRACT

Of the many factors influencing winter survival of plants, freezing tolerance is one of the most important. Cold acclimation increases freezing tolerance and is involved in changes of gene expression. This study was conducted to determine the relationship between *in vitro* freezing tolerance and winter survival of canola (*Brassica napus* L.) and to determine the responses of two cold-regulated genes from *B. napus*, BN28 and BN115, to cold acclimation. Six winter cultivars and one spring cultivar that had previously exhibited a broad range of winter survival were examined both in a controlled environment chamber and under field conditions.

Freezing tests showed that cold acclimation increased the freezing tolerance of both winter and spring cultivars, and that the differences in freezing tolerance among cultivars

were due to their ability to acclimate. Freezing tolerance measured by freezing tests was highly correlated with winter survival, suggesting that this test can be used to predict the comparative winter survival of canola cultivars.

Southern blot analysis revealed that the seven cultivars examined had the same copy number of BN28 and BN115. Northern blot analysis indicated that these genes were induced within 1 day by cold temperature and turned off immediately by deacclimation. Though time-course increase in transcript in one of the genes was highly correlated with the time-course increase of freezing tolerance, no correlation was found at the cultivar level. However, this does not rule out the possible role of cold-regulated genes in freezing tolerance of canola.

INTRODUCTION

In most winter crop producing areas, freezing temperature is the single most important factor limiting winter survival in the field (Steponkus, 1978). Freezing tolerance is the ability of a plant to survive subfreezing temperatures, and constitutes a major aspect of winter survival. Screening for differences in freezing tolerance among cultivars or breeding lines has been of great concern to plant breeders for many years.

Field survival is the usual method of evaluating winter hardiness. However, such results are often inconclusive because of variability in weather conditions from year to year (Levitt, 1980). Because of these yearly variations, trials must be conducted for a number of years, and are therefore extremely time-consuming (Andrew, 1960). The development of rapid techniques for screening plants for high acclimation potential has led to the development of indirect screening methods (Steponkus, 1978). These methods have arisen largely as a result of empirical correlations between some physiological or anatomical characteristics of plants. One of the most common methods for determining freezing tolerance is the measurement of leakage of ions from plant cells after

freezing stress (Palta et al., 1977; Palta and Li, 1980; Stephen et al., 1989; Sulc et al., 1991; Arora and Palta, 1991). The advantage of this method is that it is simple, repeatable, rapid, and imposes a realistic freeze-thaw stress on intact tissue (Teutonico et al., 1993).

Some genetic and phenotypic markers which segregate for winter hardiness also have been used for screening. Prior exposure of plants to low nonfreezing temperatures (cold acclimation) increases freezing tolerance. Many physiological changes are known to occur in plants during cold acclimation (Levitt, 1980; Sakai and Larch, 1987; Steponkus and Lynch, 1989), leading Weiser (1970) to suggest that they are mediated by altered gene expression. Using recent molecular biology techniques, several genes induced by cold temperature were isolated and studied for their possible role in cold acclimation (Mohapatra et al., 1989; Lin et al., 1990; Cativelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Dunn et al., 1990; Houde et al., 1991; Nordin et al., 1991; Gilmour et al., 1992; Orr et al., 1992; Bihn et al., 1992; Weretilnyk et al., 1993). In addition to functional studies of these genes, the link between the expression of cold-regulated genes and freezing tolerance can be used to aid in the breeding for winter hardiness, provided that the expression of those molecular markers is highly correlated with winter hardiness and/or freezing tolerance (Mohapatra et al., 1989).

The objectives of this study were to

1. Study the correlation between freezing tolerance and winter survival and evaluate the reliability of *in vitro* freezing tests for predicting winter survival of canola (*B. napus*).
2. Study the expression of two cold-regulated genes from *B. napus*, BN28 and BN115, during cold acclimation and deacclimation.
3. Determine whether the expression of cold-regulated genes at the transcript level is correlated with freezing tolerance, and eventually with winter survival in the field.

MATERIALS AND METHODS

Six winter cultivars, CDH3, WRG86, Duobul, Ceres, KWC4113, Accord, and one spring cultivar, Pactol, were used for freezing tests while only winter cultivar was used for field experiments.

Evaluation of Winter Survival

Seeds of the six winter cultivars were planted at the rate of 5.6 kg/ha in a completely randomized block design with 3 replications on the Michigan State University Agronomy Farm at East Lansing. Each replication consisted of a five-row plot 6 m long and 0.92 m wide. Seeds were planted Sept. 7, 1992, and Sept. 6, 1993. The number of live plants in each subplot (3 row x 1 m long each) was counted in the fall of each year (Oct. 10, 1992, and Oct. 15, 1993) and again in the spring of the next year (Apr. 15, 1993, and Apr. 15, 1994). Percent survival was recorded as (no. of plants per plot in spring / no. of plants per plot in fall) X 100.

Plant Materials, Growth and Cold Treatment for Freezing Tests

Plants were grown in plastic pots in a 1:1:1 mixture of perlite:peat moss:fine vermiculite moistened with a nutrient

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solution as described by Sommerville and Ogren (1982). Plants were maintained in a controlled environment chamber at 22 to 24°C under constant illumination from cool white fluororescent lights. Plants were irrigated with distilled water every 2 days. Twenty-day-old seedlings were moved to the 2°C cold room with constant fluorescent light for cold acclimation (Gilmour et al., 1988). Time-sequence experiments of freezing tolerance were done with the nonacclimated control plants and with plants acclimated for 1, 5, and 10 days. For the deacclimation experiment, 10-days. For deacclimation experiment, plants acclimated for 10 days were transferred to room temperature and deacclimated for 1 and 3 days before the freezing tests.

For RNA extraction from plants at each acclimation stage, leaves and stems were excised at the soil level, frozen in liquid N_2, pulverized using a mortar and pestle, and stored at -80°C prior to extraction. Genomic DNA was isolated from control plants for southern blot analysis. Freezing tests and RNA extraction were performed at three different times as three replications.

Tests for Freezing Tolerance

Freezing tolerance of leaves was determined by the method of Sukumaran and Weiser (1972). Samples of three excised leaves were placed in a stoppered culture tube maintained in a low temperature bath (Masterline Model 2095,

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Forma Scientific) at 2°C. Freezing was initiated by the addition of ice chips to each tube. After a 2 hour equilibration period, the bath temperature was lowered manually in 1°C increments every 30 min. Samples were withdrawn at 1 hour intervals until the temperature reached -18°C, and placed on ice for several hours, then removed and thawed overnight in a cold room at 2°C. Freezing damage was estimated by the electrolyte leakage test. Five ml of distilled water were added to each tube and the samples were shaken gently for 3 hours. Conductivity of the resulting solution was measured using a conductance meter (YSI model 35). A value for 100 % leakage was obtained by freezing each sample at -80°C overnight, then reextracted in the original solution. A plot of temperature versus percent electrolyte leakage was used to determine the value for 50 % electrolyte leakage, which was defined as LT_{50}.

Genomic DNA and Total RNA Isolation

Total RNA was isolated by the method of Gilmour et al. (1988). Frozen pulverized plant materials were extracted in a buffer containing 100 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM EGTA, 1%(w/v) SDS, 10 mM DTT, 6 % (w/v) p-aminosalicylic acid (sodium salt), and 1 % (w/v) tri-isopropyl naphthalene sulfonic acid with an equal volume of buffer-saturated phenol: chloroform: isoamyl alcohol (25:24:1, PCI). After centrifugation to separate the phases, the aqueous phase was

extracted once more with PCI, and the nucleic acids were ethanol-precipitated twice on ice with 2M LiCl, followed by a final ethanol precipitation. The pellet was redissolved in distilled water and stored at -80°C. Total DNA was prepared from the supernatant left after LiCl precipitation of RNA from total nucleic acids. The supernatant was diluted to 0.5 M LiCl with distilled water and the DNA was precipitated with ethanol. The DNA was then resuspended in a buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

Preparation of DNA Probes

Two cold-regulated cDNA clones, BN28 and BN115, were obtained from Dr. Jas Singh, Plant Research Center, Agriculture Canada, Ottawa, Ontario. Plasmid DNA was isolated by alkaline lysis, followed by polyethyleneglycol (PEG) precipitation. Whole plasmid was cut with the restriction enzyme, EcoRI, and subjected to agarose gel electrophoresis. Insert DNA from the gel slice was isolated using the Gene Clean Kit. ^{32}P-labelled probes were prepared using 64 ng of template insert DNA by the random oligonucleotide primers method.

Southern Blot Analysis

Total genomic DNA (5 ug) was incubated overnight with a five-fold excess of a restriction enzyme. The digested DNA was size-fractionated by electrophoresis in 1.0 % agarose gel

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for 640 V-h. After electrophoresis, the gels were treated with 0.25 N HCl for 15 min, denatured with 0.5 N NaOH, 1.5 M NaCl for 20 min, and finally neutralized with three washes in 25 mM $NaPO_4$ buffer (pH 6.5). DNA was transferred to Nytran filter (Schleicher and Schuell) by capillary action with 25 mM $NaPO_4$ buffer, pH 6.5. After transfer, the DNA was cross-linked to the filters by UV irradiation. Filters were hybridized for 16 to 24 hours according to Church and Gilbert (1984), then washed three times at 45°C in 0.1 X SSC, 0.1 % SDS for 20 min each. Autoradiography was done using AR5 x-ray film (Kodak) and Cronex intensifying screens (Dupont) at -80°C.

Northern Blot Analysis

Northern blot analysis was done as previously described with some modification (Hajela et al., 1990). Fifty ug of total RNA dissolved in 100 uL RNA loading buffer. From the 100 ul of sample solution, only 15 uL (7.5 ug of total RNA) were loaded on the gel. Total RNA was fractionated on 1.2 % denaturing formaldehyde agarose gel and northern blot was prepared on a Nytran membrane (Schleicher and Schuell) using 20x SSPE as the transfer buffer. As a check for RNA integrity, efficiency of transfer, and equivalency of loading, the EtBr stained gel and membrane were carefully examined visually under UV light. The RNA in the membrane was immobilized by cross-linking with UV light. Prehybridization and hybridization was done according to manufacturer's

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instructions (Schleicher and Schuell). The transcript amount was quantified using a Betagen 603 Blot Analyzer (Betagen Corp.). Filters were stripped for subsequent rehybridization by submerging in boiling 5 mM Tris-HCl (pH 7.8), 1 mM EDTA, and 0.05 % SDS for 15 min. Stripped filters were rehybridized with the radish rDNA, pRE12, and quantified as mentioned before for the standardization of total RNA present in the membrane. The northern blot experiments were conducted three times as replications using three different RNA samples.

Statistical Analysis

Analysis of variance (AOV), least significant difference (LSD), Duncan's multiple range test (DMRT), and single correlation analysis were used to analyze the data using the statistical package MSTAT (Michigan State University, East Lansing, Michigan)

RESULTS

Winter Survival of Canola

In 1992-1993, winter survival of the most hardy cultivar, Accord was significantly greater than that of the other 5 cultivars. All except CDH3 had winter survival higher than 80 % (Table 1). In 1993-1994, winter survival was lower, and differences among cultivars were more pronounced than in 1992-1993.

Tests for Freezing Tolerance

Freezing assay was based on ion leakage as a measure of freezing damage. The relationship between temperature and percent ion leakage (freezing curves) for each of seven cultivars is shown in Figs. 1 to 7. Freezing curves for the overall mean values of seven cultivars are shown in Fig. 8. The shapes of these curves were similar for all winter cultivars as well as the spring cultivar, and in each case acclimation shifted the entire freezing curve to lower temperatures, although the specific temperature range varied among cultivars. Freezing tolerance of all cultivars was increased within 1-day of exposure to cold temperature, and appeared to be at a maximum within 5 days.

Table 1. Winter survival of six winter canola cultivars in 1992-1993 and 1993-1994.

Cultivars	Winter survival (%)	
	1992-1993	1993-1994
ACCORD	100 a†	72 a
KWC4113	86 ab	70 a
CERES	82 b	59 b
DUOBUL	80 b	65 b
WRG86	81 b	57 bc
CDH3	75 b	41 c

† Means followed by the same letter within a column are not significantly different at the 0.05 probability level

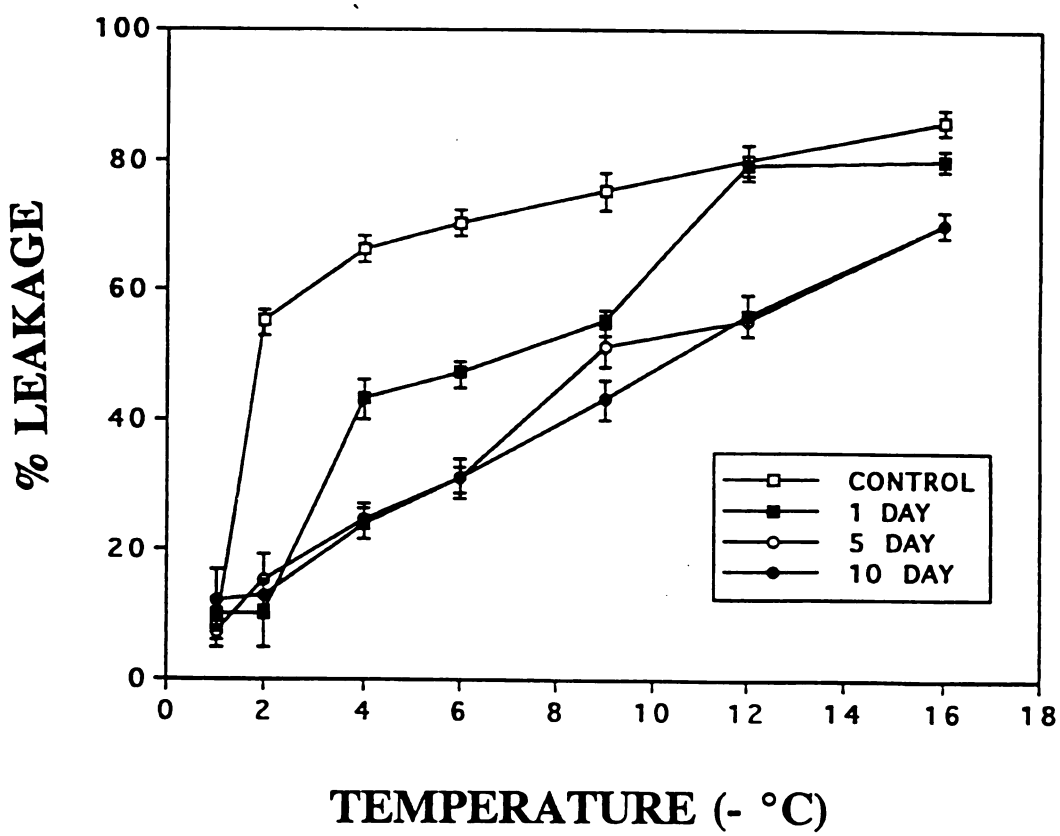

Figure 1. Freezing curves of winter canola cv. Accord acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

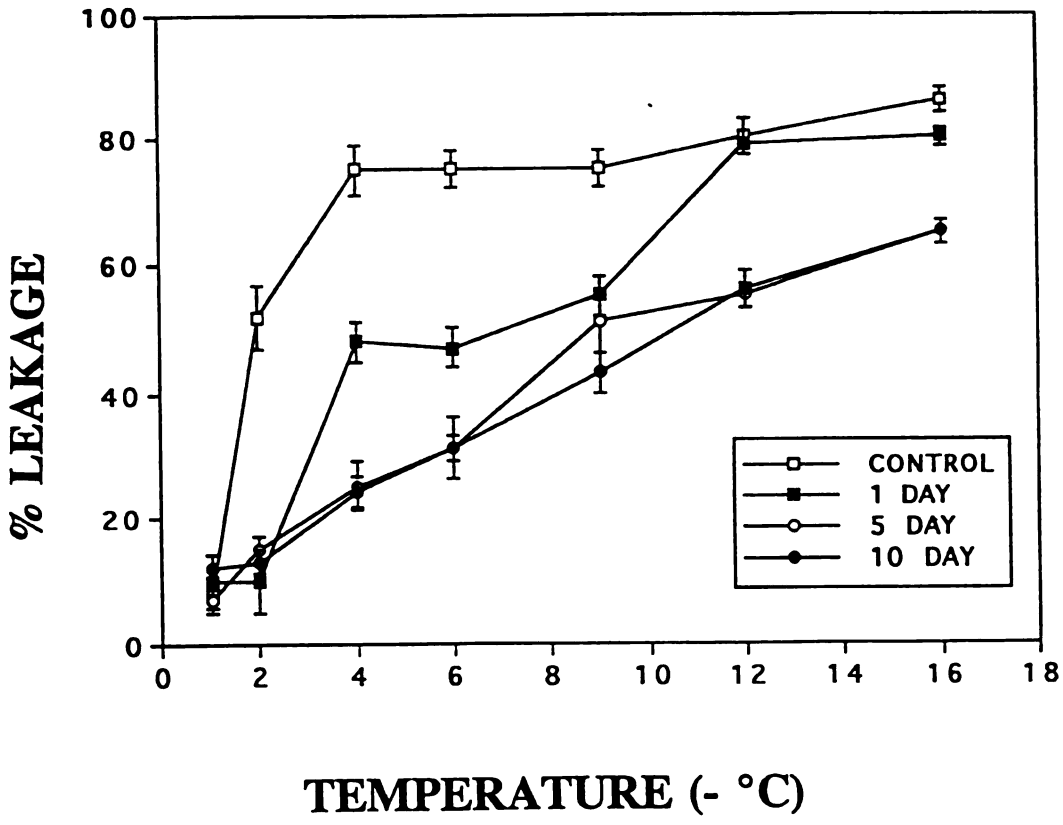

Figure 2. Freezing curves of winter canola cv. KWC4113 acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

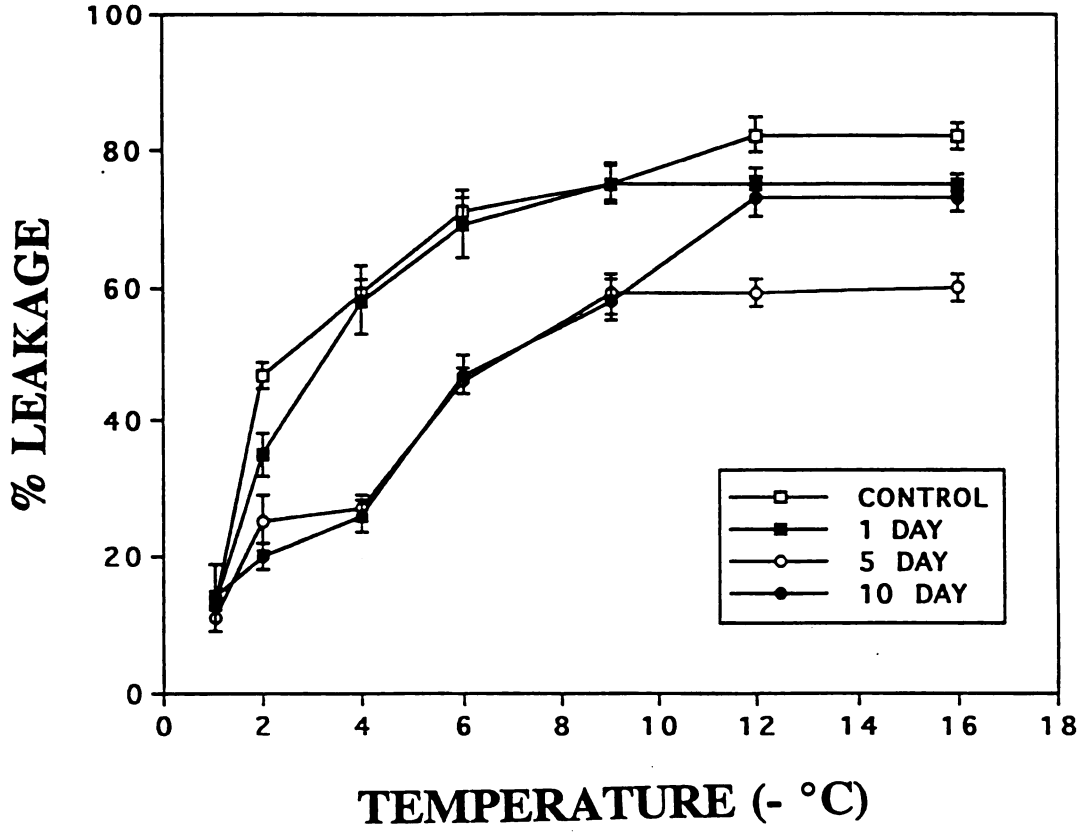

Figure 3. Freezing curves of winter canola cv. Ceres acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

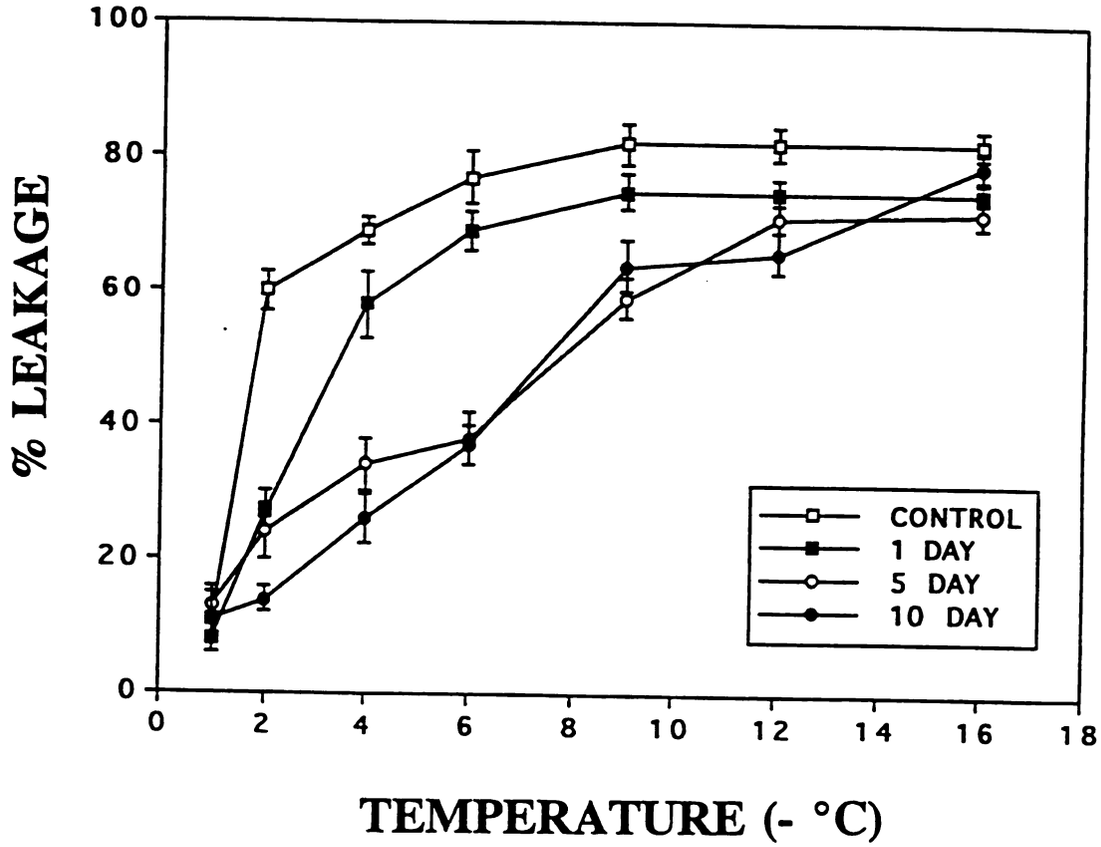

Figure 4. Freezing curves of winter canola cv. Duobul acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

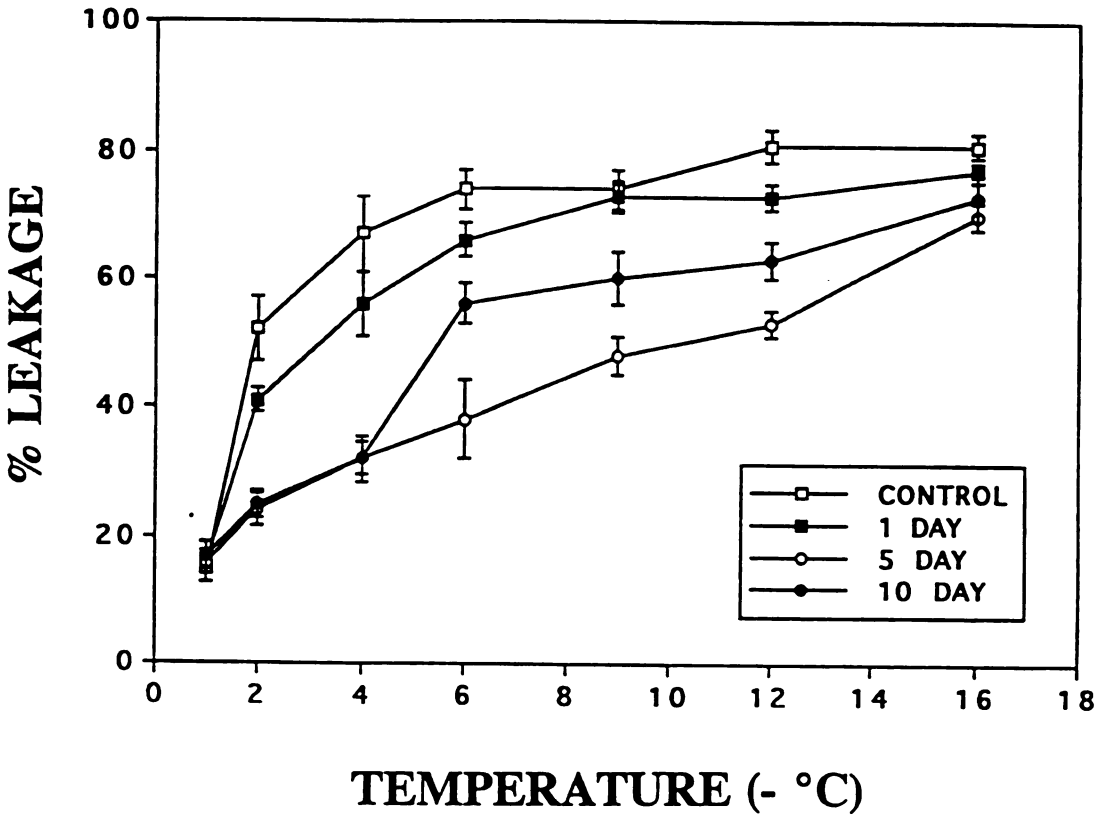

Figure 5. Freezing curves of winter canola cv. WRG86 acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

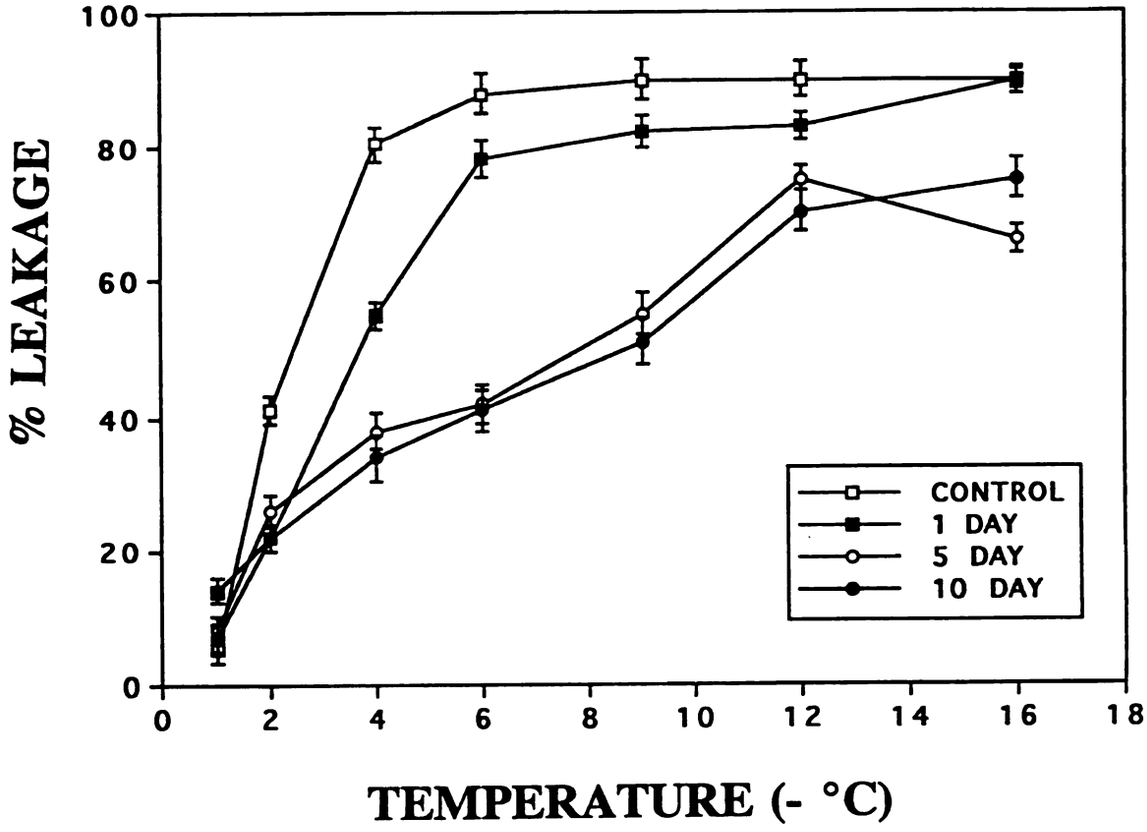

Figure 6. Freezing curves of winter canola cv. CDH3 acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

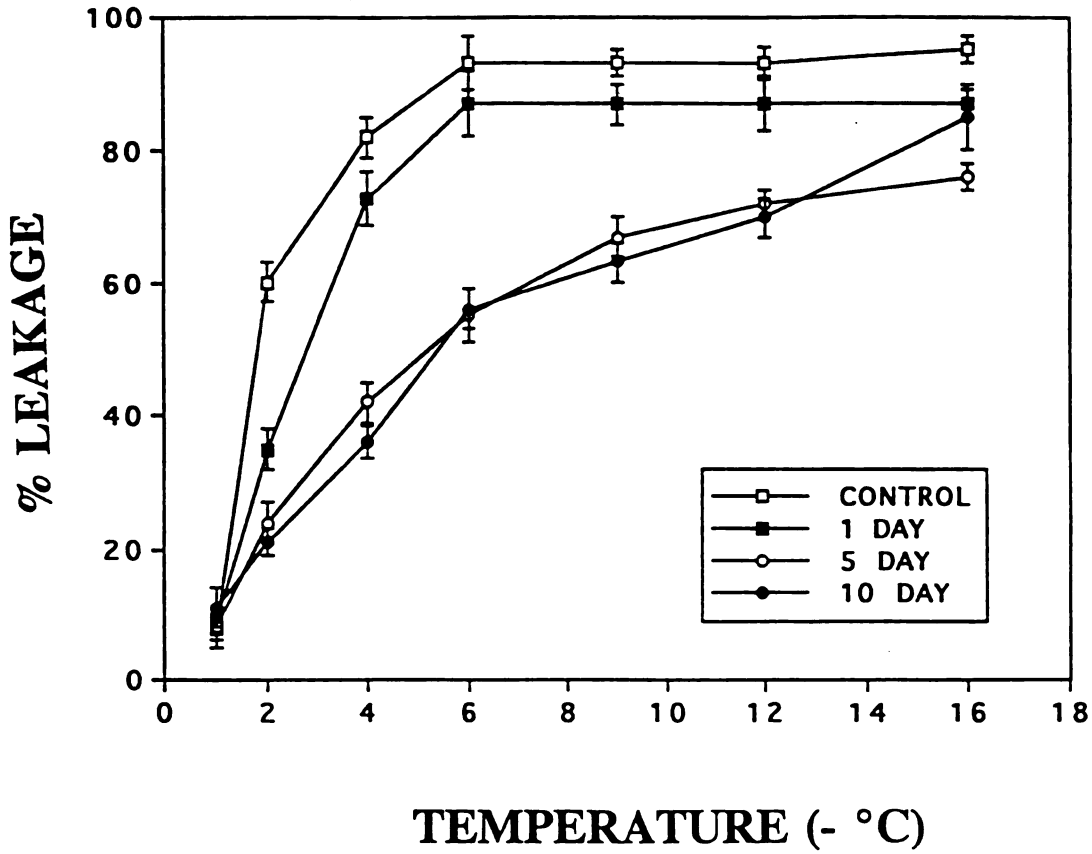

Figure 7. Freezing curves of spring canola cv. Pactol acclimated for 0, 1, 5, and 10 days, respectively. Each data point is a mean of three replications with nine sample leaves in a replication. Vertical bar indicates mean ± SE.

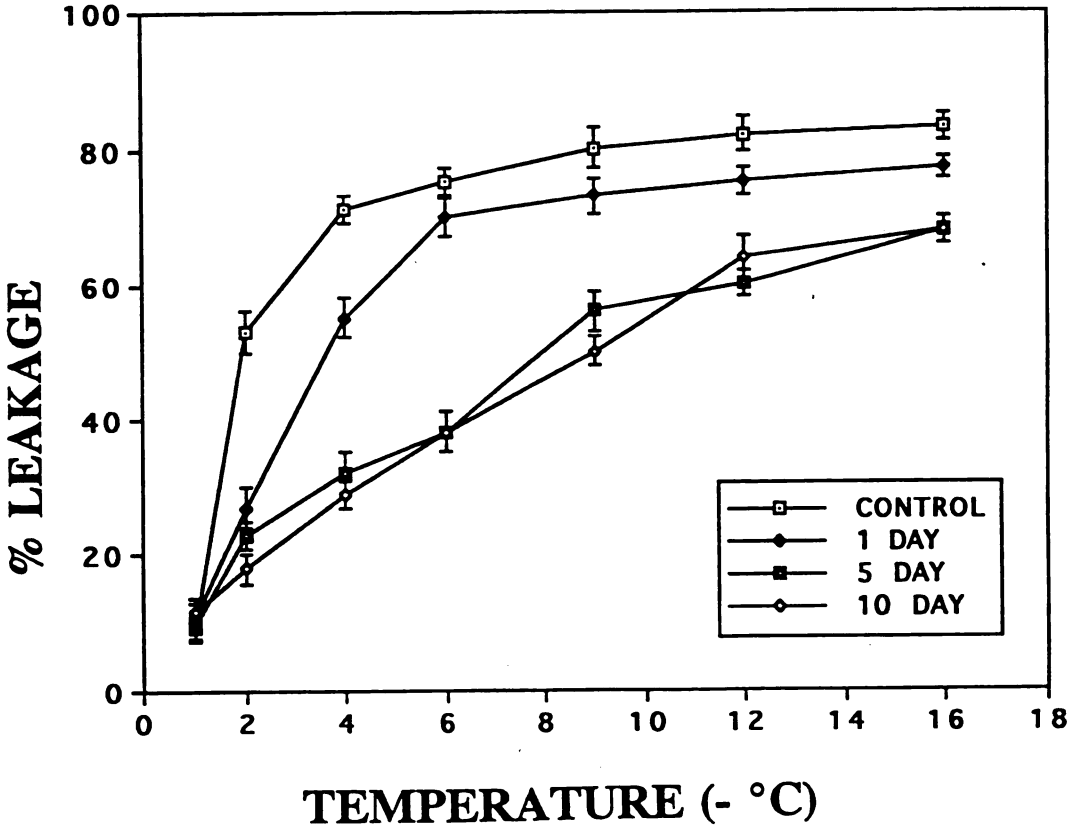

Figure 8. Freezing curves of canola acclimated for 0, 1, 5, and 10 days, respectively. Each data point represents the overall mean of seven cultivars tested in Figs. 7. Vertical bar indicates mean ± SE.

LT_{50} values were determined from the freezing curve of each cultivar (Fig. 9). Nonacclimated control plants had an LT_{50} value of approximately -2°C, while LT_{50} values in plants exposed to cold temperature were increased 1-2°C per day. LT_{50} values of control plants did not differ among cultivars. However, as soon as acclimation began, three groups of cultivars having different LT_{50} values had appeared. Although significant differences in LT_{50} value occurred among groups, no differences occurred within groups. KWC4113 and Accord belonged to the most hardy group, having the minimum LT_{50} values of -10°C and -11°C, respectively, when fully acclimated, and the spring variety, Pactol, belonged the least hardy group having a minimum LT_{50} value of -5.5°C. Another spring canola cultivar, Legend, had the same LT_{50} value as Pactol (data not shown). Other cultivars, Ceres, Duobul, WRG86, and CDH3, belonged to the medium group having LT_{50} values of -7°C to -8°C.

Nonacclimated Freezing Tolerance and Cold Acclimation Capacity

Freezing tolerance can be divided into two separate factors: Nonacclimated freezing tolerance and acclimation capacity (Table 2). Nonacclimated freezing tolerance is LT_{50} values of plants which were not acclimated. Acclimation capacity is the ability to increase freezing tolerance by cold acclimation (LT_{50} *value of the acclimated stage* - LT_{50}

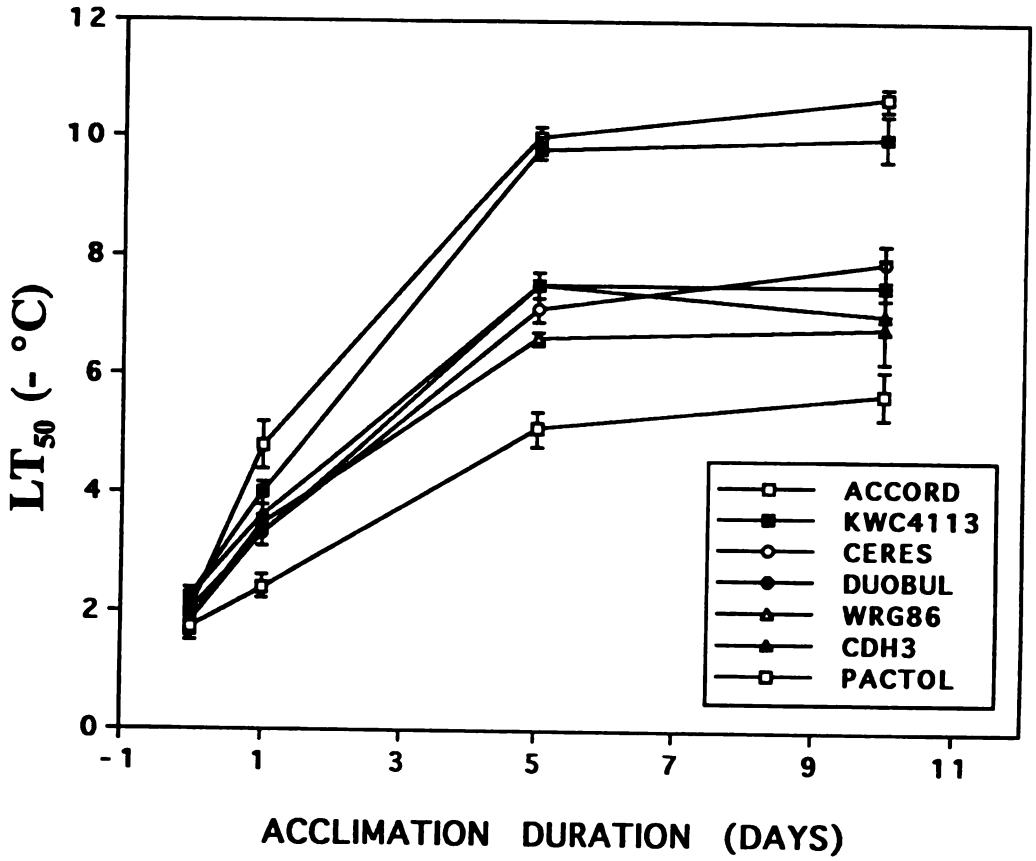

Figure 9. LT_{50} value changes in six winter canola cultivars and one spring canola cultivar (Pactol) during cold-acclimation. The plants were acclimated for 0, 1, 5, 10 days. Vertical bar indicates mean ± SE.

Table 2. Means of nonacclimated freezing tolerance and acclimation capacity of six winter cultivars and one spring cultivar at different acclimation stages.

Cultivars	Nonacclimated freezing tolerance (°C)	Acclimation capacity(°C)		
		1 day	5 day	10 day
ACCORD	-2.0 ab	2.8 a	8.0 a	8.8 a†
KWC4113	-2.2 a	1.8 ab	7.6 a	7.8 a
CERES	-2.0 ab	1.3 b	5.8 b	5.9 b
DUOBUL	-1.8 ab	1.6 ab	5.7 b	5.7 b
WRG86	-1.8 ab	1.6 ab	4.7 bc	5.0 bc
CDH3	-2.2 a	1.5 ab	5.4 b	4.8 bc
PACTOL	-1.7 b	0.7 b	3.4 c	4.0 c

† Means followed by the same letter within a column are not significantly different at the 0.05 probability level.

value of the nonacclimated stage). Nonacclimated freezing tolerance showed no significant differences among cultivars except for the spring cultivar, Pactol. However, acclimation capacity at 5 and 10 days showed clear differences among cultivars. Accord had the highest increase in LT_{50} value, 8.8°C in 10 day acclimation, while that of spring variety, Pactol, increased by only 4°C.

Correlation between Freezing Tolerance and Winter Survival

High correlations were found between *in vitro* freezing tolerance and winter survival of cultivars in all three years (Table 3). Nonacclimated (control) freezing tolerance was not correlated with winter survival in any year, however, freezing tolerance at 1, 5, and 10 days of acclimation was highly correlated with winter survival every year. Futhermore, the value of the correlation coefficient increased with acclimation time. The correlation coefficient between freezing tolerance and winter survival in 1993-1994 increased from 0.89(**) after 1 day of acclimation to 0.95(***) after 10 days of acclimation.

Effect of Deacclimation on Freezing Tolerance

Comparison of changes in LT_{50} value of cultivars from nonacclimated control plants, 10-day acclimated plants, and 3-

Table 3. Simple correlation coefficients between freezing tolerance and winter survival of six winter canola cultivars in 1992-1993 and 1993-1994.

Freezing tolerance	Winter survival	
	1992-1993	1993-1994
Control	0.33ns	0.54ns
1-day acclimation	0.93**	0.89**
5-day acclimation	0.86**	0.90**
10-day acclimation	0.92**	0.95**

† (*) and (**) simple correlation coefficient significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

day deacclimated plants are shown in Fig. 10. Generally, deacclimation decreased freezing tolerance as rapidly as acclimation increased it. More hardy cultivars lost freezing tolerance more rapidly, but still retained higher LT_{50} values than less hardy cultivars after 3 days of deacclimation, with a 3.0°C difference between the hardiest cultivar and the least hardy cultivars.

Southern Blot Analysis of BN 28 and BN 115

Autoradiograms for genomic DNA of seven cultivars cut with several restriction enzymes and probed with BN28 (Fig. 11) and BN115 (Fig. 12) showed five to ten bands, depending on the restriction enzyme and probes used. Two of the seven cultivars that were digested with EcoRI and proved with BN28 showed a restriction fragment length polymorphism in one of the minor band loci. However, based upon various restriction enzyme-digestion patterns, there was no differences in copy number or position of bands among the cultivars tested, even between the spring and winter cultivars.

Northern Blot Analysis of BN28 and BN115

Total RNA isolated from control, 1-day, 5-day, and 10-day acclimated plants of each cultivar was fractionated in denaturing formaldehyde agarose gel. RNA was transferred to a Nytran membrane and probed with BN28 and BN115 (Fig. 13). The

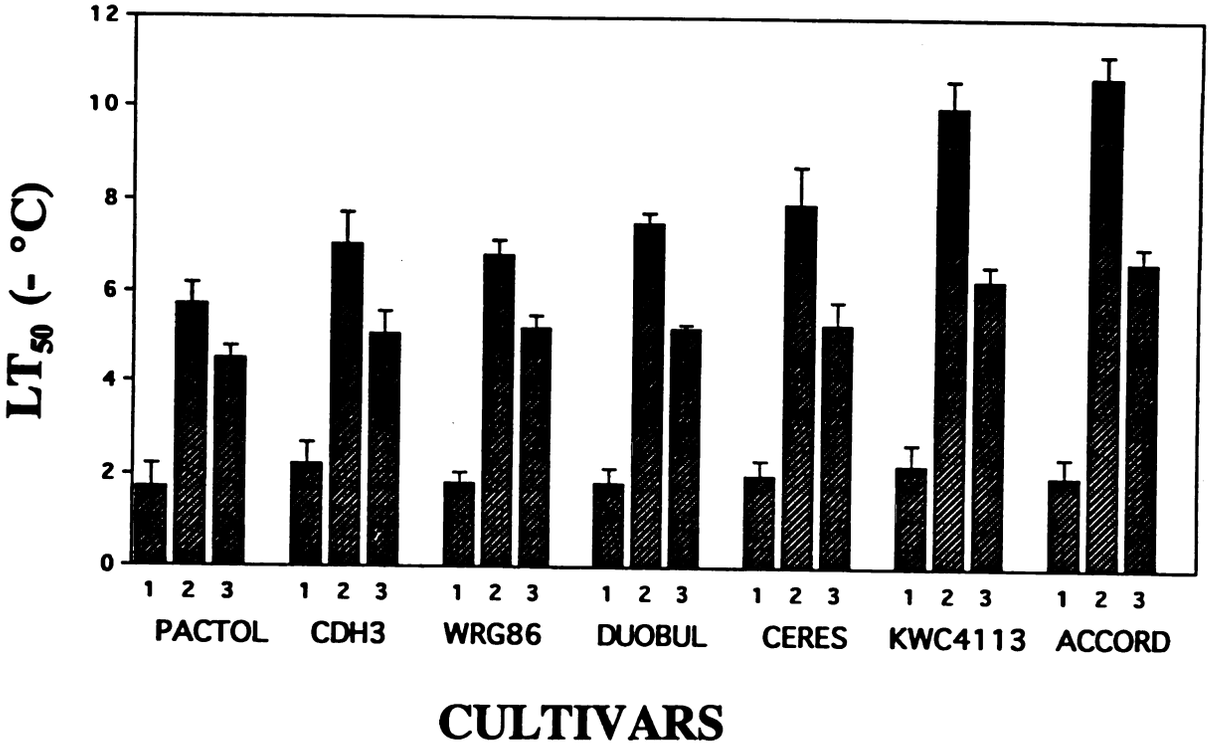

Figure 10. Effect of deacclimation on LT_{50} values of seven canola cultivars. Plants were cold-acclimated for 10 days, then deacclimated for 3 days. Vertical bar indicates mean ± SE. Lane 1: control (nonacclimated); 2: 10-day acclimation, 3: 3-day deacclimation.

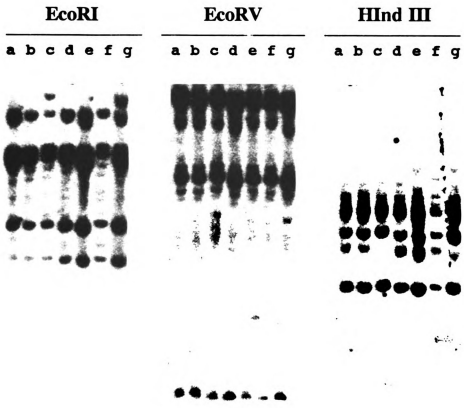

Figure 11. Southern blot analysis of the three restriction enzymes, EcoRI, EcorV, and Hind III,-digested genomic DNA from seven canola cultivars hybridized to BN28. Lane a:WRG86; b:CDH3; c:Duobul; d:Ceres; e:KWC4113; f:Accord; g:Pactol

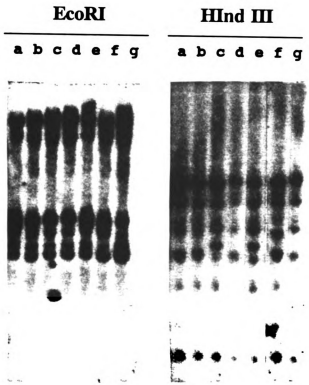

Figure 12. Southern blot analysis of the two restriction enzymes, EcoRI, and Hind III,-digested genomic DNA from seven canola cultivars hybridized to BN115. Lane a:WRG86; b:CDH3; c:Duobul; d:Ceres; e:KWC4113; f:Accord; g:Pactol.

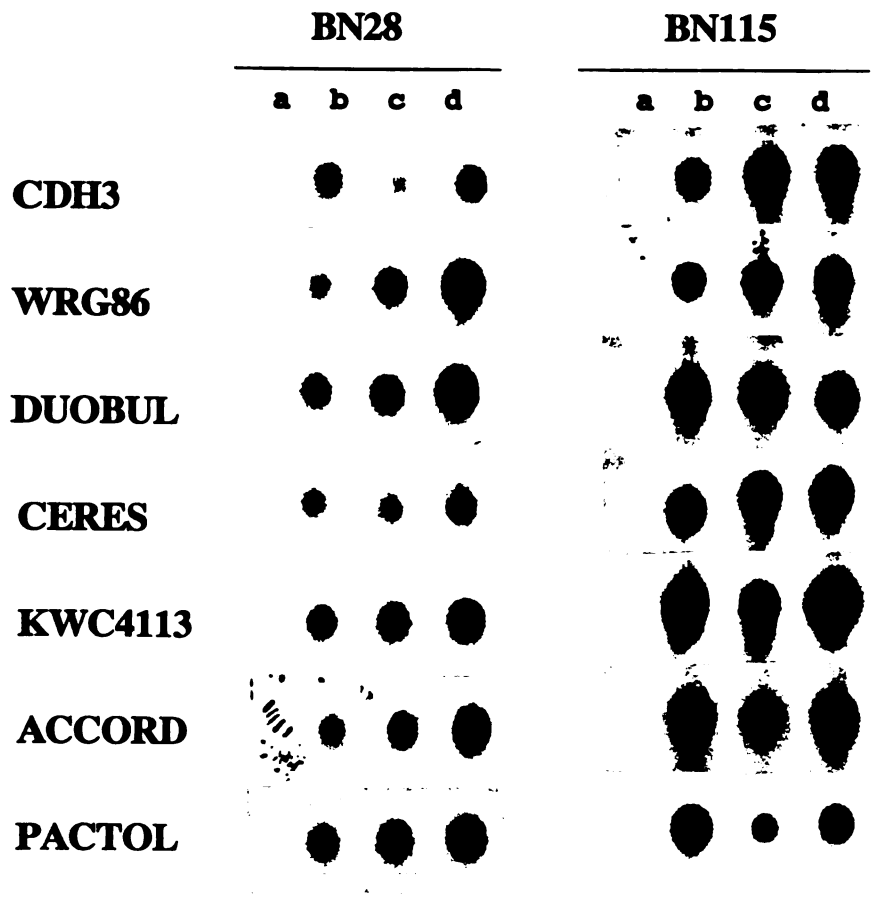

Figure 13. Northern blot analysis of total RNA from leaves of seven canola cultivars isolated at 0-day (lane a), 1-day (lane b), 5-day (lane c), and 10-day (lane d)-acclimation stages and probed with BN28 and BN115.

radioactivity bound to the membrane was measured by a Betagen Blot Analyzer. For the standardization of RNA quantity in Northern blot, the blot was stripped and rehybridized with rDNA, pRE12, which is expressed constitutively in plants (Fig. 14). Changes of transcript amount (representing the overall means of seven cultivars) by cold acclimation are shown in Fig. 15. RNA transcripts for both genes were undetected in control plants; however, in plants exposed to cold temperature, the amount of transcript increased rapidly and remained at elevated levels as long as the plants remained at cold temperatures. The two genes showed differences in their expression pattern. BN28 showed a rapid increase in the amount of transcript at 1-day (60 % of that of maximum expression) and a steady increase until 10 days of acclimation. BN115 reached its maximum expression level within 1 day of exposure to cold temperature and maintained that elevated level as long as plants remained at cold temperature.

Effect of Deacclimation on Expression of BN28 and BN115

Even 1 day of deacclimation decreased the expression of BN28 and BN115 in most of seven cultivars examined, although some cultivars with 1 day of deacclimation showed some activity remaining. Three days of deacclimation totally eliminated the transcript of both genes in all cultivars (Fig. 16), showing that both were completely turned off by deacclimation and that degradation of the transcripts occurred

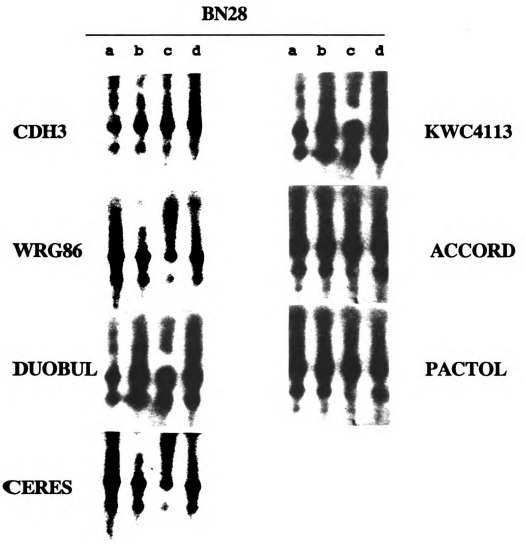

Figure 14. Standardization of Northern blot analysis by hybridizing with rDNA probe, pRE12. The blot was the membrane presented in Fig. 14, which was stripped and rehybridized with the rDNA probe.

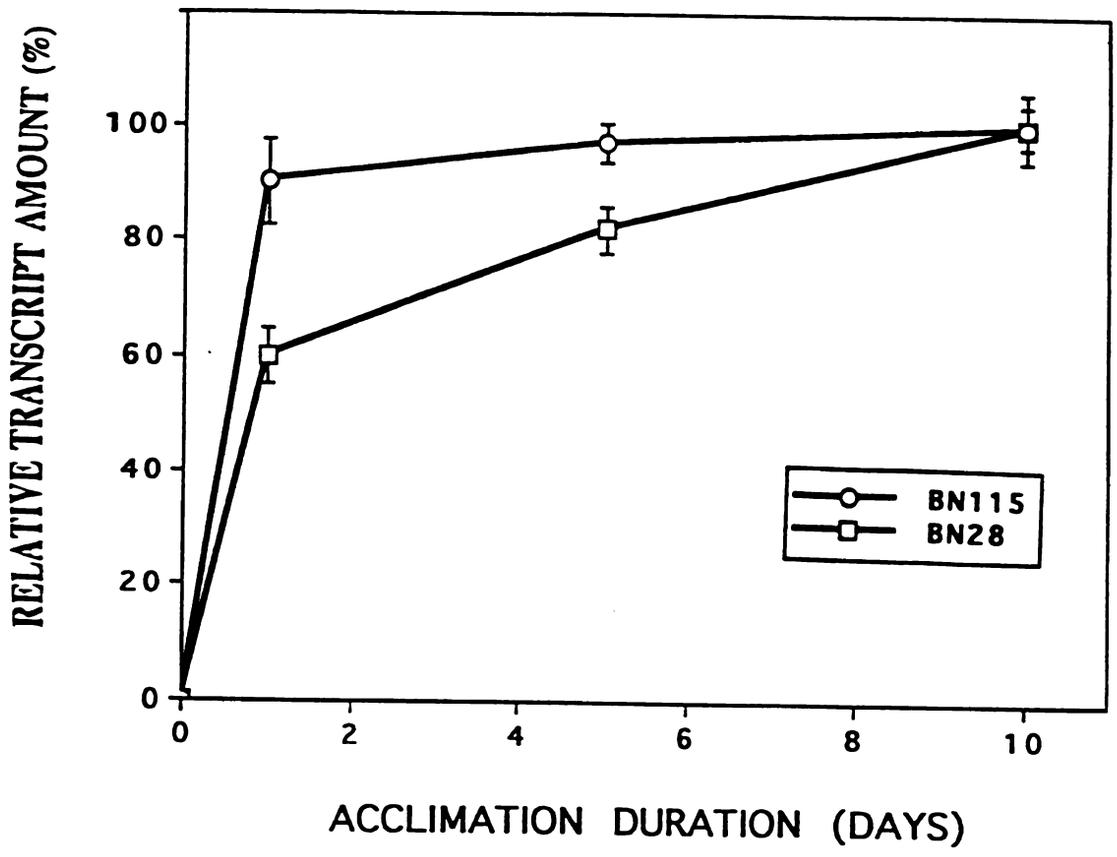

Fig. 15. Changes in transcript amount of BN28 and BN115 during cold-acclimation. The data is a graphical presentation of Fig. 13 and represents overall mean values of seven cultivars. Vertical bar indicates mean ± SE.

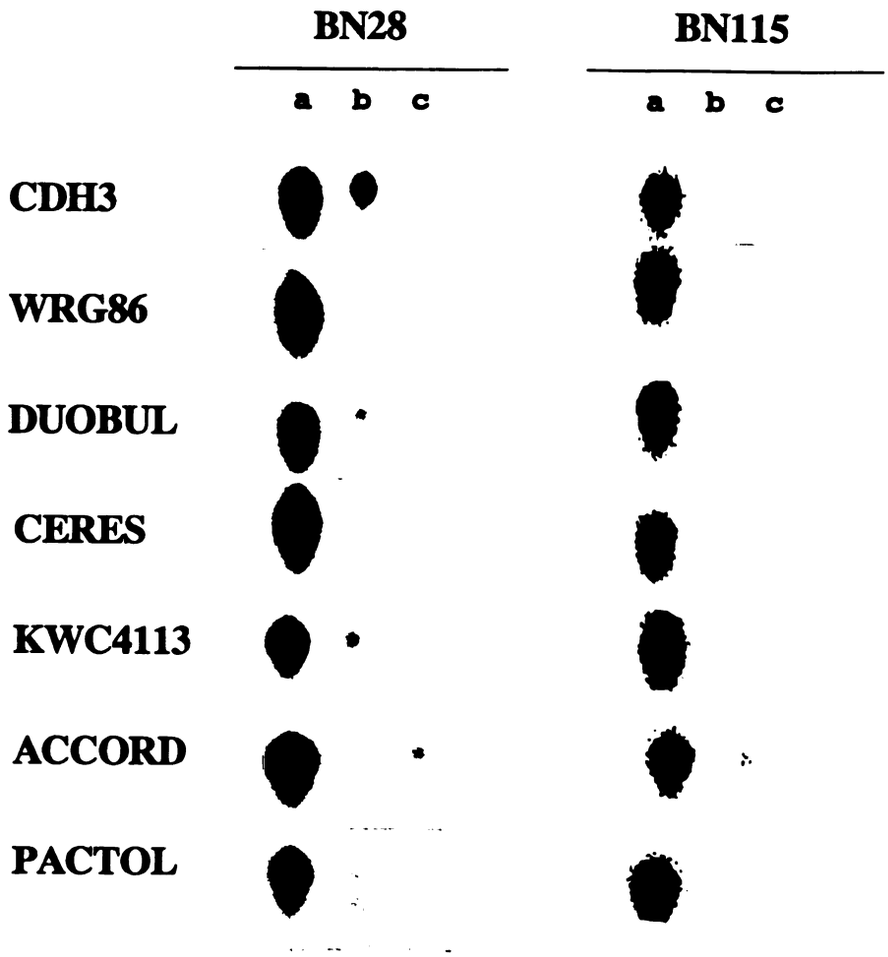

Figure 16. Northern blot analysis for effect of deacclimation on the expressions of BN28 and BN115 in seven canola cultivars. Lane a: 10-day acclimation; b: 1-day deacclimation after 10-day acclimation; c: 3-day deacclimation after 10-day acclimation.

very rapidly at room temperature.

Correlation between Freezing Tolerance and Expression of BN28 and BN115

Correlation between time-course increase in LT_{50} values for seven cultivars and time-course increase in relative transcript amount of BN28 and BN115 was examined (Fig. 17). Each point is the overall mean of seven cultivars. Time-course increase in transcript amount of BN28 was highly correlated with time-course increase in LT_{50} value (r=0.92**). As the transcript of BN28 increased due to longer acclimation period, the LT_{50} value also increased. However, the increase in transcript amount of BN115 was not significantly correlated (0.79ns) with the time-course increase in LT_{50} value.

Correlations were also examined for each cultivar (Fig. 18). Expression of BN28 and BN115 at a specific acclimation stage was not significantly correlated with freezing tolerance at that time or with winter survival at the cultivar level. The relative transcript amount of BN28 at 10 days of acclimation was Duobul > Pactol > WRG86 > Accord > Ceres > CDH3 > KWC4113, while the freezing tolerance at this time was Accord > KWC4113 > Ceres > Duobul > WRG86 > CDH3 > Pactol.

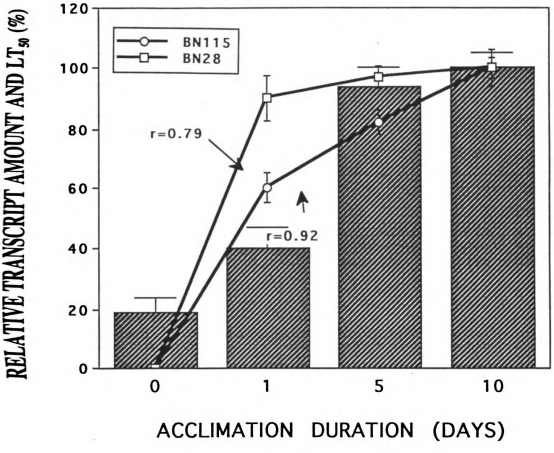

Figure 17. Comparison of time-course changes of LT_{50} value and transcript amount of BN28 and BN115 during cold acclimation. The bar indicates LT_{50} value and the line indicates the transcript amount. The data are overall mean value for seven cultivars. Vertical bar indicates mean ± SE.

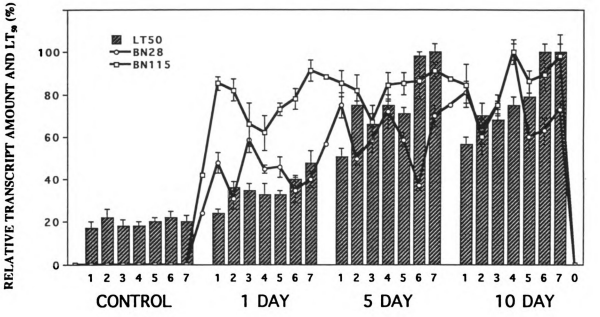

Figure 18. Correlation between freezing tolerance and transcript amount of BN28 and BN115 by cold acclimation in cultivar level at specific time of measurement. The bar indicates LT_{50} value and the line indicates the transcript amount. Vertical bar indicates mean ± SE. Lane 1: Pactol; 2: CDH3; 3: WRG86; 4: Duobul; 5: Ceres; 6: KWC4113; 7: Accord.

Tissue Specific-Expression of BN28 and BN115

The tissue-specific expression of BN28 and BN115 was examined in two representative winter cultivars, the hardy cultivar, KWC4113, and the tender cultivar, WRG86 (Fig. 19). Both genes were expressed more strongly in the leaf than in the stem, and there was no detectable expression in root tissue.

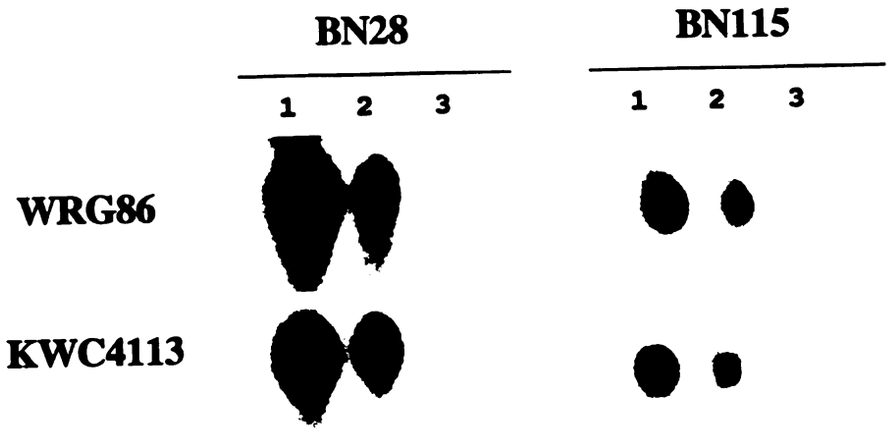

Figure 19. Northern blot analysis for tissue-specific expression of BN28 and BN115 by cold acclimation in two winter cultivars, WRG86 and KWC4113. Lane 1: leaf; 2: stem; 3: root.

DISCUSSION

The primary observations from freezing tolerance studies are as follows: (1) cold acclimation increases the freezing tolerance of both spring and winter cultivars with different acclimation capacities. (2) There is no difference in nonacclimated freezing tolerance among cultivars, however, differences in acclimation capacity among cultivars determine their freezing tolerance after cold acclimation. (3) Freezing tolerance highly correlates with winter survival, though small differences in freezing tolerance of cultivars with similar winter survival were not separated by freezing tests. (4) Deacclimation decreases the freezing tolerance very rapidly; however, different cultivars still have different levels of freezing tolerance after deacclimation.

In this study, three were three groups of cultivars with different levels of freezing tolerances (Fig. 9). This included two groups of winter cultivars and one spring cultivar. Although cultivars within a group had different winter survivals, their freezing tolerance to freezing tests was not significantly different. This suggests a lack of precision in freezing tests for predicting differences in winter survival of cultivars with a narrow differences in

freezing tolerance. It may also indicate that factors other than freezing tolerance itself are involved in winter survival under field conditions (Thompson and Hughes, 1986). However, differences in freezing tolerance between groups, and their correlation with winter survival, suggest that freezing tests can be used to detect differences in winter survival of cultivars with broad differences in freezing tolerance. This agrees with observations by Teutonico et al. (1993).

The observation that freezing tolerance in the nonacclimated state and acclimation capacity are not correlated, and that only acclimation capacity has a high correlation with winter survival, suggests that they are regulated independently. It also suggests that acclimation ability is more important to winter survival. This agrees with observations reported for these two traits in potato (Palta, 1992) and *Brassica* species (Teutonico et al., 1993).

All cultivars showed a rapid decrease in freezing tolerance during a short period of deacclimation. However, differences in freezing tolerance among cultivars occurred after deacclimation. Winter survival may be determined by how much a certain cultivar retains freezing tolerance after the occurrence of warm temperatures in the middle of winter and the speed of reacclimation prior to subsequent lethal freezing temperatures. As shown in this experiment, the hardy cultivars increased more rapidly in freezing tolerance during cold acclimation and retained a higher freezing tolerance after

deacclimation than less hardy cultivars. This suggests that the retention of freezing tolerance after deacclimation and the speed of reacclimation may play a role in winter survival.

BN28 and BN115 are two genes from *Brassica napus* induced by cold temperature (Orr et al., 1992; Weretilynk et al.,1993). The reason for cultivar differences in the level of expression are not known. If the regulation of this expression occurs at the levels of transcription, then the possible reason may include differences in (1) copy number of genes involved; (2) promoter sequences resulting in different transcriptional efficiency; (3) the availability and efficiency of promoter-binding protein and other transcriptional factors; and (4) changes in mRNA processing and stability (Mohapatra et al., 1989).

Southern blot data (Fig. 11, 12) showed that these genes are a multigene family. This multigene family may consist of either the same genes having multiple copies in the genome or slightly different copies of genes having very similar molecular weight of transcript as discussed by Weretilynk et al. (1993). The lack of difference in copy number and even in restriction fragment length polymorphism (RFLP) of these two genes among cultivars is not surprising considering the narrow genetic bases of breeding stocks and the recent development of canola cultivars (Sahhidi, 1990).

Expression of these two genes was induced within 12 hours of exposure to cold temperature and maintained their

expression as long as plants were kept at cold temperatures. The expression pattern of the two genes was slightly different: BN28 was induced to 60 % of full expression with a 1-day exposure and increased steadily to full expression with 10 days of acclimation, while BN115 was expressed fully with a 1-day exposure and maintained full expression with cold temperature. These results are consistent with those reported for *Brassica napus* cv. Jet neuf (Weretilnyk et al., 1993) and *Arabidopsis thaliana* L. Heyn (Hajela et al.,1990) for BN115, but inconsistent with that of *Arabidopsis* for BN28.

Transcript level of the genes was not positively correlated with freezing tolerance in cultivars at the specific time of acclimation (Fig. 18). However, the observations that these genes are turned on in response to cold temperature and maintain the expressions under cold temperature (Fig. 13) and the time-course increase of BN28 expression is coincident with the time-course increase in freezing tolerance (Fig. 17) do not allow us to rule out the possible role of the genes in freezing tolerance. One possible explanation is that these genes represent a minimum requirement for the plants to gain freezing tolerance, though the difference in freezing tolerance among cultivars can be explained by other genes associated with these two genes. Considering that the gene expression was determined in the transcription level in this experiment, examination of the translation expression level may provide further information.

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CHAPTER II

EFFECTS OF PLANTING DATE ON FREEZING TOLERANCE, WINTER SURVIVAL, AND RESPONSES OF TWO COLD-REGULATED GENES, BN28 AND BN115, IN CANOLA (*Brassica napus* L.)

ABSTRACT

Planting date is the most important factor in determing winter survival of crop plants. The objective of this study was to explore the effect of planting date on the development of freezing tolerance and winter survival of canola. In addition, the expression of two cold-regulated genes, BN28 and BN115, under field condition was also explored.

Six winter cultivars were planted at three different dates during the fall on the Michigan State University Research Farm at East Lansing, MI. Freezing tolerance was determined by ion leakage tests, while expression of the genes was determined by northern blot analysis of total RNA taken at the same times.

Planting date as well as cultivar treatment had a significant effect on freezing tolerance and winter survival. A different pattern in development of freezing tolerance was observed for different planting dates. There was a high

correlation between freezing tolerance and winter survival suggesting that freezing tolerance is the single most important factor in determining winter survival. The expression of cold-regulated genes was detected within 15 days after planting. Although time-course increase in expression of the cold-regulated genes was matched with increase in freezing tolerance, the difference of expression in cultivar level at specific times of measurement was not correlated with freezing tolerance.

The effect of fall-applied nitrogen and seeding rate combined with planting date on winter survival was also examined.

INTRODUCTION

Each crop should be planted at the time of the year when its stages of development will be appropriate for the expected environments. Temperature and moisture are of particular importance, and in many species, light is also very important. Numerous studies have been conducted to determine the best planting dates for various overwintering crops. In general, the earlier a crop can be planted, the better will be its productivity (Auld et al., 1983, 1989).

Although there is considerable information available on the effect of planting date on the performance of winter crops (Canvin, 1965; Noderstaggard, 1972; Huber, 1973; Kondratowicz, 1974; Kondra, 1977; Degenhardt and Kondra, 1981; Christensen et al., 1984), most are concerned with agronomic factors such as yield, crop quality, and winter survival. Limited information is available on the mechanism of cold hardiness and increase in freezing tolerance as affected by different planting dates. Differences in planting date and exposure of plants to different environmental conditions at different stages of growth influence their capacity for winter hardiness. An understanding of how plants respond to different environmental factors (e.g., photoperiod and temperature, and

fluctuation of temperature during the day and night) at different growth stages provides us clues for improving cold hardiness by both cultural practice and breeding strategies.

Recently, a number of genes have been described that respond to severe environmental stresses such as drought, heat shock, low temperature, or high salt concentration (Mundy and Chua, 1988; Close et al., 1989; Bartel et al., 1990; Hajela et al., 1990; Kukela and Frank, 1990; Skriver and Mundy, 1990; Bray, 1991; Pla et al., 1991; Lang and Palba, 1992; Yamaguchi-Shinozaki, 1994). Though laboratory manipulation of these genes provides some clues for their regulation and possible functions, little is known as to how they are expressed under field condition. A few investigators have explored the expression of heat shock protein genes under field conditions (Burk et al., 1985; Kimpel and Key, 1985; Chen et al., 1990; DeRocher et al., 1991; Lorrain et al., 1993), however, there is no information on how cold-regulated genes respond to field environments. Data from field experiments combined with what has been learned from laboratory experiments provide some insight in the functions and regulations of these genes.

The objectives of this research were 1) to determine the effect of planting date on the development of freezing tolerance and eventually, on winter survival of canola and 2) to study the response of two cold-regulated genes, BN28 and BN115, isolated from *B. napus* (Orr et al., 1992; Weretilynk et al., 1993) under field conditions at different planting dates.

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MATERIALS AND METHODS

The same six winter cultivars used in the previous study (WRG86, CDH3, Duobul, Ceres, Accord and KWC4113) were used in this study.

Planting, Management and Experimental Design

This study was conducted at the Michigan State University Agronomy Farm in East Lansing, Michigan. A split-plot design was used with three replications. Three planting dates, Aug. 25, Sept. 10, and Sept. 25 in 1993, were assigned to the main plot. Six cultivars were assigned to the subplots of each main plots. Seed of each cultivar was planted at the rate of 5.6 kg/ha in a five-row plot 6 m long and 92 cm wide.

Evaluation of Freezing Tolerance

Six cultivars planted on Aug. 25 and three of the six cultivars planted on Sept. 10 and Sept. 25 were subjected to freezing tests. Freezing tests were performed 15 (Sept. 10), 30 (Sept. 25), 45 (Oct. 10), and 75 (Nov. 13) days after planting (DAP) for the Aug. 25 planting. For the Sept. 10 planting, freezing tests were performed at 15 (Sept. 25), 30 (Oct. 10), and 60 DAP (Nov. 13), and for the Sept. 25

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planting, at 15 (Oct. 10) and 45 DAP (Nov. 14).

Three different plants were collected from each plot in the field and transferred to the laboratory immediately. One leaf disc was taken from each of the 3 plants and the 3 discs were placed in a stoppered culture tube as a replication. Freezing tests were performed as previously described (Chapter I).

Evaluation of Winter Survival

Winter survival was evaluated as previously described (Chapter I).

RNA Extraction and Northern Blot Analysis

Sampling was done at the same time as for freezing tests. Leaf and stem parts of plants were cut in the field, frozen in liquid N_2, then kept in a box with dry ice before storage at -80 °C in the laboratory. RNA extraction and northern blot analysis with BN28 and BN115 were performed as previously described (Chapter I). Three different northern blot analyses were conducted with different RNA samples extracted from each replication.

Nitrogen Rate and Seeding Rate Experiment

To explore the effect of fall-applied nitrogen and seeding rate on winter survival of canola, the susceptible cultivar, Duobul, was planted at the same dates as in the

previous experiment. For each planting time, 0, 20, and 40 kg/ha of actual nitrogen (as urea) was applied. Winter survival was evaluated as previously described. Another experiment was established with seeding rates of 4, 8, and 12 kg/ha at each of three planting dates. The winter survival of all treatments was evaluated.

Statistical analysis

Analysis of variance, least significant difference, Duncan's multiple range test, and simple correlation analysis were used to analyze the data using the statistical package MSTAT (Michigan State University, East Lansing, Michigan).

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RESULTS

Weather Conditions

The winter of 1993-1994 was colder than that of 1992-1993, with a minimum air temperature of -29.0°C (Jan. 19, 1994), compared with a low of only -22.0°C for the winter of 1992-1993 (Fig. 1). The coldest 10-day mean daily minimum temperature in 1992-1993 was -16.5°C (end of Feb. 1993) compared to -22°C in 1993-1994 (middle of Jan. 1994).

The overall pattern of air temperature changes in the 1993-1994 growing season was as follows: warm temperatures (above 10°C of 10-day mean daily minimum temperature) were maintained until the middle of the September. Then, the temperature decreased slowly from 10°C to freezing through the middle of September to the beginning of November. Freezing temperatures occurred at the beginning of November, developed to around -2°C until there was a sudden temperature drop to -14.2°C in the end of December. The coldest time of the year occurred in mid-January, and these severe conditions lasted until the end of February. In March, the temperature was still maintained at freezing levels through the end of the month, then slowly increased to 0°C until the beginning of April.

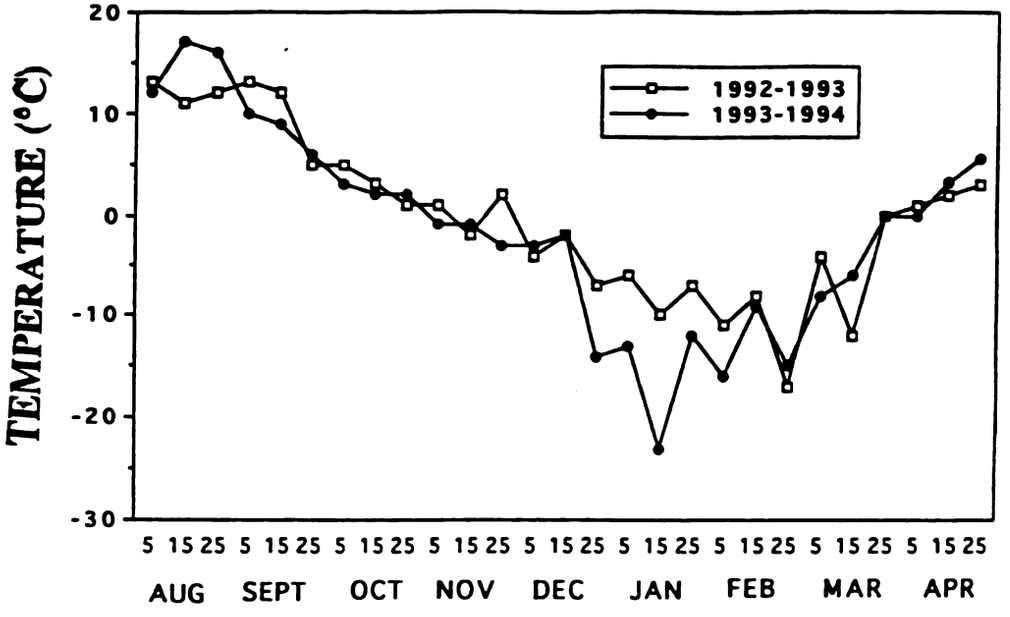

Figure 1. Ten-day mean daily minimum temperatures at East Lansing, MI during the winter in 1992-1993 and 1993-1994.

Increase of Freezing Tolerance under Field Conditions

For the canola planted Aug. 25 (Fig. 2), no increase in freezing tolerance was detected until 30 DAP (Aug. 25 - Sept. 25). Thereafter, a rapid increase in freezing tolerance occurred during 30 to 45 DAP (Sept. 25 - Oct. 10) followed by gradual increase until 75 DAP (Oct. 10 - Nov. 10). There were 3 distinct groups of cultivars with different LT_{50} values. CDH3 and WRG86 belonged to the least hardy group, Duobul and Ceres belonged to medium hardy group, and Accord and KWC4113 belonged to the most hardy group. The very hardy cultivars, KWC4113 and Accord, attained their maximum LT_{50} values of -20°C, while that of the susceptible cultivar, CDH3 was -15°C on Nov. 13. However, whether the increase in freezing tolerance would be continued to the next growth period (after Nov. 13) was not determined in this experiment.

For the Sept. 10 planting (Fig. 3), a rapid increase in freezing tolerance occurred during 15 to 30 DAP (Sept. 25 - Oct. 10) followed by very steady increase during 30 to 60 DAP (Oct. 10 - Nov. 10). However, the maximum freezing tolerances of cultivars in these plots were much less than that of the same cultivars planted Aug. 25. For example, KWC4113 planted Sept. 10 attained an LT_{50} value of -15°C whereas the same cultivar planted Aug. 25 attained -20°C.

For the Sept. 25 planting (Fig. 4), most of the freezing tolerance retained at the end of hardening process was gained

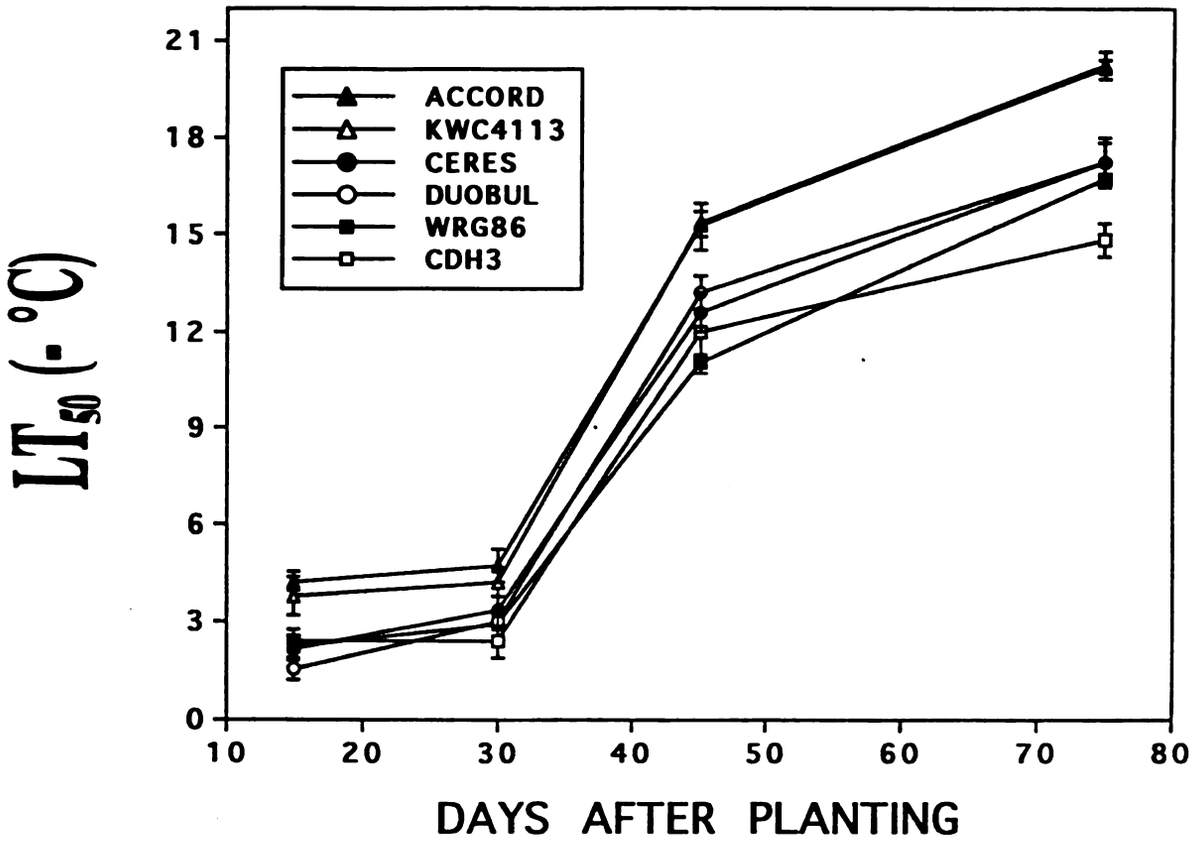

Figure 2. Changes in freezing tolerance of six winter canola cultivars planted Aug. 25, 1993. Freezing tests were conducted at 15, 30, 45, and 75 days after planting. Bar indicates mean ± SE.

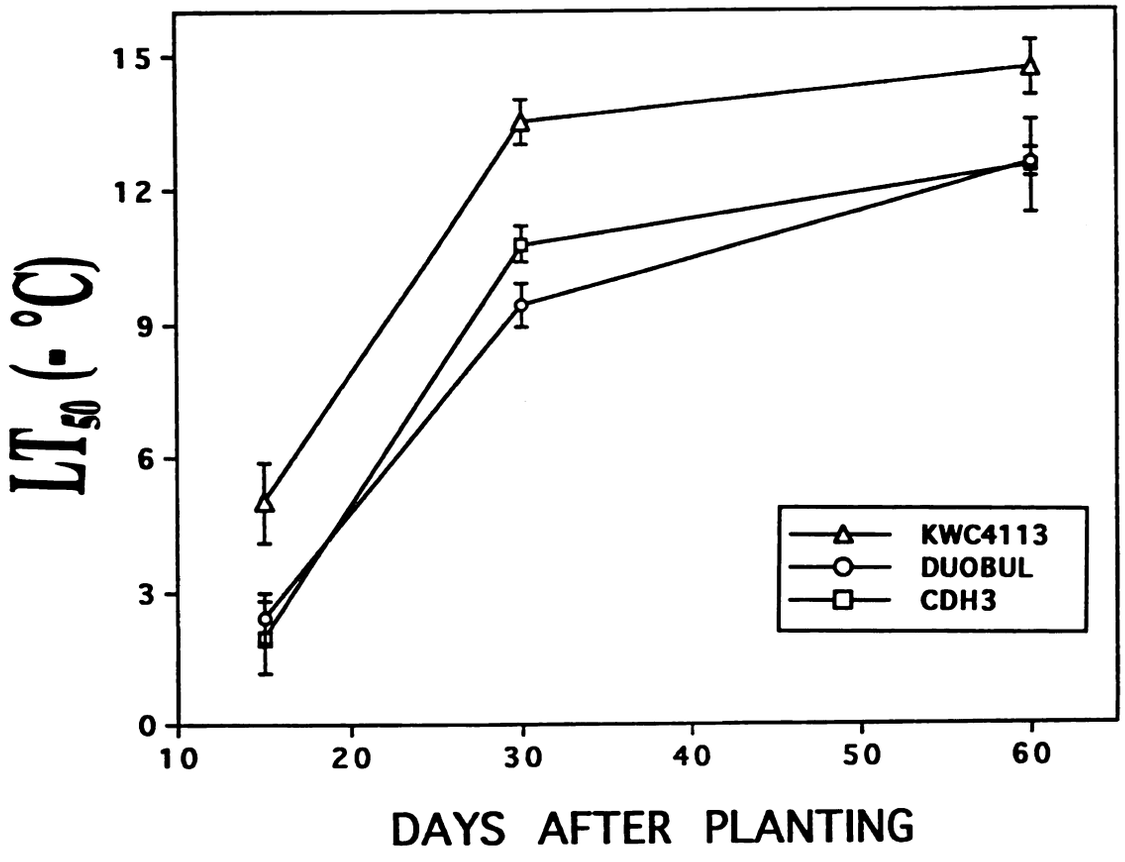

Figure 3. Changes in freezing tolerance of three winter canola cultivars planted Sept. 10, 1993. Freezing tests were conducted at 15, 30, and 60 days after planting. Bar indicates mean ± SE.

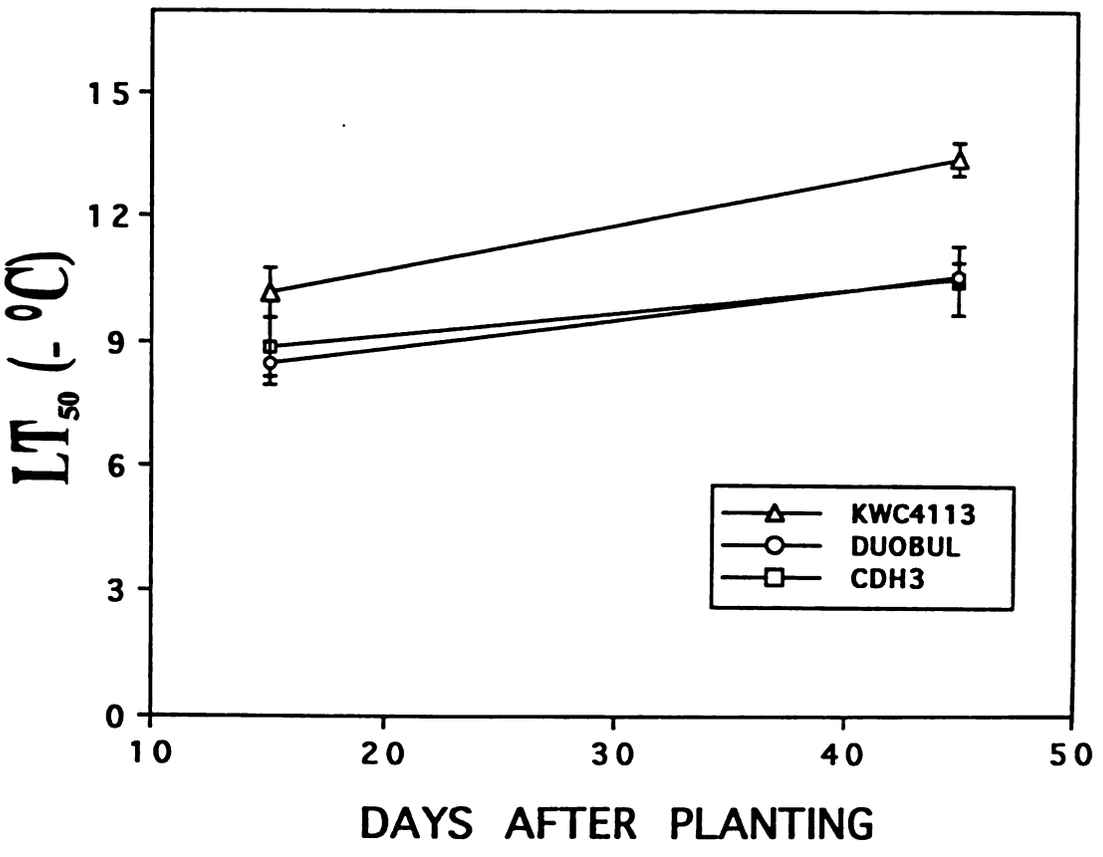

Figure 4. Changes in freezing tolerance of three winter canola cultivars planted Sept.25, 1993. Freezing tests were conducted at 15 and 45 days after planting. Bar indicates ± SE.

during the first 15 DAP (Sept. 25 to Oct. 10), followed by steady increase to a maximum of -13°C for the most hardy cultivar, KWC 4113, and only -8.5°C for less hardy cultivars.

The effect of planting date on freezing tolerance is shown in Fig. 5. Each data point represents the overall means of six or three cultivars shown in Fig. 2, Fig. 3, and Fig. 4. A dramatic increase in freezing tolerance in all three planting dates occurred during the period of Sept. 25 to Oct. 10 during which the 10-day mean daily minimum temperature dropped from 8.9°C to 2.7°C. Before that time, no significant increase in freezing tolerance occurred in either of the plots planted Aug. 25 or Sept. 10. Though all three planting dates resulted in a very steady increase in freezing tolerance between Oct. 10 and Nov. 15, the increase in the Aug. 25 planting was more rapid. Planting date significantly affected freezing tolerance with maximum LT_{50} values of -18°C, -14°C, and -12°C for plantings made Aug. 25, Sept. 10, and Sept. 25, respectively.

Responses of Two Cold-Regulated Genes, BN28 and BN115, under Field Conditions

To determine the response of two-cold regulated genes under field conditions, total RNA from six cultivars planted Aug. 25 were extracted from 15, 30, 45, and 75-day old plants. Northern blot analysis was performed with BN28 and BN115

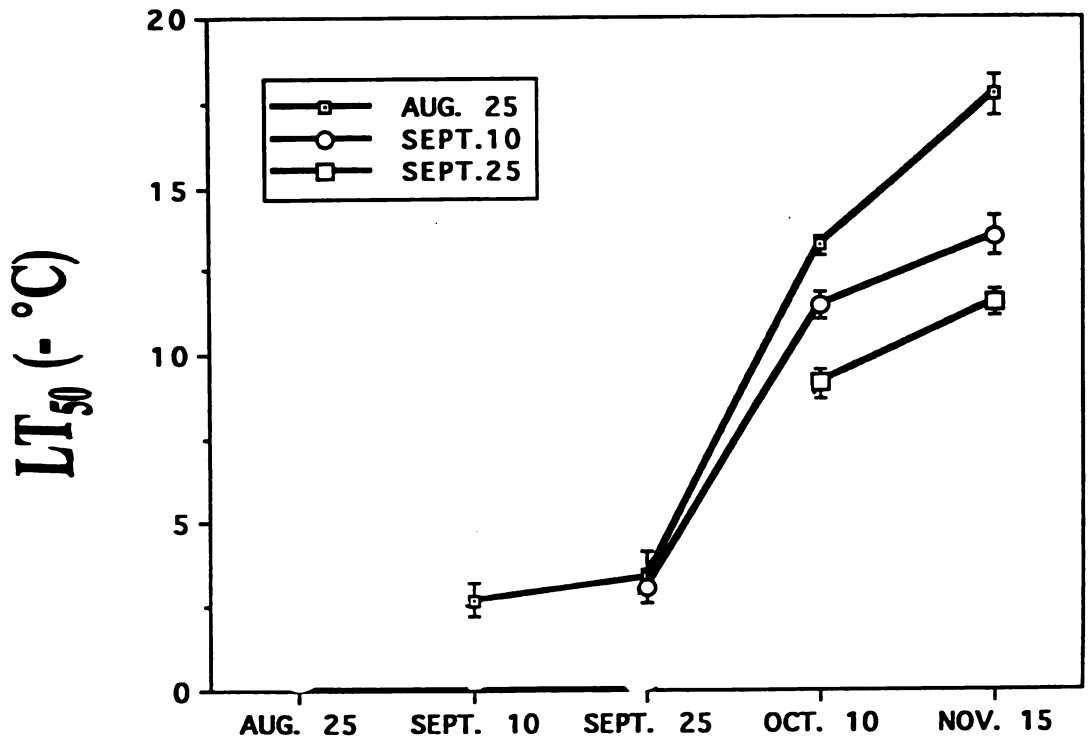

Figure 5. Changes in freezing tolerance of winter canola affected by different planting dates. Data represent overall means of six cultivars planted Aug. 25, and overall means of three cultivars planted Sept. 10 and Sept. 25. Freezing tests were performed at every 15-day or 30-day interval, depending on planting date. Vertical bar indicates mean ± SE.

probes (Fig. 6). The general pattern of expression of the two genes is shown in Fig. 7. Each data point is the overall mean for six cultivars. Both genes were turned on within 15 days of planting; however, the expression patterns of the two genes were different. The transcript amount of BN28 increased gradually throughout the experimental period, while that of BN115 increased rapidly within 15 DAP followed by a steady increase until the end of the experiment (Nov. 13). At this point, both genes retained their full transcript level. One interesting observation is that both the genes were turned on well before the onset of increase in freezing tolerance.

The expression of these genes in each cultivar was examined to determine the correlation between the freezing tolerance of a specific cultivar and transcript level (Table 1 and Table 2). There were no specific patterns of the gene expression to explain the difference in freezing tolerance among cultivars. Though most cultivars showed increases in BN28 expression over time, the differences among cultivars on a specific sampling date were inconsistant. In the case of BN115, there was no difference in gene expression at either among sampling dates or cultivars on a specific sampling date.

Figur

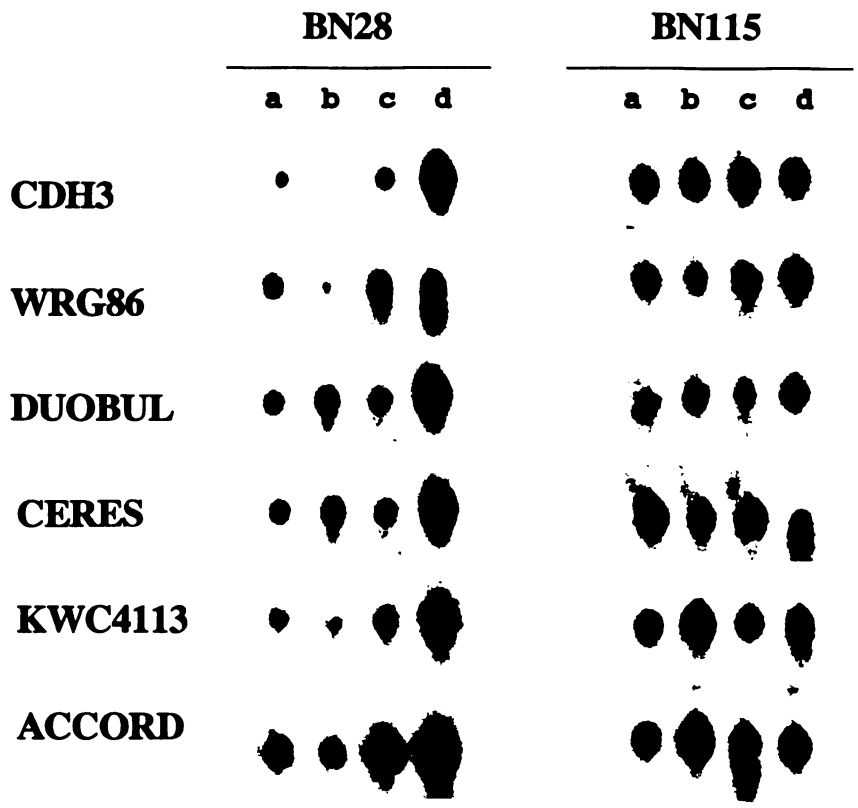

Figure 6. Northern blot analysis of six winter canola cultivars planted Aug. 25, 1993 in the field. Total RNA were isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d). The membrane was hybridized with BN28 and BN115.

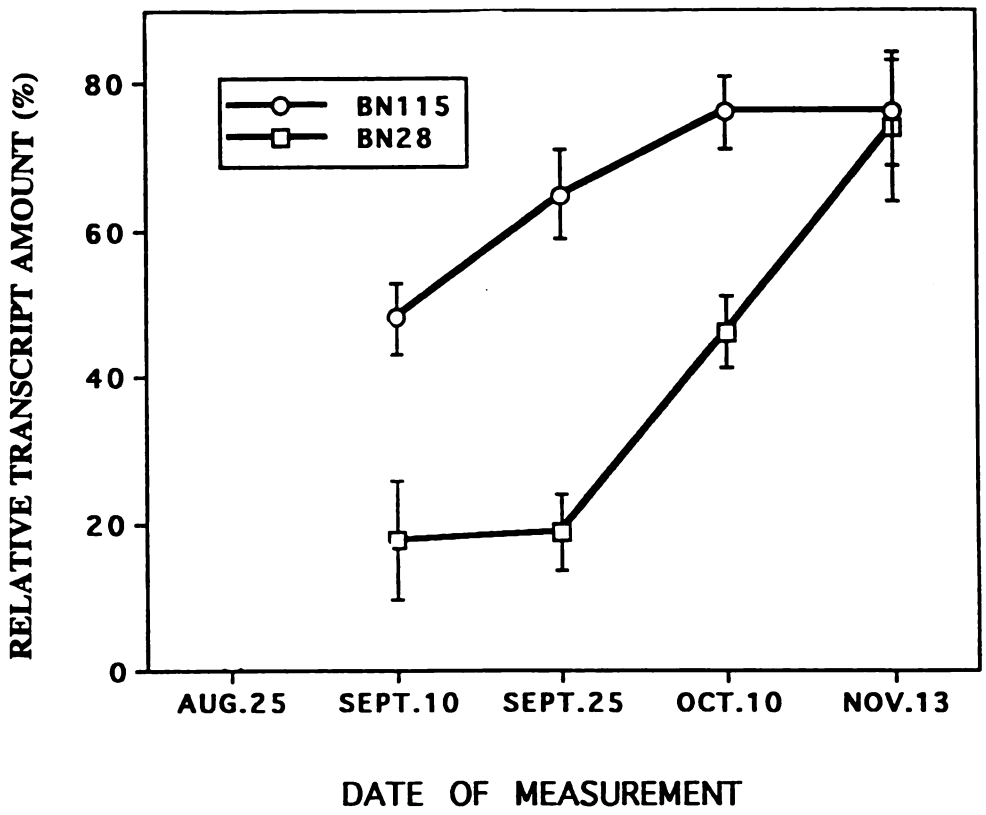

Figure 7. Time-course changes in transcript amount of BN28 and BN115 in canola planted Aug. 25, 1993. The data represent the overall mean of six cultivars and is a graphical representation of Fig. 6. Vertical bar indicates ± SE.

Table 1. Changes in transcript amount of BN28 in 6 winter canola cultivars planted Aug. 25, 1993. Data are numerical presentation of Northern blot in Fig. 6.

Cultivars	Relative transcript amount (%)			
	Sept. 10	Sept. 25	Oct. 10	Nov. 13
ACCORD	37a†	28ab	68a	100a
KWC4113	19a	16b	62ab	100a
CERES	20a	13b	37b	66c
DUOBUL	10a	18b	33b	82b
WRG86	10a	13b	35b	78c
CDH3	13a	31a	20c	80bc

† Means followed by the same letter within column are not significantly different at the 0.05 probability level.

Tabl

Cult

ACCO
KWC4
CERE
DUCB
WRG8
CDH3

Table 2. Changes in transcript amount of BN115 in 6 winter canola cultivars planted Aug. 25, 1993. Data are numerical presentation of Northern blot in Fig. 6

Cultivars	Relative transcript amount (%)			
	Sept. 10	Sept. 25	Oct. 10	Nov. 13
ACCORD	43b†	58b	58bc	50bc
KWC4113	46b	35c	47c	55b
CERES	35b	44c	46c	46c
DUOBUL	57a	100a	100a	70ab
WRG86	49a	95a	95a	100a
CDH3	60a	56ab	72ab	72ab

† Means followed by the same letter within a column are not significantly different at the 0.05 probability level.

Effect of Planting Date on the Expression of Cold-Regulated Genes, BN28 and BN115.

The effect of planting date on the expression of BN28 and BN115 was examined by northern blot analysis of total RNA from 3 winter cultivars at 3 different planting dates (Aug. 25, Sept. 10, and Sept. 25). Total RNA was isolated at 15 (Sept. 10), 30 (Sept. 25), 45 (Oct. 10), and 75 DAP (Nov. 13) for the Aug. 25 planting; 15 (Sept. 25), 30 (Oct. 10), and 60 DAP (Nov. 13) for the Sept. 10 planting; and 15 (Oct. 10) and 45 DAP (Nov. 13) for the Sept. 25 planting.

Total RNA was probed with BN28 (Fig. 8) and BN115 (Fig. 9). The graphical representation of the gene expression affected by different planting dates is shown in Fig. 10 (BN28) and Fig. 11 (BN115). There was both a slow and rapid phase of increase in expression of BN28. In the Aug. 25 planting, the increase in expression of BN28 was slow until 30 DAP, then increased rapidly from 30 to 45 DAP. Both in the Sept. 10 and Sept. 25 plantings, the rapid increase phase was coincident with that of the Aug. 25 planting regardless of plant ages. The maximum expression was attained at the end of the experiment (Nov. 13) and there was no difference in transcript amount of BN28 at the end of experiment among the three planting dates. BN115 reached its full expression within 15 DAP in two planting dates and later planting induced a higher expression than earlier planting at the end of the experiment (Nov. 13).

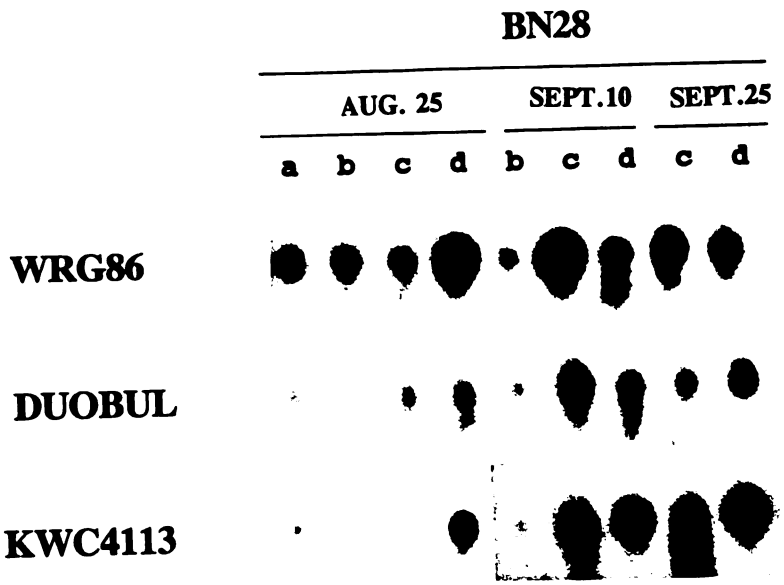

Figure 8. Northern blot analysis of 3 winter canola cultivars planted Aug. 25, Sept. 10, and Sept. 25, 1993, respectively. Total RNA from leaves of plants was isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d), respectively. The membrane was hybridized with BN28.

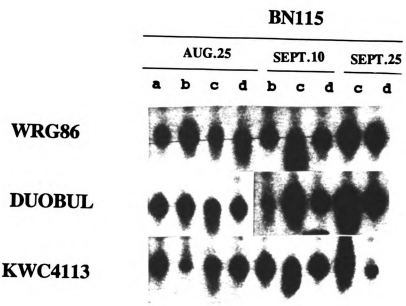

Figure 9. Northern blot analysis of 3 winter cultivars planted Aug. 25, Sept. 10, and Sept. 25, 1993, respectively. Total RNA from leaves of plants was isolated Sept. 10 (lane a), Sept. 25 (lane b), Oct. 10 (lane c), and Nov. 13 (lane d), respectively. The membrane was hybridized with BN115.

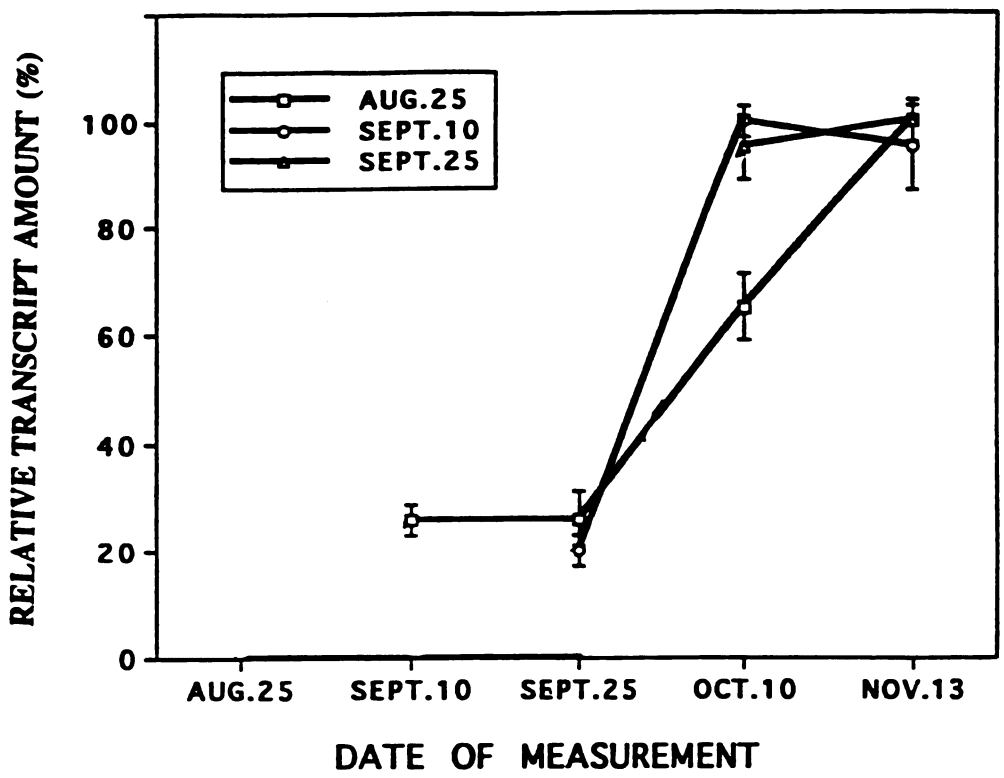

Figure 10. Changes in transcript amount of BN28 affected by different planting dates. Data represent overall means of three cultivars and a graphical presentation of the northern blot in Fig. 8. Vertical bar indicates mean ± SE.

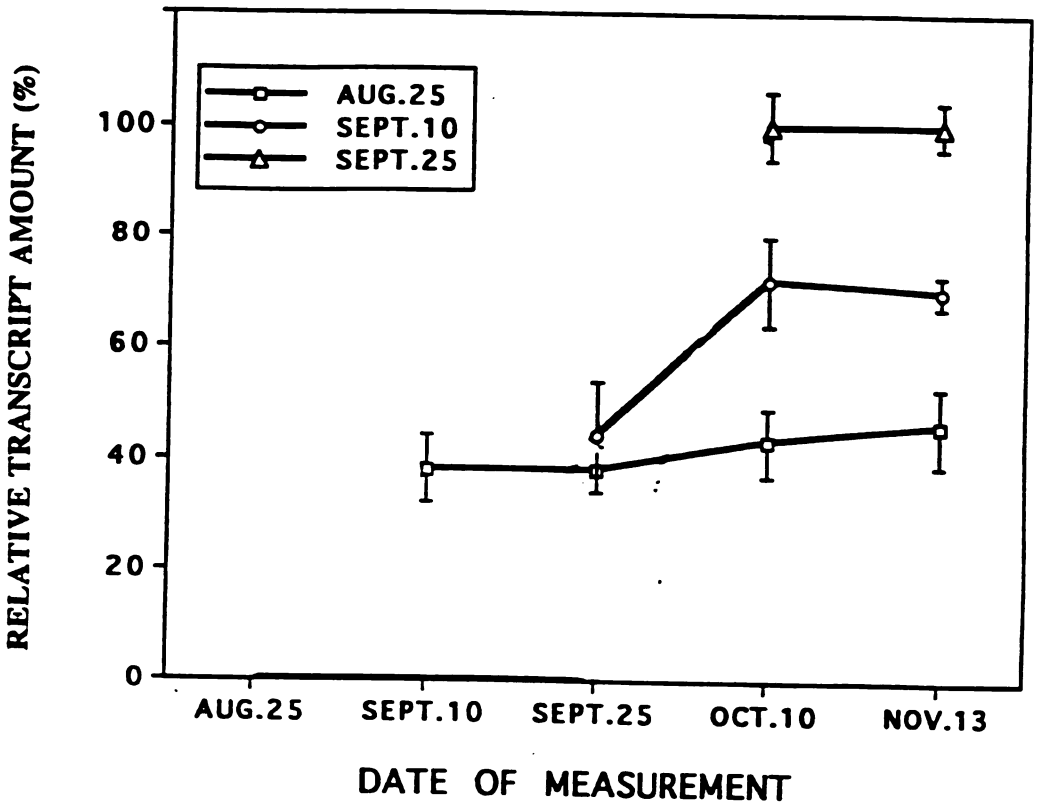

Figure 11. Changes in transcript amount of BN115 affected by different planting dates. Data represent overall means of three cultivars and a graphical presentation of the northern blot in Fig. 9. Vertical bar indicates mean ± SE.

Effect of Planting Date on Winter Survival of Canola

Although both cultivar and planting date significantly affected winter survival, the effect of planting date was greater than that of cultivar (Table 3). Earlier planting (Aug. 25) resulted in above 80 % survival except for the least hardy cultivar (66 %), while later planting (Sept. 25) resulted in no survival for four of the six cultivars.

The hardy cultivars, Accord and KWC4113, showed 40 and 20 % survival, respectively, even when planted Sept. 25, while other cultivars showed no survival. However, plant growth prior to freezing was much poorer than that of earlier plantings.

Correlation between Freezing Tolerance, Winter Survival, and Expression of BN28 and BN115

A significant correlation was observed between freezing tolerance and winter survival of cultivars in these studies (Table 4) except for the Sept. 10 sampling date from the Aug. 25 planting. Correlation coefficients ranged from 0.83(*) to 0.96(**).

Though the time-course increase in transcript amount of BN28 was correlated with time-course increase in freezing tolerance, no significant correlation occurred between the gene expression and freezing tolerance (Table 5), and eventually, with winter survival (Table 6) in cultivar level

Table 3. Winter survival of six winter canola cultivars, planted Aug. 25, Sept. 10, and Sept. 25, 1993.

Cultivars	Planting date		
	Aug. 25	Sept. 10	Sept. 25
	--winter survival (%)--		
ACCORD	100.0a†	72.0a	40.0a
KWC4113	100.0a	70.0a	20.0a
CERES	84.0b	59.0b	6.0b
DUOBUL	93.0b	65.0b	0.0c
WRG86	79.0bc	57.0b	0.0c
CDH3	66.0c	41.0c	0.0c

† Means followed by the same letter within a column are not significantly different at the 0.05 probability level.

Table 4. Simple correlation coefficients between freezing tolerance and winter survival of canola cultivars planted Aug. 25, Sept. 10, and Sept. 25, 1993.

Planting Date	Sampling Date	r
Aug. 25	Sept. 10	0.54ns
	Sept. 25	0.89*
	Oct. 10	0.85*
	Nov. 15	0.94**
Sept.10	Sept. 25	0.88*
	Oct. 10	0.96**
	Nov. 15	0.83*
Sept.25	Oct. 10	0.87*
	Nov. 15	0.93**

† (*) and (**) simple correlation coefficient significant at 0.05 and 0.01 probability levels, respectively.
(ns) not significant at the 0.05 probability level.

Table 5. Simple correlation coefficients† between transcript amount of BN28 and BN115 and freezing tolerance of canola cultivars planted Aug. 25, Sept. 10, and Sept, 25, 1993.

	r	
Planting Date	BN 28	BN 115
Aug. 25	0.59ns†	-0.37ns
Sept.10	0.47ns	-0.33ns
Sept.25	0.60ns	-0.40ns

† Correlation coefficient was estimated between transcript amount at Nov. 13 and freezing tolerance at Nov. 13.

‡ (*) and (**) simple correlation coefficient, significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

Table 6. Simple correlation coefficients† between transcript amount of BN28 and BN115 and winter survival of canola cultivars planted on Aug. 25, Sept. 10, and Sept. 25.

	r	
Planting date	BN 28	BN 115
Aug. 25	0.49ns‡	-0.27ns
Sept.10	0.15ns	-0.33ns
Sept.25	0.30ns	-0.34ns

† Correlation coefficient between winter survival and transcript amount at Nov. 13.

‡ (*) and (**) simple correlation coefficient, significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

at specific time of measurement. Transcript amount of BN115 was negatively correlated with freezing tolerance and winter survival, but no values were significant.

Effect of Planting Date and Seeding Rate on Winter Survival of Canola

Planting date and seeding rate had significant effects on winter survival of Duobul in both 1992-1993 and 1993-1994 (Table 7, 8). As the seeding rate increased, the winter survival decreased. However, there was no survival from the Sept. 25 planting from any of the three seeding rates during 1993-1994.

Effects of Planting Date and Fall-Applied Nitrogen on Winter Survival

The effects of planting date and fall-applied nitrogen on winter survival were examined in the less hardy cultivar, Duobul, in both 1992-1993 and 1993-1994 (Table 2.9). Twenty and forty kg/ha of nitrogen (as urea) were applied after seedling emergence. Only planting date had a significant effect on winter survival in both years; nitrogen had no significant effect.

Table 7. Effect of planting date and seeding rate on winter survival of canola cultivar, Duobul, in 1992-1993.

Planting date	Seeding rate (kg/ha)			
	4	8	12	Mean
	---winter survival (%)--			
Aug. 25	81	80	46	69a†
Sept. 10	83	80	39	67a
Sept. 25	69	60	46	58b
Mean	77a	73a	43b	

F- test:		
	Planting date (A)	8.7 **‡
	Seeding date (B)	10.6 **
	Interaction (A X B)	0.5 ns

† Means followed by the same letter within a column and rows are not significantly different at the 0.05 probability level.

‡ (*) and (**), significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

Table 8. Effect of planting date and seeding rate on winter survival of canola cultivar, Duobul, in 1993-1994.

Planting date	Seeding rate (kg/ha)			
	4	8	12	Mean
	---winter survival (%)--			
Aug. 25	87	80	56	74a†
Sept. 10	55	47	39	47b
Sept. 25	0	0	0	0c
Mean	47a	42a	31b	

F- test:		
	Planting date (A)	21.7 **‡
	Seeding rate (B)	12.6 **
	Interaction (A X B)	0.5 ns

† Means followed by the same letter within a column and rows are not significantly different at the 0.05 probability level.

‡ (*) and (**), significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

Table 9. Effect of fall-applied nitrogen and planting date on winter survival of canola cultivar, Duobul, in 1992-1993 and 1993-1994.

Nitrogen (kg/ha)	Winter survival(%)							
	----1992-1993---				-----1993-1994-----			
	Aug. 25	Sep. 10	Sep. 25	Mean	Aug. 25	Sep. 10	Sep. 25	Mean
Control	88	91	45	74	90	73	0	54
20	90	81	42	71	87	68	0	51
40	86	90	53	76	93	55	0	49
Mean	88a	87a	46b		90a	65b	0c	

F-test:		
Planting date (A)	31.5(**)	114.8(**)
Nitrogen (B)	0.4(ns)	1.2(ns)
A x B	0.8(ns)	0.8(ns)

‡ (*) and (**), significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

DISCUSSION

At all three planting dates, a phase occurred when a rapid increase in freezing tolerance occurred regardless of plant age. Between Sept. 25 and Oct. 10, the minimum air temperature decreased from 10°C to 4°C and freezing tolerance of plants increased rapidly. This is consistent with the findings of Kacperska-Palacz (1978) showing that alfalfa and rapeseed initiated cold-hardening at a temperature of 5°C, apparently without the transition to a state of readiness to harden seen in woody plants (Sakai and Larcher, 1978).

However, earlier planting resulted in greater freezing tolerance compared to later planting. The most hardy cultivar planted Aug. 25 reached an LT_{50} value of -20°C in mid-November, while that of the Sept. 25 planting reached only -13°C. In addition, planting date greatly affected how plants attained freezing tolerance. For all three planting dates the first phase of rapid increase in freezing tolerance was followed by a steady increase from mid-October to mid-November (Fig. 5). However, the pattern during the second phase differed among planting dates. The earlier planting showed a gradual increase in freezing tolerance until mid-November, which presumably continued until December. However, the later planting showed

less increase in freezing tolerance than the earlier planting in this period. This may be explained by differences in growth; earlier plantings grow more and accumulate more photosynthate reserves which increase cold acclimation. This suggestion is supported by published reports that reduction of carbohydrate reserves reduced cold hardiness in over-wintering plants (Levitt, 1956). Another possibility is that the gradual exposure to cold temperature is more effective than the sudden exposure. This is supported by a report by Pomeroy et al. (1974) that high levels of hardiness in wheat could be rapidly induced in 4-6 days if the hardening temperature was preceded by warm temperature, while previous reports (Pomeroy and Fowler, 1973; Andrew et al., 1974) showed that 6-8 weeks of hardening temperature were required for maximum hardiness without previous exposure to warm temperature.

Growth itself may affect the winter survival of canola. Earlier planting promotes greater fall foliar growth, which covers the growing point, thus protecting it. Later planting does not provide adequate foliar coverage; thus, the growing point remains vulnerable to freezing damage. This is consistent with data that snow cover increases winter survival of canola under severe winter conditions in Michigan's Upper Peninsula (Copeland, unpublished data).

The process of cold acclimation and development of freezing tolerance is a prime example of the interaction between genetic factors and the environment. Aside from the

effect of planting date, different cultivars showed different freezing tolerance and winter survival. The most hardy cultivars showed acceptable levels of winter survival when planted at dates too late for less hardy cultivars.

The study on the expression of the two cold-regulated genes, BN28 and BN115, revealed no clue about their roles in cold acclimation. Though there is no correlation between freezing tolerance and expression of these genes at the cultivar level at specific sampling times, observations that these genes are turned on in response to cold temperature in the field and that the time-course increase in BN28 expression is coincident with increase in freezing tolerance (Fig. 7 and Fig. 10) suggests that they may have a role in winter hardiness. Expression of the genes was examined only on the transcript level in this study. Future studies on translational level expression may explain cultivar differences in freezing tolerance.

In conclusion, planting date is the most important factor determining the freezing tolerance of canola, and freezing tolerance is highly correlated with winter survival. However, in case of unavoidable late planting, a choice of a hardy cultivar may compensate for the loss of freezing tolerance due to later planting. The possibility of the two cold-regulated genes for use as molecular markers for selection for winter hardiness is not promising.

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CHAPTER III

EFFECTS OF TWO PLANT GROWTH REGULATORS, CERONE AND TERPAL-C, ON FREEZING TOLERANCE AND WINTER SURVIVAL OF CANOLA (*Brassica napus* L.)

ABSTRACT

One of the most attractive short-term solutions postulated for increasing freezing tolerance of winter crops may be application of chemicals. This research was conducted to determine the effects of two plant growth regulators, Terpal-C and Cerone, as well as fall-applied phosphorus, on freezing tolerance and winter survival of canola. Three cultivars were planted on the Michigan State University Agronomy Farm at East Lansing, MI. on Sept. 10, 1992 and 1993. Chemicals were applied to one-month-old plants when they reached the 5 leaf stage. Ion leakage tests were conducted 25 days after chemical treatment. Winter survival was evaluated by counting the plant standing in the fall and spring.

Neither the chemicals nor the phosphorus have any effect in increasing freezing tolerance and winter survival. Instead, one of the chemicals, Terpal-C, inhibited natural cold hardening.

INTRODUCTION

Numerous efforts have been made to overcome the freezing problems of crop plants in agriculture, including breeding efforts to develop more hardy cultivars and in modification of cultural practices to ensure winter survival. Most of the hardy cultivars of wheat grown today contain major genes for cold hardiness (Gusta et al., 1990). Consequently, little or no progress has been made in enhancing the genetic potential of cold hardiness in winter wheat. Searches for cost-effective strategies in plant freezing protection have focused on short-term solutions because of the limited gene pool and difficulties in breeding procedures for cold hardiness (Fowler and Gusta, 1979).

One of the most attractive short-term methods for increasing freezing tolerance may be the application of chemicals (Marth, 1965; Chen and Li, 1976; Paquin et al., 1976; Proebsting and Mills, 1976; Raese, 1977; Ketchie and Miller, 1978; Gusta et al., 1982; Dunner and Gianfagna, 1988; Morrison and Andrews, 1993). One group of chemicals, called cryoprotectants, increases hardiness, *per se*, regardless of stage of development, while a second group, primarily growth regulators, modifies the stage of development, thus indirectly

modifying freezing tolerance. The basic idea is that because of the general negative correlation between growth rate and cold hardiness, one might expect growth-inhibiting chemicals to favor the development of hardiness and growth promoters to reduce hardiness (Howell and Dennis, 1980).

The development of freezing tolerance is controlled by plant hormones. ABA is known to be involved in cold acclimation of plants (Irving, 1969; Rikin, 1975; Waldman, 1975; Chen et al., 1979; Orr et al., 1986; Reany and Gusta, 1987; Lang et al., 1988; Mohapatra et al., 1989) and, coincidently, with expression of certain cold-regulated genes (Thomashow, 1990). Another plant hormone involved in cold hardiness is gibberellic acid (GA) which has an antagonistic effect on freezing tolerance. Waldman et al. (1975) suggested that ABA induces cold acclimation by inhibiting the synthesis of GA, and demonstrated that the exogenous application of ABA dramatically reduced GA level in cold hardy alfalfa cultivars. The primary synthetic plant growth regulators that increase winter hardiness have either antigibberellin or ethylene-producing characteristics. Though the role of these plant growth regulators in winter hardiness at the molecular and cellular level is uncertain, it is assumed that they prevent the loss of freezing tolerance (Gusta et al., 1990).

The objective of this study was to determine the effects of two plant growth regulators, Terpal-C and Cerone, on freezing tolerance and winter survival of canola.

MATERIALS AND METHODS

Three winter canola (*Brassica napus* L.) cultivars, WRG86, Duobul, and KWC4113, were used in this study.

Planting, Management and Experimental Design

This study was conducted at the Michigan State University Agronomy Farm in East Lansing, Michigan. A split-plot design with cultivar as the main plot and growth regulator as subplots was used with three replications. Seed was planted at the rate of 5.6 kg/ha in a 5-row plot 6 m long and 92 cm wide. The planting date for the first year was Sept. 5, 1992 and for the second year, Sept. 6, 1993.

Application of Plant Growth Regulators

Two plant growth regulators, Terpal-C (BASF) and Cerone (Union Carbide), were used for this experiment. Chemicals were applied with a compressed CO_2 sprayer one month after planting (Oct. 13, 1993) when most of the plants reached the 5 leaf stage. The chemicals were diluted with distilled water at the rate of 300:1, then applied at the rate of 1840 g/ha for Terpal-C and 620 g/ha for Cerone. Control plots were sprayed with distilled water.

Evaluation of Freezing Tolerance

In 1993-1994, freezing tests were conducted on plant samples collected just before chemical application (Oct. 10) and 25 days after chemical application (Nov. 5). Five different plants were collected from each plot in the field and immediately transferred to the laboratory. One leaf disc was taken from each plant and 3 leaf discs from the same plot were placed in a stoppered culture tube representing one replication. Freezing tests were conducted as described in Chapter I.

Evaluation of Winter Survival

Winter survival for both 1992-1993 and 1993-1994 studies were evaluated as described in Chapter I.

Phosphorus Experiment

The same cultivars were planted on the same date at a different experimental site. At planting time, 0, 100, and 200 kg/ha of actual phosphorus (as form of 46 % ingredient of fertilizer) were applied to provide 3 different P_2O_5 levels. Winter survival was evaluated as previously described.

RESULTS AND DISCUSSIONS

Effect of Plant Growth Regulators, Terpal-C and Cerone on Freezing Tolerance and Winter Survival of Canola

Typical freezing curves were developed from the two sensitive cultivars, WRG86 (Fig. 1) and Duobul (Fig. 2), and the hardy cultivar, KWC4113 (Fig. 3). All three cultivars showed similar responses to the growth regulator treatment.

Cerone had little or no effect in increasing freezing tolerance. However, Terpal-C decreased the freezing tolerances of all cultivars compared to that of control on Nov. 10 (Table 1). Interestingly, this treatment seemed to inhibit the normal development of freezing tolerance.

Both Terpal-C and Cerone treatment had no significant effect on the winter survival of canola in either year (Table 2 and Table 3). The only effect on winter survival was among cultivars, and only during the second year. No interaction occurred between growth regulator treatment and cultivar in either year.

The results from freezing tests are not consistent with those of Morrison and Andrews (1992) who reported that Terpal-C increased freezing tolerance. Terpal-C application to canola in the field increased freezing tolerance of canola by 1-2 °C

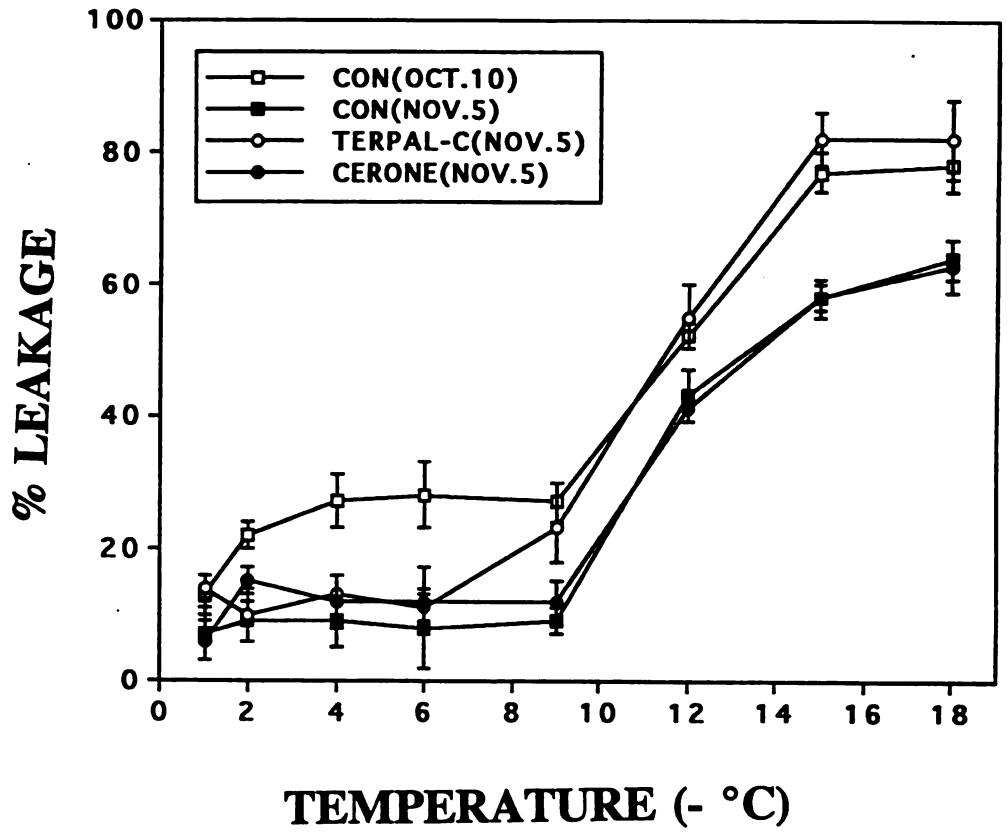

Figure 1. Effect of plant growth regulators, Terpal-C and Cerone, on freezing tolerance of winter canola cv. WRG86. Freezing tests were conducted before chemical treatment (Oct. 10) and 25 days after chemical treatment (Nov. 5). Vertical bar indicates mean ± SE.

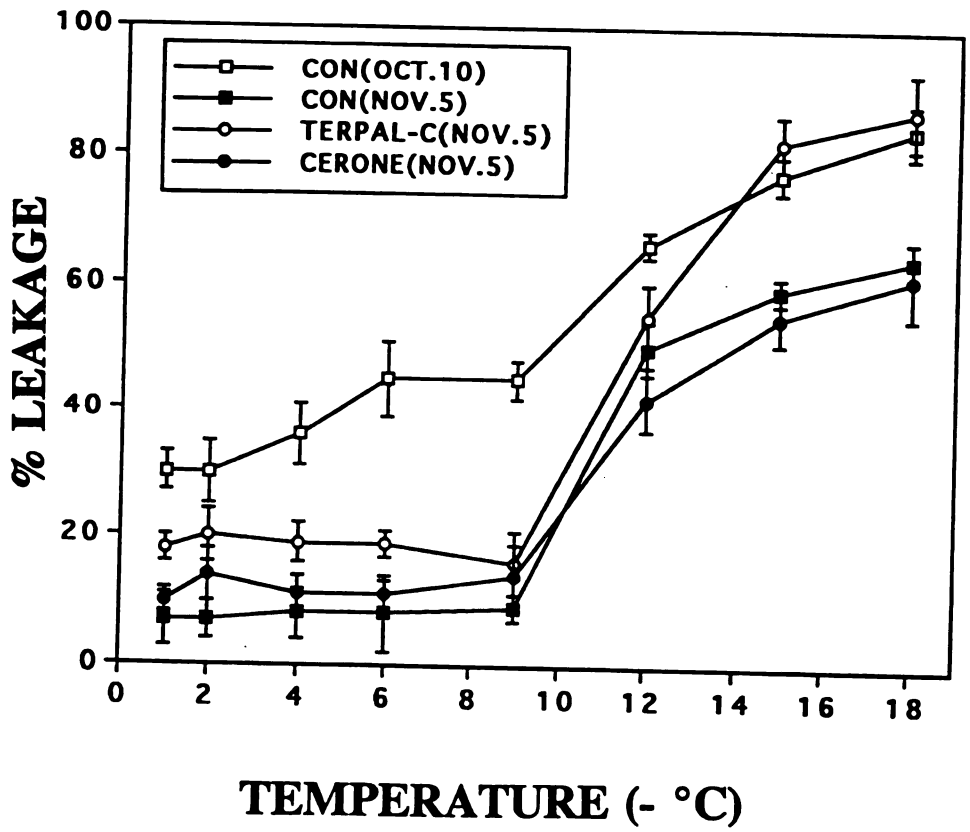

Figure 2. Effect of plant growth regulators, Terpal-C and Cerone, on freezing tolerance of winter canola cv. Duobul. Freezing tests were conducted before chemical treatment (Oct. 10) and 25 days after chemical treatment (Nov. 5). Vertical bar indicates mean ± SE.

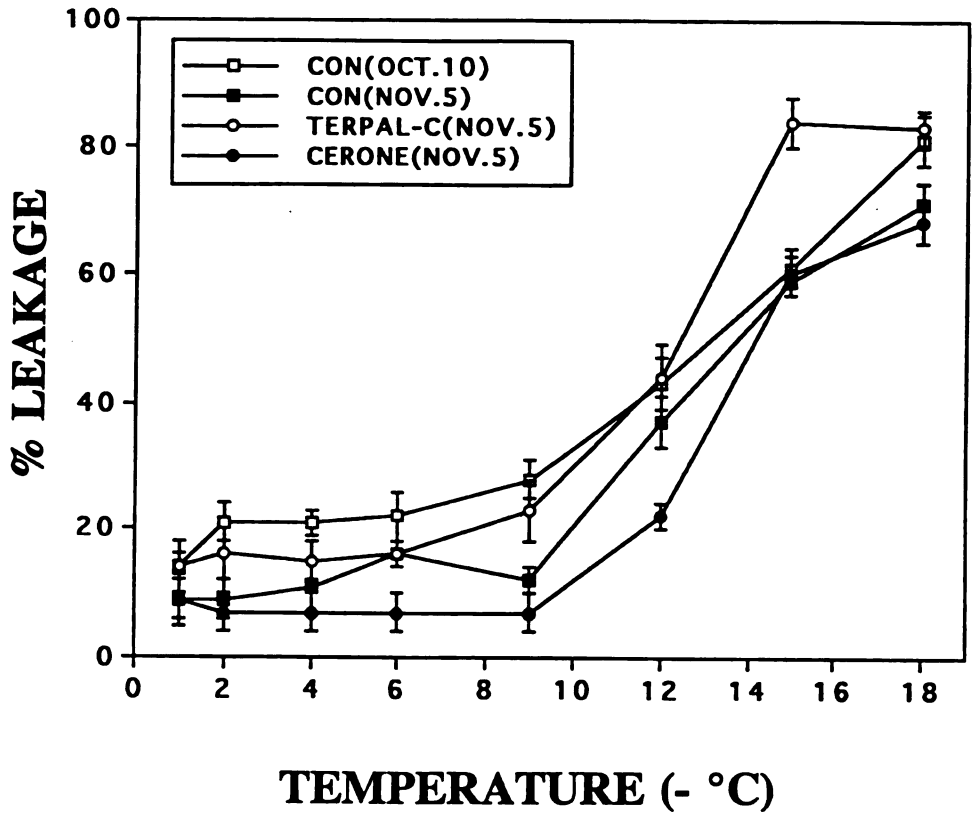

Figure 3. Effect of plant growth regulators, Terpal-C and Cerone, on freezing tolerance of winter canola cv. KWC4113. Freezing tests were conducted before chemical treatment (Oct. 10) and 25 days after chemical treatment (Nov. 5). Vertical bar indicates mean ± SE.

Table 1. Effect of plant growth regulators, Terpal-C and Cerone, on freezing tolerance of three canola cultivars, WRG86, Duobul, and KWC4113.

Chemical	LT_{50} (-°C)			
	WRG86	Duobul	KWC4113	Mean
Control†(Oct. 10)	10.3	9.5	12.1	10.6b
Control (Nov. 5)	11.9	12.0	13.8	12.6a
Terpal-C (Nov. 5)	9.2	10.4	11.4	10.3b
Cerone (Nov. 5)	11.7	12.6	13.6	12.3a
Mean	10.8c	11.2b	12.7a	

F-test:		
	Variety (A)	11.74 (**)‡
	Chemical (B)	12.75 (**)
	A x B	0.75 (ns)

† Two controls as before chemical treatment (Oct. 10) and after chemical treatment (Nov. 5).

‡ (*) and (**); significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at 0.05 probability level.

in December compared to the control. Whether decrease in freezing tolerance by Terpal-C treatment in this experiment is due to its effect on the retention of physiological immaturity or the inhibition of natural cold-hardening by the chemical application should be elucidated in future.

In spite of the decrease of freezing tolerance by Terpal-C, it did not affect winter survival. This may be additional evidence that its effect is not the increase in freezing tolerance but the prevention in loss of freezing tolerance during the middle of severe winters (Gusta et. al, 1992).

Effect of Fall-Applied Phosphorus on the Winter Survival of Canola

In the 1993-1994 experiments, the effect of phosphorus on freezing tolerance of three canola cultivars was examined by ion leakage tests on Nov. 15. Phosphorus treatment did not affect freezing tolerance (Table 4) and winter survival of canola in either year (Table 5).

The experimental evidence of an association between phosphorus and winter hardiness is limited and somewhat controversial. Levitt (1980) indicated that there were as many investigators who found no association between phosphorus and winter hardiness as there were who found a positive association. The role of phosphorus in winter hardiness is

Table 2. Effect of plant growth regulators, Terpal-C and Cerone, on winter survival of three canola cultivars in 1992-1993.

Chemical	Winter survival(%)			
	WRG86	Duobul	KWC4113	Mean
Control	82.0	80.3	86.0	83.0
Terpal-C	92.0	81.0	90.3	88.0
Cerone	83.0	91.0	95.6	89.0
Mean	80.5	84.3	90.8	
F-test:	Variety (A)		1.160 (ns)†	
	Chemical (B)		0.270 (ns)	
	A x B		1.470 (ns)	

† (*) and (**); significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at 0.05 probability level.

Table 3. Effect of plant growth regulators, Terpal-C and Cerone on winter survival of three canola cultivars in 1993-1994.

Chemical	Winter survival(%)			
	WRG86	Duobul	KWC4113	Mean
Control	60.0	65.0	76.5	67.0
Terpal-C	63.5	58.0	73.5	65.0
Cerone	58.0	59.0	78.5	65.1
Mean	60.3b	60.6b	76.1a	
F-test:	Variety (A)		3.200 (*)†	
	Chemical (B)		0.270 (ns)	
	A x B		1.470 (ns)	

† (*) and (**); significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at 0.05 probability level.

Table 4. Effect of fall-applied phosphorus on freezing tolerance of three winter cultivars in 1993-1994

Treatment	Cultivars			
K_2PO_4 (kg/ha)	WRG86	DUOBUL	KWC4113	
		---LT_{50} at Nov. 15---		
Control	11.5	11.2	13.0	11.9
100	10.9	11.8	12.9	11.8
200	9.4	11.1	11.9	10.8
Mean	10.6c	11.4b	12.6a	
F-test : Variety (A)		4.34 (*)†		
Phosphorus (B)		0.78 (ns)		
A x B		0.94 (ns)		

† (*) and (**); significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

Table 5. Effect of fall-applied phosphorus on winter survival of three winter cultivars in 1992-1993 and 1993-1994.

K_2PO_4 (kg/ha)	Winter survival (%)							
	----1992-1993---				-----1993-1994-----			
	WRG 86	Duo-bul	KWC4 4113	Mean	WRG 86	Duo-bul	KWC 4113	Mean
Control	81	80	86	83	56	52	73	60
100	97	97	87	93	42	65	82	63
200	91	83	79	84	55	56	75	63
Mean	89	86	84		51b	57b	76a	
F-test:								
Variety		(A)		1.35(ns)†			4.8(*)	
Phosphorus		(B)		0.88(ns)			1.2(ns)	
A x B				0.73(ns)			0.8(ns)	

† (*) and (**), significant at 0.05 and 0.01 probability levels, respectively. (ns) not significant at the 0.05 probability level.

also controversial. Phosphorus is known to favor accumulation of photosynthate, which can be used in the hardening process. Phosphate accumulated in the plant also enhances the regrowth of roots in the spring to enable recovery of the damage by winter freezing (Robertson et al., 1992).

Either phosphorus does not have any relation to freezing tolerance and winter survival of canola, or the winters were not adequately severe to reveal the difference induced by phosphorus treatment. Finally, the effect of phosphorus may have been masked by natural cold-hardening.

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