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Production of mutants with high cold tolerance in spring canola (*Brassica napus*)

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Abstract A major factor affecting spring canola (*Brassica napus*) production in Canada is killing frosts during seedling development in the spring and seed maturation in the fall. The objective of this study was to explore the possibility of producing spring canola lines with mutations that have altered biochemical pathways that increase cold tolerance. The approach was to generate UV point mutations in cultured microspores followed by chemical in vitro selection of individual mutant microspores or embryos resulting in measurable alterations to various biochemical pathways with elevated levels of key defense signaling molecules such as, salicylic acid (SA), *p*-Fluoro-D,L-Phenyl Alanine (FPA), and jasmonic acid (JA). In addition, since proline (Pro) is known to protect plant tissues in the cold-induced osmotic stress pathway, mutants that overproduce Pro were selected in vitro by using three Pro analogues: hydroxyproline (HP), azetidine-2-carboxylate (A2C); and, 3,4-dehydro-D,L-proline (DP). Of the 329 in vitro selected mutant embryos produced, 74 were identified with significant cold tolerance compared to their donor parents through indoor freezer tests at –6°C, and 19 had better winter field survival than winter canola checks. All chemically selected mutant doubled haploids with increased cold tolerance compared well with parent lines for all seed quality and agronomic parameters. Development of increased frost tolerant cultivars should allow for spring canola to be produced in western Canada without compromising seed quality.

Keywords Spring canola · Cold tolerance · Jasmonic acid · Mutagenesis · Proline · Salicylic acid

Abbreviations

A_2C	Azetidine-2-carboxylate
B_5G	Solid germination medium
DH	Doubled haploid
DP	3,4-dehydro-D,L-proline
FPA	*p*-Fluoro-D,L-Phenyl Alanine
HP	Hydroxyproline
JA	Jasmonic acid
LD_{50}	Lethal dose at 50% kill
NLN	Liquid embryo initiation medium
Pro	Proline
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	System acquired resistance
SD	Spontaneous diploid
UV	Ultra violet

Introduction

Of the three main oil-producing crops in the world, canola, soybean and palm, canola is the only one grown exclusively in the temperate zone. Therefore

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the major factor affecting canola production in western Canada is the short frost-free period that ranges from 130 days in the long season zone in southern Manitoba to 100 days in the short season of northern Alberta. Incidences of early or late frosts during the growing season have serious detrimental effects on canola yield and seed quality. A spring frost kills plants leading to uneven stands with variable maturities affecting the quality of harvested seed while fall frost stops the enzymatic activity that breaks down chlorophyll during maturation. Chlorophyll retained in the seed seriously downgrades canola oil by turning the crude oil from a desired golden colour to green and contributes to an off-flavour of the oil if processed using conventional refining methods. It was estimated that only 35% of the Saskatchewan canola crop was graded No.1 Canada compared to the 10 year provincial average of 79% (DeClercq 2004) due to green seed. The cost of removing chlorophyll from canola oil is up to $30/t at the 10% green seed level. Producers can potentially lose millions of dollars annually in crop productivity and market quality due to frost damaged crops (Canola Council of Canada 2004).

The majority of previous research in canola frost and cold tolerance applied genetic engineering approaches to crop improvement. Transformation of spring canola with five genes from bromegrass and fall rye associated with frost tolerance has produced breeding lines with some improvement in cold tolerance (Gusta et al. 2001). Anti-sense gene (complement) transformations, which targeted a specific gene that produces a protein that binds chlorophyll and makes it stable, produced transgenic lines that contained no green seed (Johnson-Flanagan et al. 1999), while Thomashow (1998) identified a family of genes that encode proteins, which turn on freezing tolerance genes. To date, there have been no transgenic, cold tolerant spring canola varieties commercially released.

Increased levels of specific phenolics have been reported to physically protect plants against cold and frost stresses (Solecka and Kacperska 1995). Fletcher and Kott (1999) used doubled haploid, ultra-violet mutagenesis combined with in vitro chemical selection with inhibitors of phenylalanine ammonia-lyase (PAL) and proline (Pro) biosynthesis to identify increased phenolic levels in plants with subsequent improved cold tolerance. Furthermore, Johnson-Flanagan and Singh (1991) applied ABA to haploid *Brassica napus* embryos and found no green seed in the harvested plants, since abscisic acid (ABA) has been shown to have a role in promoting the degradation of chlorophyll and preventing green seed formation after frost stress (Green et al. 1998).

Maximum genetic potential of freezing tolerance in plants greatly improves with a prior period of cool temperature acclimation and an optimal 3-day acclimation period was reported in susbsequent indoor freezing tests (Rife and Zeinali 2003). Research has shown that during cold acclimation several genes are induced, lipid membrane composition is changed, Pro and other cryoprotectants accumulate and active oxygen species are detoxified (Hincha 2002; Iba 2002).

Oxidative stress contributes to cold temperature injury in plants, which occurs when there is an excess of free radicals within the cells (McKersie and Bowley 1997; Halliwell and Gutteridge 1999). Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, and hydroxyl radicals, damage cell membrane components, degrade polysaccharides, and denature enzymes (Halliwell et al. 1988; Monk et al. 1989). Plants have developed very efficient antioxidant systems to scavenge ROS to provide protection against oxidative stress (Allen 1995). Antioxidant enzymes (e.g. catalase, superoxide dismutase) are the most active and protective mechanism against such oxidative stress (Allen 1995; Halliwell et al. 1988). For example, small increases of hydrogen peroxide occur in response to minor environmental stresses that signal the plant to modify gene expression that activates plant defense responses, and with the exception of catalase, all genes for antioxidant enzymes increased during cold acclimation in wheat (Baek and Skinner 2003).

Murata and Los (1997) found that cold stress in plants is sensed by changes in membrane fluidity and cytoskeletal reorganization (Örvar et al. 2000), which alters the calcium channels and calcium levels are cited as the primary stimulus for sensing cold stress (Plieth 1999). Therefore, it is essential for plants to stabilize membranes during cold acclimation to prevent freezing damage. Phenylpropanoid metabolism also increases in plants under cold stress by producing phenolics, which are strong antioxidants and also increases cell wall lignification or suberization which protect plant tissues (Chalker-Scott and

Fuchigami 1989). The Pro and phenolic pathways are closely linked as Pro metabolism provides the precursors for the production of phenylalanine, as the precursor for phenylpropanoid biosynthesis (Hare and Cress 1997).

Many plants accumulate free Pro, which is an organic osmolyte, in response to abiotic stresses such as low temperature, drought, and high salinity (Hare et al. 1998). When plants are exposed to environmental stresses, Pro mediates a number of cellular and subcellular processes. Proline accumulates in plants under stress conditions by activating Pro biosynthesis and inhibition of Pro degradation (Nakashima et al. 1998) by osmoregulation and is quickly consumed after the stress has dissipated (Trotel-Aziz et al. 2003). It is thought that high levels of Pro stored under stress conditions may be very beneficial during stress recovery as Pro degradation can provide carbon and nitrogen and is capable of high energy output required of plant cells to recover from stress (Peng and Verma 1996; Hare and Cress 1997). Despite the overall protective role of Pro in response to environmental stress, degredation intermediates of Pro have been reported to induce plant toxicity due to incomplete or deficient enzyme activity (Hellman et al. 2000; Deuschle et al. 2001).

Recent studies have demonstrated the valuable effects of salicylic acid (SA) on cold tolerance in major economically important crops, including maize, rice, tomato and wheat (Janda et al. 1999; Kang and Saltveit 2002; Senaratna et al. 2000; Tasgin et al. 2003). Salicylic acid based signaling mechanisms were shown to occur in response to chilling tolerance and to pathogens (Scott et al. 2004), illustrating common pathways and components driven by biotic and abiotic stresses (Pastori and Foyer 2002). SA plays a key role as a signaling molecule involved in both the induction of systemic acquired resistance (SAR) and in local defense reactions when under abiotic stress. During stress, the SA increases which signals and maintains a SAR. Elevated levels of endogenous SA function as antioxidants reducing oxidative damage which are direct scavengers of ROS (Halliwell et al. 1988) and are associated with mediating oxidative burst that lead to hypersensitive response lesions. SAR, which is a long lasting, broad-based resistance that occurs in plants exposed to stresses in which a signal derived from the initial affected tissue travels to defend unaffected plant tissues (Borsani et al. 2001).

Jasmonic acid enhances the expression of plant defense genes (Creelman and Mullet 1997) and with SA, these acids are both thought to be involved in the transportation of chemical signals in plants (Sasaki et al. 2001). Jasmonic acid is derived from linolenic acid released from plasma membrane lipids (Ryan 1992) or plastid membranes (Vick and Zimmerman 1987) converted to JA by enzymatic action. JA biosynthesis is initiated by pathogens or insect pests, which produce elicitors and systemic signaling molecules that interact with plasma membrane receptors (Ryan 1992). Past research has shown that the primary interaction between the JA and SA pathways are mutually antagonistic (Kunkel and Brooks 2002). This relationship works by one pathway inducing defense genes against a particular stress while down regulating defense activities of the other pathway.

An effective in vitro microspore culture, UV mutagenesis system, and chemical selection exists for the production of mutants of *Brassica napus* with improved quality and disease resistance traits (Kott 1995; Kott et al. 2002; Beaith et al. 2005). Haploid and doubled haploid tissues and plants express all traits, including native or induced mutations. Genetically fixed, homozygous beneficial mutations generated in vitro and identified in doubled haploid lines are fully expressed and directly inherited, allowing for rapid commercial exploitation. The objective of this research was twofold: To develop a method for improving frost tolerance in spring type *Brassica napus*, and to develop canola germplasm that is more tolerant to frost than existing commercial Canadian canola spring varieties without sacrificing agronomic or seed quality.

Materials and methods

Plant materials

The five spring canola breeding lines used in this study were: Lolinda, SC990155, SC990221 (University of Guelph), PR6450 and PR6450 (Monsanto Canada Inc.). All Guelph lines are doubled haploids while the others are F6 generation pedigree bred Monsanto lines. Lolinda and PR6450 are low

linolenic cultivars while PR6327 is Roundup™ tolerant with the RT73 transgenic insertion.

Plant culture method

Six donor plants per line were grown in 2L pots in LA4 Professional Growing Mix maintained in a growth room at 22°C/18°C D/N, with an18h photoperiod (200 μmol $m^{-2}s^{-1}$ PAR). Plants were watered daily and fertilized with 20–20–20 (N–P–K) every second day. When flowering began, buds were harvested for isolated microspore culture, mutagenesis, in vitro selection and mutant doubled haploid production. The standard doubled haploid production protocol was followed (Kott 1995). In short, buds 3.5–4.5 mm were harvested, surface sterilized (10 min in 5.6% sodium hypochlorite), rinsed and macerated in a mechanical blender (20 s) in 5 ml of 13% sucrose sterile water. The spore homogenate was filtered through nested Nytex filters (63 and 44 μm), filtrate was poured into 50 ml conical centrifuge tubes, the volume was brought up to 50 ml with sterile13% sucrose water and centrifuged for 5 min at 900*g*. Centrifugation was repeated two more times, changing the supernatant each time, then the spore pellet was suspended in 13% sucrose NLN liquid medium with the spore concentration adjusted to 80,000 microspores per ml of medium. About 15 ml of the microspore suspension was pipetted into each standard size Petri plate and sealed with a double layer of Parafilm.

Mutagenesis

In vitro mutagenesis was done after plating, by exposing plates of microspores to 280 nm short-wave ultraviolet light (model C81, Ultra-Violet Prod. Inc., San Gabriel, CA). The duration of UV exposure was between 75 s and 90 s and was determined for each line based on irradiation tests that delivered a 50% reduction in embryogenesis (LD_{50}). One plate per culture was not irradiated and was maintained as the control. Plates were cultured at 30°C in a light-proof box placed in an incubator for 14 days. Cell divisions and tiny globular embryos were observed after seven days. On Day 14 the light-proof box was placed on a gyratory shaker (model G10, New Brunswick Scientific, Edison, NJ) set at 52 rpm at room temperature (21°C) for embryo maturation (Day 28).

Plant regeneration

Ten to 15 embryos per plate were transferred to B_5G solid germination medium, 28–35 days after culture (Fletcher et al. 1998) and placed in a 4°C chamber for 10 days to stimulate plantlet regeneration. The plates were moved to a 25°C, 12-h photoperiod chamber for 30 days for plant development and then plantlets were transplanted to 72 cell insert KORD flats (LA4 Professional Growing Mix) in the growth room. Flats were covered with a clear plastic lid for the first week for growth room acclimation.

Development of in vitro chemical selection

Preliminary experiments were conducted to determine the chemical concentration of specific compounds to impose adequate selection pressure on the microspores in liquid medium (NLN) or on germinating embryos/plantlets on solid B_5G medium. These preliminary experiments were conducted on non-mutagenized microspores in order to eliminate any mutagenic effects on chemical selection rates. Microspores and embryos were screened with a wide range of concentrations of the various selection agents to identify the optimal concentration for selection pressure. The concentrations of chemical selection agents tested ranged from 0.05 mM to 10.0 mM for the following agents: 5-chlorosalicylic acid (SA); JA; *p*-fluoro-D,L-phenylalanine (FPA); and, three Pro analogues: hydroxyproline (HP); azetidine-2-carboxylate (A2C); and, 3,4-dehydro-D,L-proline (DP). Duration of chemical selection ranged from 2 days to 24 days after culture either in NLN or flooded over the solid B5G medium. Chemical selection agents remained in/on the medium until surviving mutants were identified and transferred. Control plates with no chemical selection, were carried through the in vitro and solid germination media for comparison. Chemical selection of microspores in vitro was optimal when the culture was between 2 days and 10 days old. Chemical selection of plantlets on solid B_5G was effective during root development between 54 days and

70 days in culture. Since ethanol and methanol were used to dissolve SA and JA, respectively, control plates were exposed to similar concentration percentages of ethanol or methanol to determine whether these solutions altered selection capacity.

Mutagenized microspores were selected at the single cell stage in vitro (1–3 days old) with SA, JA, FPA and Pro analogues HP, A2C and DP. Selection by flooding the B5G solid was done using SA, FPA, A2C and DP when the plantlets were 24 days and 40 days old.

Preparation of specific selection agents

5-chlorosalicylic acid (SA)

A 5.8 mM SA stock solution (molecular weight of SA = 172.57 g/mol) was prepared by dissolving 10 mg of SA in 1 ml of 95% alcohol. The volume was made up to 10 ml with double distilled sterile water or NLN. A 10 ml syringe fitted with a 0.22 μm pore size filter (Fisher Scientific, Whitby, ON) was used to filter sterilize the 5.8 mM SA stock solution. A 10% ethanol solution was prepared by adding 1 ml ethanol to 9 ml of double distilled sterile water or NLN and filter sterilized. The 5.8 mM SA stock and ethanol stock solutions were stored at 4°C. The following formulas were used to determine the volume required to treat the microspores at the various concentrations of SA and to calculate the final ethanol concentration in the treated plates, respectively.

$$\text{Treatment volume required} = \frac{\text{Concentration of SA} \times \text{volume of medium}}{\text{Concentration of stock SA}}$$

$$\text{Quantity of ethanol in SA treatment} \times 100 = \frac{\%\ \text{Final concentration}}{\text{Volume of medium/Petri plate}}$$

Jasmonic Acid (JA)

A 95.17 mM JA stock solution (molecular weight of JA = 210.3g/mol) was prepared by dissolving 100 mg of JA in 5 ml of methanol. A 5 ml syringe fitted with a 0.22 μm pore size filter was used to filter sterilize the 95.17 mM JA stock solution which was stored at –20°C in a freezer. Example of concentration derivations: 0.00788 ml of 95.17 mM JA was used to treat microspores in a plate at a 0.05 mM JA chemical selection rate.

p-*fluoro-D,L-Phenyalanine (FPA)*

A 5.46 mM FPA stock solution (molecular weight of FPA = 183.20g/mol) was prepared by dissolving 10 mg of FPA in 1 mL of 95 % ethanol. The volume was made up to 10 ml with double distilled sterile water or NLN. The 5.46 mM FPA stock solution was sterilized through a 0.22 μm pore size filter and stored at 4°C. The required treatment volume formula above was used to determine the volume required to treat the cultures at the various concentrations of FPA.

Azetidine-2-Carboxylate (A2C)

A 9.89 mM A2C stock solution (molecular weight of A2C = 101.1 g/mol) was prepared by dissolving 10 mg of A2C in 1 ml of double distilled sterile water. The volume was made up to 10 ml with double distilled sterile water or NLN and filter sterilized using a 0.22 μm pore size filter. The 9.89 mM A2C stock solution was stored at 4°C. Using the formula cited above, treatment volumes were calculated for various concentrations of A2C.

Proline inhibitor: 3,4-dehydro-D,L-Proline (DP)

A 8.84 mM DP stock solution (molecular weight of DP = 113.1 g/mol) was prepared by dissolving 10 mg of DP in 1 ml of double distilled sterile water. The volume was made up to 10 mL with double distilled sterile water or NLN and filter sterilized using a 0.22 μm pore size filter. The 8.84 mM DP stock solution was stored at 4°C. Using the formula cited above, treatment volumes were calculated for various concentrations of DP.

Proline analogue: Trans-4-hydroxy-L-proline (HP)

A 91.56 mM HP stock solution (molecular weight of HP = 131.1 g/mol) was prepared by dissolving

12 mg of HP in 1 mL of double distilled sterile water. The volume was made up to 10 mL with double distilled sterile water or NLN and filter sterilized using a 0.22 μm pore size filter. The 91.56 mM HP stock solution was stored at 4°C. Using the formula cited above, treatment volumes were calculated for various concentrations of HP.

Colchicine doubling and doubled haploid (DH) seed increase

Haploid mutant plants were chromosome doubled when flowering began. Roots were washed and immersed in 0.34% (w/v) solution of colchicine (Sigma-Aldrich Canada Ltd., Oakville, ON) kept under fluorescent lights for 2 h (Fletcher et al. 1998). After thorough rinsing plants were replanted into KORD (24 cells/flat) with pre-watered LA4 Professional Growing Mix. At bolting, plants with doubled sectors were individually covered with pollination bags (Cryovac^{TM}, Crawford Packaging, London, ON) to produce selfed doubled haploid (DH) seed. Spontaneous diploids (SD) (appearing at ~10%) identified by larger flowers with fertile anthers and abundant pollen were not doubled, but transferred to 4″ pots and covered with pollination bags.

Three high quality selfed seeds harvested from mutant DH or SD plants were grown out for selfed seed increase under the same growing environment as the donor plants. Each DH line was assigned an ID number 997, followed by a mutant line code. These selfed DH lines were used for the indoor/outdoor cold tolerance screens.

Cold tolerance freezer test screening

Increased selfed seed of all in vitro selected plants, were used for screening for cold tolerance. For each mutant line 18 plants were grown to the 3–4 leaf stage in 2, 3 × 3 flat inserts (9 cells each, 8 inserts per flat) as two replications. Two 72-cell flats were tested together in a large chest freezer, including replicates of one control spring line (Lolinda) and one control winter line (Otrad 18) as checks. Each 9-cell pack was an experimental unit with 9 plants each.

Flats were watered before acclimation in a 4°C, 8-h photoperiod growth chamber for 48 h. Inside the freezer with computer controlled temperature probes set at –6°C, separated 9-cell packs were randomly placed on a free-standing bench 40 cm above the bottom of the freezer with an 8 cm gap around all sides. The temperature probes were set on aluminum brackets on the bench at the same height as the plants. Two fans mounted underneath the bench provided continuous airflow with a consistent temperature throughout the freezer. The duration of the freezing test was 12 h.

After freezing, plants were returned to the acclimation chamber for 24 h before being moved back to the growthroom. After a 3-week recovery period, plant survival was scored as: dead, stressed (surviving but very weak plants with no new growth), or living (plants that have developed and maintained photosynthetically actively growing leaf tissue). Mutant lines were evaluated by comparing their freezing tolerance to the spring control, Lolinda. The winter check having strong frost tolerance was used for validation of the experiment and for mutant performance comparisons.

All high scoring DH mutant lines were retested in a second freezer screen test along with their parental lines, for performance confirmation. Mutant line survival scores in the second freezer screen tests were subjected to and analysis of variance (ANOVA) using the software Statistica^{R} (version 5). Significance of differences between pairs of sample means was estimated by a two-tailed t test. Excel^{R} was used to calculate means and standard errors.

Proline assay

Four chemically selected mutants that showed enhanced freezing tolerance were evaluated for leaf Pro content together with the parent lines. One mutant line each from four chemical selections (997-48 [DP], 997-201 [A2C], 997-8 [JA], 997-121 [SA]) was analysed. Twenty-one-day old plants were moved to a 4°C acclimatization chamber for 48 h prior to Pro testing. Plants were exposed to the 4°C for the same 48-h time period at 21 days of age. Proline content was determined using a revised ninhydrin method (Huguet-Robert et al. 2003). Eight 7 mm diameter leaf discs per plant were obtained using a single hole punch and placed into pre weighed glass vials on a bed of ice and weighed to obtain the sampled leaf weight. Two milliliters of

distilled water were added and placed in a boiling water bath for 30 min then cooled to room temperature. One hundred microliters of the sample that contains hot water soluble compounds was mixed with 400 µl of distilled water and 1 ml of acid ninhydrin (0.25 g ninhydrin, 15 ml acetic acid, and 10 ml of distilled water) into a new vial. Separate vials were prepared in duplicate with 1 mM L-Pro (100 µl 1 mM L-Pro, 400 µl distilled water, 1 ml acid ninhydrin) solution as a standard. A blank sample (500 µl distilled water, 1 ml acid ninhydrin) was prepared to tare the spectrophotometer at 520 nm. These mixtures were boiled for 20 min in a boiling water bath and then cooled on a bed of ice. Three milliliter of toluene was added to each vial and shaken vigorously. The vials were placed in the dark for 2 h and the chromophore formed was transferred into a quartz cuvette and absorbance was measured at 520 nm on the spectrophotometer.

The absorbance readings were divided by 0.2164 to convert into mM then divided by 1000 to convert into µM. The µM was divided by 1000 and multiplied by two to give µmol Pro value in 2 ml then divided by the leaf weight (grams) to give µmol/g fresh weight which was multiplied by 10 to give the dry leaf weight µmol/g of Pro.

Multi year field screening for frost tolerance of selected mutant lines

Winter field screening in 2003/2004 (80 DHs) and 2004/05 (284 DHs) was conducted to test the spring DH mutants that displayed the strongest indoor cold tolerance to validate the stability of the mutations and to allow direct comparisons with parents and winter checks. The increased seed from 80 available in vitro selected DH mutants were planted September 9, 2003 into 2 m long rows with 0.30 g/row at the Monsanto station field in Listowel, Ontario. Parent lines of the mutants, Lolinda, and breeding lines SC990155, SC990221, PR6450 and PR6327 were seeded along with winter type *B. napus* cultivar Arctic, and four genetically diverse breeding lines WC06, BOL DH, Otrad 18, Otrad 21 as checks were seeded every fifth row. Winter check data was derived as the mean of all rows for each cultivar.

On September 29, 2004 at the Listowel Monsanto field station, a total of 284 mutant lines were planted in single rows (0.3 g seed/row). These included 74 mutants that showed strong cold tolerance in the freezer tests, mutants with strong cold tolerance in the previous year's field trial, all remaining new mutant lines and a number of parent lines and checks. Plants that survived the winter were counted on May 26 in both years and percentage winter survival was calculated by dividing the surviving plants by the total number of plants per row. Vigour notes were conducted in the fall with a rating scale of 1–5 in 2003, 1–9 in 2004 where 1 is poor and 5 or 9 is excellent, respectively.

Agronomic and quality evaluation of selected mutant doubled haploids

All available DH mutant lines (284) were also seeded in April of 2004 in 4-m long rows in Listowel, Ontario and evaluated for agronomic and seed quality traits. Monsanto commercial canola varieties DKL 32-75, and DKL 34-55 were checks along with the five parent lines used for this study. Agronomic traits evaluated were vigour, days to flower (DTF), days to maturity (DTM), lodging, height, and general impression notes were recorded at flowering and maturity. Plants in the center of every row were cut by hand and dried in paper bags for 1 week before hand threshing. Cleaned seed was analyzed for oil and moisture content using the NMR (Minispec Mq 10 Nuclear Magnetic Resonance; Monsanto lab certified by CGC). The methyl esters of fatty acids were separated and determined quantitatively by a Varian Star 3600CX Gas Chromatograph (GC). The simultaneous determination of oil, protein, and glucosinolate quality data was obtained using whole seed by Near-Infrared Reflectance (Foss 5000 NIR). All field data was compared to the original donor lines and to the Monsanto commercial varieties.

Results

Selection of mutant lines using various chemical agents

Clearly the chemical selection levels used varied with the sensitivity of genotypes and microspore/

Table 1 Final concentration chemical selection rates (mM) in vitro liquid medium (NLN) or in solid germination media (B_5G) across 5 donor lines

Chemical selection	Donor lines				
	PR6327	PR6450	SC990221	SC990155	Lolinda
SA in NLN	0.20			0.15	0.10
SA in BSG	0.25, 0.375, 0.50	0.25, 0.375, 0.50, 0.75	0.25, 0.375, 0.50	0.25, 0.375	0.375, 0.50, 0.75
JA in NLN	0.05, 0.10	0.10, 0.20	0.15, 0.20	0.05, 0.10	0.05, 0.075, 0.20, 0.30
				0.10, 0.15,	
FPA in NLN	0.125, 0.15				
A2C in NLN	0.25, 0.375			0.175, 0.25	
	0.50				
A2C in B5G		0.25, 0.50			
DP in NLN	0.10, 0.375, 0.50			0.375	
DP in B5G	0.50, 1.0	0.50, 1.00	0.50, 1.00		1.00
HP in NLN	5.0, 7.5, 10.0	10.0			7.5, 10.0

SA, salicylic acid; JA, jasmonic acid; FPA, *p*-fluoro-D,L-phenylalanine; A2C, azetidine-2-carboxylate; DP, 3,4-dehydro-D,L-proline; HP, hydroxyproline

Spaces indicate selection was not effective

embryo age (Table 1). A total of 329 unique mutant microspore derived lines were produced from 35 cultures through the in vitro chemical selection system. These mutants were derived from five parent lines, and were exposed to six different selection agents. The media in control plates were augmented with equivalent concentrations of alcohol solvents (ethanol for SA and FPA; methanol for JA) to determine whether alcohol presence alone interfered with the selection process. No damaging effects to cultures of neither ethanol nor methanol were found. Not all lines, nor selection agents were equally competent in producing surviving mutants (Table 2).

Indoor cold tolerance screening

In vitro selected mutants showed a wide range of cold tolerance when subjected to freezer tests. All chemical selection agents, with the exception of *p*-fluoro-D,L-phenylalanine (FPA), produced mutants with enhanced cold tolerance compared to their respective spring parent lines.

Of the 329 DH lines produced, the 74 mutants demonstrating strongest tolerance during the preliminary freezer test were re-screened for confirmation of survival levels. Many mutants showed stronger cold tolerance than their respective spring parent lines during the confirmation screening, while 19 mutants

Table 2 Summary of total number of mutants produced by in vitro chemical selection that were subsequently screened in the freezer for cold tolerance

Line	Total	JA	SA	DP	A2C	HP	FPA
PR6327	98	11	24	34	22	4	3
PR6450	66	29	15	8	14		
SC990221	60	20	28	12			
SC990155	36	17	10	2	7		
Lolinda	69	45	19	1		4	
Total	329	122	96	57	43	8	3

Treatments: Jasmonic acid (JA), Salicylic acid (SA), 3,4-dehydro-D,L-proline (DP), azetidine-2-carboxylate (A2C), hydroxyproline (HP), *p*-fluoro-D,L-phenylalanine (FPA)

had statistically significant cold tolerance enhancement over their parents (Table 3). Most importantly, 17 spring mutants had noticeably higher, but not significantly higher, survival rates than the winter canola check, Otrad 18.

Proline assay

In order to determine if there was a measurable change in Pro levels in a sample of four mutants each from a different chemical selection, leaf discs from 4 mutants and their respective parent lines were analysed for Pro levels. Results showed moderate levels of Pro under normal growth room environmental conditions, while the 4 mutants tested had a slightly higher Pro level than the parent lines. Two mutant lines from selections with Pro analogues, 997-48 and 997-201, accumulated substantially more Pro when exposed to 4°C as compared to the parents, PR6450 and SC990155 respectively (Fig. 1). The 997-8, Lolinda JA mutant, also had increased Pro leaf content when exposed to the 4°C environment, while 977-121, a SA mutant, did not.

Field agronomic and quality evaluation of *B. napus* chemically selected mutant doubled haploids

In studies such as these wherein germplasm improvement relies on mutation it is essential that other

Table 3 Summary of 19 mutants with statistically significantly higher cold tolerance compared to their respective spring parent in the indoor freezer screening

Line	Parent and selection	Mutant mean survival[a]	Parent mean survival[a]	Mutant survival compared to parent survival (+/–)	*P* value	Winter check survival[a]	Mutant survival compared to winter check survival (+/–)
997-121	327-0.375 DP	33	15	+50[c]	0.010	18	+42
997-219	327-0.25 A2C	31	17	+39[b]	0.045	21	+28
997-246	327-0.50 A2C	28	22	+17[b]	0.045	35	–20
997-251	327-0.25 SA	28	12	+44[b]	0.022	32	–11
997-315	327-0.25 SA	21	7	+39[b]	0.045	20	+3
997-327	327-0.50 SA	19	33	+44[b]	0.045	11	+23
997-48	450-0.50 DP	24	12	+33[b]	0.036	13	+31
997-55	450-0.50 A2C	19	7	+33[b]	0.036	20	–4
997-69	21-050 DP	21	9	+33[b]	0.035	14	+20
997-16	55-0.05 JA	31	11	+56[b]	0.035	27	+11
997-21	55-0.05 JA	26	4	+61[c]	0.001	22	+11
997-25	55-0.05 JA	27	13	+39[c]	0.001	25	+6
997-150	55-0.10 JA	27	17	+28[c]	0.007	30	–8
997-151	55-0.10 JA	30	10	+56[b]	0.035	27	+7
997-200	55-0.25 A2C	13	5	+22[b]	0.017	16	–9
997-201	55-0.175 A2C	28	4	+67[c]	0.007	25	+7
997-7	LL-0.05 JA	33	19	+39[b]	0.017	35	–5
997-8	LL-0.05 JA	35	19	+44[c]	0.008	35	0
997-189	LL-0.15 JA	26	14	+33[b]	0.035	31	–14

Parent lines: Lolinda (LL), SC990155 (55), PR6450 (450), PR6327 (327) and SC990221 (21)

Selection treatments were: Jasmonic acid (JA), Salicylic acid (SA), 3,4-dehydro-D,L-proline (DP), azetidine-2-carboxylate (A2C), hydroxyproline (HP)

[a] All tests were done on 36 double haploid plants per line

[b, c] denote statistical significance at the 5% and1% level of probability, respectively

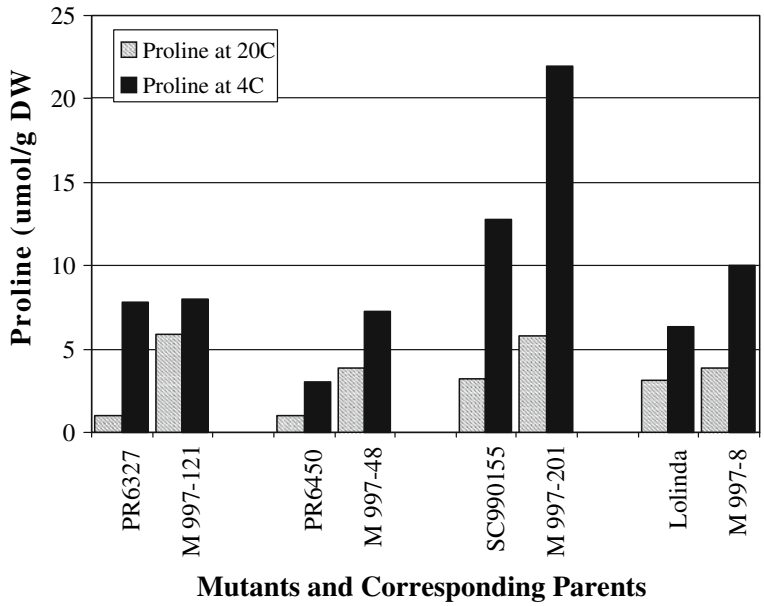

Fig. 1 Proline level in canola leaf discs from in vitro selected mutants with enhanced cold tolerance determined by the freezer test with corresponding parent lines. Proline values were recorded from canola leaf discs under normal growth room (20°C) and acclimation chamber (4°C)

valuable traits, already existing in the genotypes are not altered or weakened. Therefore, all available doubled haploid selected mutant lines (284), 5 parental checks, and two commercial canola varieties (DKL32-75; DKL34-55) were planted in Listowel, Ontario in spring 2004 to evaluate agronomic and seed quality characteristics of the selected mutants. Agronomic notes such as DTF, standability (lodging index = LGI) and DTM were recorded, along with the analysis of oil, protein, glucosinolate and fatty acid quality traits on the harvested seeds. The major seed quality and agronomic traits of the mutant lines are summarized in Table 4 and show that all mutants tested fall into the normal range for canola.

The quality and agronomic traits for the top 19 mutants, pre-selected in the freezer test, are presented individually in Table 5. No serious quality or agronomic deficiencies appear to be associated with the improved cold tolerance among the mutants. Total saturates were expected to be higher in mutants derived from PR6327 and SC990221, since the parents both have total saturates over 8 percent (Table 5). Similarly, mutants derived from Lolinda and SC990221 had elevated glucosinolate levels due to the higher glucosinolate content in the parents. Furthermore, Lolinda and PR6450 are cultivars with greatly reduced linolenic fatty acid (~2–3%) and therefore as expected the derived mutants from these

Table 4 Summary of means of some seed quality and agronomic parameters of 284 chemically selected mutants and parent lines grown in the field 2004

	Percent Oil NIR	Percent protein NIR	Glucosinolates µm/g whole seed	C18:3 Linolenic acid	Total saturates	DTF[a] 2004	LGI[b] 2004	DTM[c] 2004
Maximum	50.09	52.81	23.57	11.80	9.00	56.0	7.0	109.0
Minimum	32.98	38.79	3.10	1.58	5.52	48.0	2.0	100.0
Mean	45.50	46.65	11.55	6.68	7.32	52.5	5.1	1.03
SD	2.19	2.56	3.46	3.42	0.73	1.17	1.06	1.56

[a] DTF days to flowering

[b] LGI lodging index (1upright-9 down flat)

[c] DTM days to maturity

Table 5 Means of some seed quality and agronomic parameters of 19 mutants produced by in vitro chemical selection with superior cold tolerance over their respective parent in the indoor freezer test

Mutant line or parent	Percent oil NIR	Percent protein NIR	Glucosinolates µm/g whole seed	C18:3 Linolenic	Total saturates acid	DTF[a] 2004	LGI[b] 2004	DTM[c] 2004
997-121 (327)	46.00	43.88	11.00	9.05	8.27	52	6	102
997-219 (327)	44.59	41.77	6.72	8.71	8.32	52	6	102
997-246 (327)	44.89	43.29	13.15	9.66	7.93	54	4	102
997-251 (327)	46.94	44.24	8.90	9.73	7.89	54	6	104
997-315 (327)	45.20	46.02	5.72	6.88	6.85	NA	NA	NA
997-327 (327)	44.42	45.54	6.24	7.29	7.70	NA	NA	NA
PR6327	**45.44**	**43.62**	**11.50**	**9.67**	**8.03**	**52**	**5**	**103**
997-48 (450)	49.68	46.28	10.10	1.81	7.04	52	5	102
997-55 (450)	48.65	47.56	9.38	1.97	6.46	52	4	104
PR6450	**46.86**	**46.32**	**10.09**	**2.35**	**6.68**	**53**	**5**	**104**
997-69 (21)	44.01	49.35	15.98	9.87	8.10	52	6	104
SC990221	**43.62**	**50.25**	**19.95**	**10.19**	**8.02**	**53**	**5**	**105**
997-16 (55)	43.01	48.06	12.72	11.21	7.36	52	4	104
997-21 (55)	44.05	47.20	15.14	11.17	7.37	52	5	104
997-25 (55)	45.52	48.42	10.84	10.44	6.96	52	5	105
997-150 (55)	46.76	48.34	9.85	9.90	6.91	52	7	106
997-151 (55)	48.55	48.04	7.28	10.09	6.83	53	4	106
997-200 (55)	45.31	47.56	7.72	10.27	7.24	52	5	104
997-201 (55)	48.01	46.89	9.20	10.21	6.85	52	5	102
SC990155	**45.16**	**49.30**	**13.23**	**9.89**	**7.25**	**53**	**5**	**105**
97-7(LL)	46.01	48.45	14.95	3.16	6.27	52	5	104
997-8 (LL)	44.61	49.52	20.66	3.91	6.40	52	4	104
997-189 (LL)	45.61	48.39	15.99	3.06	6.15	52	4	104
Lolinda	**46.12**	**48.07**	**14.57**	**3.46**	**6.63**	**52**	**5**	**104**

Five parent lines are bolded

[a] DTF days to flowering

[b] LGI lodging index (1upright-9 down flat)

[c] DTM days to maturity

lines also show this trait. This trial was important to identify and eliminate any chemically selected mutants that no longer contained acceptable or desirable canola agronomic or quality traits.

Multi year winter field cold tolerance screening of chemically selected mutant doubled haploids

All available cold tolerant doubled haploid mutants along with parent lines and winter *B. napus* checks were planted in the fall of 2003 and 2004. The goal of these trials was to identify the level of cold tolerance of the mutant lines in respect to their spring parents and winter lines.

The differences between plant counts in the fall and late spring were used to evaluate the cold tolerance performance of the mutants in the field. All available DH mutants (80) with positive indoor freeze results were planted in a field trial in early September 2003. Because of the mild fall and warm winter of 2003/04 plant establishment was excellent. Twenty DH lines that showed some survival are shown in Fig. 2 along with the survival results of the parents, while all other mutants did not survive. Only two DH lines (997-48, 997-71) both selected with 0.50 mM

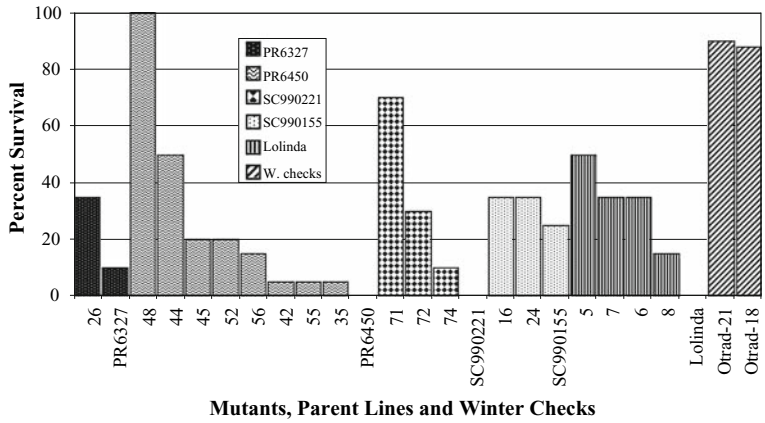

Fig. 2 Percent winter survival of in vitro selected mutant doubled haploids, parent lines and two winter check varieties grown in the 2003/2004 winter season. Total mutant lines planted was 80; only mutant doubled haploids with some survival are shown

DP on solid medium showed excellent survival at 100% and 70%, and distinctly higher survival rates than their parental lines. In comparison, the two winter checks showed a 90% and 88% survival in the winter of 2003/2004. Replanted in 2004, 997-48 and -71 again displayed higher plant survival than their parental checks and slightly higher than the same winter field checks at 25% and 20% (Fig. 3). Although 18 mutant lines had various levels of survival out of the 80 planted, only 2 mutants were considered for further field testing.

The cool fall of 2004 resulted in very small plants going into the winter season, and together with an excessively wet and harsh winter, the 2004/2005 trial yielded valuable survival results. Field survival results varied considerably from the indoor tests, especially among the 19 mutants with the best freezer scores (Table 3, Fig. 3) although 6 mutants were strong in both survival tests. There was high cold tolerance correlation for three SC990155 mutants 997-150, 997-151, and 997-200 between the indoor cold tolerance and field percent survival. Of the 284 mutant lines planted in 2004, 21 performed as well as the winter checks and survived much better than their parents. Clearly the 2004/2005 trial was much more valuable for differentiating the level of cold tolerance among these lines, than the previous year wherein the winter check varieties had only 13% and 10% survival.

Discussion

Microspore/embryo mutagenesis and chemical selection

The in vitro mutagenesis of *B. napus* microspores with short-wave UV light followed by selection with chemicals important in plant defense biochemical pathways, generated 329 mutants. Historically, in

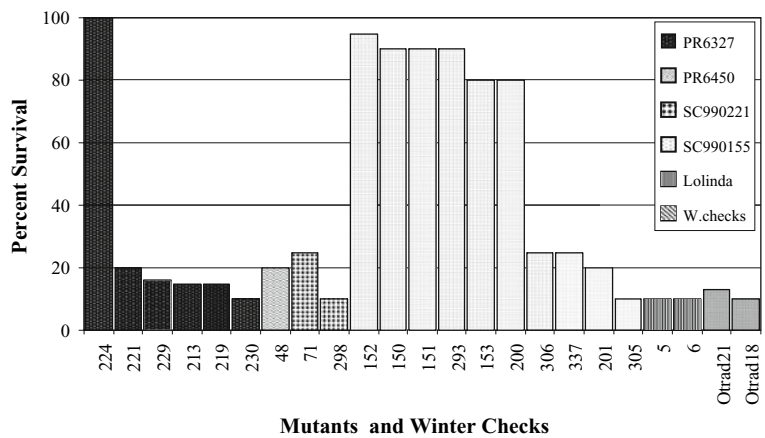

Fig. 3 Percent winter survival of in vitro selected mutant doubled haploids and two winter check varieties grown in the 2004/2005 winter season. Selection treatments were: Jasmonic acid (JA), Salicylic acid (SA), 3,4-dehydro-D,L-proline (DP), azetidine-2-carboxylate (A2C). Patterns indicate the parent line from which the mutants were derived

vitro UV irradiation has generated mutants in canola by producing novel point mutations in surviving haploid microspores (Ahmad et al. 1991; Kott 1995) and in this study had successfully induced changes without causing deleterious effects, imposing no associated morphological or physiological damage in the most cold tolerant DH lines (Table 5). Various economically important traits such as herbicide tolerance, enhanced quality and disease resistance have been achieved through induced microspore mutagenesis and chemical selection in *B. napus* (e.g. Swanson et al. 1989; Kott et al. 2002; Beaith et al. 2005). This study produced many mutants without noticeable phenotypic changes, but with measurable enhanced cold tolerance. Since UV exposure can produce many random variants it was imperative to identify desirable mutants through chemical selection with positive and measurable effects on specific biochemical pathways (Kott 1995). Selection agents were chosen based on their known significant role in abiotic and biotic plant defense pathways. Proline analogues as (hydroxyproline [HP], azetidine-2-carboxylate [A2C]; 3,4-dehydro-D,L-proline [DP]) were used in selections since Pro is an organic osmolyte that readily accumulates in plants under osmotic stress in response to cold and other abiotic pressures (Handa et al. 1986; Kavi Kishor et al. 1995; Nanjo et al. 1999). This study identified 49 A2C, 57 DP, and 9 HP mutants from the Pro analogue chemical screening.

In vitro microspore and embryo screening was also done with 5-chlorosalicylic acid (SA), JA and *p*-fluoro-D,L-phenylalanine (FPA) that are key defense signaling molecules in the frost-induced oxidative stress pathway. Ninety-six SA, 122 JA and 3 FPA mutants were identified from these chemical selections. Recent studies have demonstrated valuable effects of increased SA on cold tolerance in major crops such as maize, rice, wheat, bean and tomato (Janda et al. 1999; Senaratna et al. 2000; Kang and Saltveit 2002; Tasgin et al. 2003). Field screening confirmed that three SA selected lines performed better than the winter checks (Table 5), possibly due to the action of elevated levels of 5-chlorosalicylic acid. Similarly, eight of the best mutant lines field tested over the 2004/2005 winter were derived from the JA in vitro selections. Research indicates that JA enhances the expression of plant defense genes (Creelman and Mullet 1997) and that JA may be involved in the transportation of chemical signals in plants (Sasaki et al. 2001), but the primary interaction between JA and SA pathways are mutually antagonistic (Kunkel and Brooks 2002). Gupta et al. (2000) found that mutants *eds4* and *pad4* demonstrated enhanced responses to inducers of JA gene expression and reduced SA accumulation. In the current study, these same principles may be at work, wherein an enhanced JA level greatly suppressed the SA activity with positive expression of cold tolerance.

Indoor freezing tolerance of chemically selected mutant doubled haploids

Frost tolerance field studies are difficult to replicate due to the extreme environmental variability. Winter field trials may not experience heavy frost or in severe cases completely kill all plants. Thus, the development of a rapid, affordable, repeatable indoor cold prediction test that correlated with survival in field trials was extremely important.

The controlled freezer test developed here used a medium range temperature (–6°C), used in similar tests to identify differences in plant cold tolerance (Fowler et al. 1973). Previous indoor cold tolerance studies reported inconsistent results using multiple seedlings in large flats of soil during freezing (Andrews and Morrison 1992), wherein plants around the edges were severely affected unlike plants in the middle of the flat. The results in the current study may be more consistent because the nine-cell packs were evenly spaced within the freezer outside of a flat and because doubled haploid lines were used in which all seed is genetically identical. Every test included the parent of the mutant line, Lolinda, as a DH spring check as well as OTR-18, a DH low glucosinolate extraction from an Eastern European winter hardy variety (Otradnenskji), as the winter check. Of the 329 in vitro selected mutants, 74 appeared to be more cold tolerant than the spring check, Lolinda, but showed varied survival values compared to their respective parents. The SA, JA, and Pro analogue mutants varied from +44 to –28, +61 to –17, and +67 to –11% survival compared to their respective parents. This method proved fast and effective in that the DH mutants that performed best in the field tests were among those identified in the freezer tests.

Agronomic and quality evaluation of selected mutant doubled haploids

The trial planted in spring 2004 was essential to evaluate agronomic and quality characteristics of the increased double haploid mutant seed. Some morphological differences were expected since exposure to physical mutagens could result in considerable base pair changes (Poehlman and Sleper 1995). Seventeen chemically selected mutants of the 329 assessed after the double haploid process, showed that their reproductive systems were sufficiently altered that they were unable to set any or enough seed for the quality and agronomic evaluation trial. Only two mutants, 997-10 and 997-236, were functionally damaged to the extent that they failed to grow in the 2004 observation trial. However, clearly for most mutants the alterations in agronomic traits are minor and range well within the accepted norm for canola (Table 4).

Most of the mutant lines were phenotypically very similar to their respective parents and exhibited the specific traits of their parents. For example, most PR6327-derived mutations had the most desirable agronomic package since PR6327 was a vigorous and high yielding parental line, and the PR6450 and Lolinda-derived mutants inherited the low linolenic trait from their parents (Table 5). Oil, protein, glucosinolate analysis and fatty acid profiles of the mutants compared well to parental lines planted together in the same trial for agronomic and quality comparisons (Table 5). The seed quality profiles of the mutants (data not shown) confirm that UV application can increase genetic variation in *B. napus* genotypes.

Proline assay

Improved stress tolerance has been achieved by the over-expression of osmolytes like Pro in transgenic plants. Konstantinova et al. (2002) enhanced freezing tolerance in transgenic tobacco by over-expressing the p5cs gene. Furthermore, canola leaf discs readily accumulate increased amounts of Pro under osmotic stress (Huguet-Robert et al. 2003). The Pro assay experiment conducted on a sample of four chemically selected DH mutants confirmed the critical role Pro has in plant protection against osmotic stress induced by a change from normal to cold growing environments. The 997-48 DP mutant and 997-201 A2C mutant generated a 2.55 and 1.75 fold increase in Pro, respectively, compared to corresponding parental lines when exposed to the 4°C environment (Fig. 1). The 997-8 Lolinda based JA mutant at 4°C showed a 1.56 fold increase in Pro compared to the parent, Lolinda. Since JA has a regulatory role in plant development and activates defense gene transcription in plants under abiotic and biotic stresses (Farmer and Ryan 1992), the increase in Pro in this JA mutant suggests that both chemical components have a significant role in osmotic/cold stress tolerance in *B. napus.* The only mutant that did not have a significant Pro increase at 4°C was the 997-121 SA selected mutant compared to the parent line PR6327 (Fig. 1). This SA mutant could have achieved cold tolerance through a different signaling pathway that does not require an increase in Pro to provide protection.

Field screening for cold tolerance of selected mutant doubled haploids

Multi generation winter field-testing in 2003/2004 and 2004/2005 were done on the best pre-selected freezer mutants to confirm mutation stability and to make direct comparisons to parents and winter checks. Two of the most promising cold tolerant doubled haploid mutants, 997-48 and 997-71, both had high survival percentages in winter field trials over 2 years as compared to parental and winter checks, although in the 2004/2005 trial other new and much more tolerant mutants were identified (Fig. 3). The indoor freeze test effectively eliminated mutants with no cold tolerance while identifying those with potential, as 6 out of the 21 pre-selected mutants confirmed excellent survival in the severe 2004/2005 winter.

There are two strategies that allow plants to maintain high levels of Pro under stress. The first method is to remove the feedback inhibition of P5C synthase rate limiting enzyme in plant Pro biosynthesis. Hong et al. (2000) found an increase in salt stress tolerance in transgenic tobacco that accumulated high levels of Pro with the feedback inhibition removed. Seven DH mutants selected with A2C had enhanced cold tolerance identified in the 2004/2005

field trial. These mutations may have targeted the Pro biosynthetic pathway at the P5C multifunctional enzyme, which has interfered with the conversion of high Pro levels back to glutamic acid. Two mutants, 997-200 and 997-293 selected with 0.25 A2C had 80% and 90% plant survival in the 2004 winter field trial. The winter check Otrad 21 planted between these two mutant rows only had 5% of the plants survive the winter. As well, 997-219 and 997-221 screened with 0.25 mM A2C and a third mutant, 997-213 screened with 0.375 mM A2C had 15, 20, and 15% survival, respectively, in 2004. Arctic, which was the winter check positioned between these mutants did not survive the 2004 winter.

The second method plants use to maintain high levels of Pro is by reducing Pro metabolism while under stress. Transgenic *Arabidopsis* plants with repressed Pro dehydrogenase, the first step in the Pro degradation pathway, displayed enhanced freezing tolerance as the plants constitutively accumulated Pro (Nanjo et al. 1999). DP selected mutants derived from PR6327 and SC990221, displayed the greatest cold tolerance of all selected mutants. However, 997-224, selected with 0.375 DP performed exceptionally well with 100% survival in the 2004 winter field trial (Fig. 3). It appears that the metabolism of Pro in the cold tolerant DP selected mutants may have been reduced, resulting in enhanced Pro levels and frost tolerance. Two DP selected mutants 997-48 and 997-71, survived the 2003 and 2004 winter field screening (100% and 70% [2003]; 20% and 25% [2004], respectively). Survival was impressive for these mutants in 2004/2005 considering that the two Otrad winter check rows that flanked the mutants' rows only had 20% survivability.

Chemical selection with *p*-fluoro-D,L-phenylalanine (FPA) resulted in lethal deleterious effects, which caused very few mutants to produce doubled haploid seed. The analogue of phenylalanine was used to inhibit PAL activity and identify mutants with increased phenolics, which are known to enhance the tolerance of canola to cold stresses (Fletcher and Kott 1999). Phenolic metabolism provides a very important protective layer to plant membranes with the deposition of suberins and lignins, which protect tissues from cold stresses. Phenolics also produce antioxidants that reduce cell and DNA damage by free oxygen radicals during cold stress. However, the three mutants produced which survived the FPA selection in this study did not exhibit any cold tolerance.

Salicylic acid-related pathogen mutants have been reported to have common SA based signaling mechanisms (Scott et al. 2004) and increased levels of SA have improved cold tolerance in economically important crops (Janda et al. 1999; Senaratna et al. 2000). However, it is not feasible to spray commercial canola fields with a SA solution to induce a defense response against an expected frost. Chemical screening with SA of mutant embryos produced DHs that could withstand the toxic salicylic levels and therefore, it was hypothesized that surviving embryos may have enhanced SA levels. Ninety-six SA mutants were produced with a wide variation in the cold tolerance. The three SA selected mutants with enhanced cold tolerance demonstrated the important role SA has in the cold defense response (Fig. 3). These SA mutants may have altered genes that constitutively or temporally increase the expression SA, which leads to the expression of defense genes and the development of a hypersensitive response. This, in turn, signals and maintains a SAR, which promotes tolerance to various abiotic and osmotic stresses.

Similar to SA, JA acts as a signaling molecule activating gene expression in plant responses to environmental stresses such as cold stress. Cross talk between these pathways is extremely important for plants to regulate pathogen defense gene expression (Kunkel and Brooks 2002) and also plays a key role in low temperature growth (Scott et al. 2004). Jasmonic acid and SA regulators may control and regulate each other's plant defense actions which allow the plant to prioritize the activation of one signal pathway over the other by activating different sets of overlapping genes against different stresses (Reymond and Farmer 1998). Microarray analysis discovered that three out of forty-one JA response genes are also involved in the SA signaling defense pathway (Sasaki et al. 2001).

Eight JA selected mutants derived from SC990155 and Lolinda were identified during freezer screening with increased cold tolerance. These JA mutants averaged 35% and 19% (respectively) higher survival over parental checks. Clearly, the four SC990155 mutants chemically selected with 0.10 mM JA had the greatest survival in the 2004/05 field trial (Fig. 3). Mutants 997-150, 997-151, 997-152, 997-153 had

90%, 90%, 95%, and 80% survival, respectively, in the winter trial of 2004/05. Lolinda mutants 997-5 and 997-6 screened with 0.05 mM JA had 10% survival in the same trial, which is respectable due to the poor soil conditions in their part of the field and may also be significant as Lolinda is a low linolenic variety and the elevated JA is ultimately derived from the linolenic acid pathway. The JA-selected mutants from the other patent lines did not show a significant increase in frost tolerance in the indoor or outdoor cold screening tests.

One mutant line, 997-315 with 39% greater indoor cold tolerance than its parental genotype also displayed excellent agronomic and quality traits. This selected mutant has been increased in 2005/2006 winter nursery and was advanced to the 2006 first year (private) WCC/RRC yield trials in various locations across the short, mid, and long season zones in western Canada and will potentially be eligible for registration for 2008.

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