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Genetic analysis and mapping of genes controlling freezing tolerance in oilseed *Brassica*

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Abstract

Freezing tolerance is the ability of plants to survive subfreezing temperatures and is a major component of winter survival. In order to study the genetic regulation of freezing tolerance, an F2 population of *Brassica rapa* and a doubled haploid population of *Brassica napus* were assayed *in vitro* for relative freezing tolerance of acclimated and nonacclimated plants. Linkage maps developed previously were used to identify putative quantitative trait loci (QTL). Genomic regions with significant effects on freezing tolerance were not found for the *B. napus* population, but for *B. rapa* four regions were associated with acclimated freezing tolerance (FTA) and acclimation ability (FTB), and two unliked regions were associated with nonacclimated freezing tolerance (FTN). Acclimation ability was regulated by genes with very small additive effects and both positive and negative dominance effects. The allele from the winter parent at the FTN QTL had positive additive effects, but negative dominance effects. RFLP loci detected by a cold-induced and a stress-related cDNA from *Arabidopsis thaliana* mapped near two QTL for FTA/FTB. Further tests are needed to determine if alleles at these loci are responsible for the QTL effects we detected.

Introduction

The ability of plants to tolerate frost is a major component of winter survival in herbaceous perennial or biennial crop plants, such as oilseed *Brassica*. In many crop species, freezing tolerance increases dramatically when plants are acclimated to low temperatures before freezing. The freezing tolerance of cold-acclimated plants, measured by an *in vitro* assay, correlated with the winter survival of *Brassica* cultivars, but there was no correlation between nonacclimated and acclimated freezing tolerances [42]. This suggested that the two tolerances were controlled by separate genetic mechanisms, but very little is known about the genetic control of freezing tolerance in *Brassica* species.

The genetic regulation of freezing tolerance and winter hardiness is probably complex in most or all crop species. Studies with winter wheat have suggested polygenic inheritance of winter hardiness [11, 13] and several chromosomes containing genes for frost resistance have been identified using monosomic [42] or chromosome substitution [20, 41] lines. Segregation in a cross of diploid *Solanum* species suggested that nonacclimated freezing tolerance and capacity to acclimate were controlled by only a few genes [40]. In barley, only one genomic region was associated with both winter hardiness and cold tolerance [12], however, the presence of transgressive segregants suggested that other regions were involved but were not mapped in this study. The reported gene action for frost tolerance has varied from recessive [31] to partially dominant [9] in winter wheat, largely additive [4] to partially dominant [30] in alfalfa, and partially recessive in potato [40]. These differences could be due to variability in genotypes, or to frost test conditions since the severity of the winter determined whether there was dominant or recessive control of hardiness in oats [24].

Cold acclimation induces many changes in plants, including altered gene expression, and cold-induced cDNA clones have been isolated from several species, including *Arabidopsis thaliana* [10, 18, 22, 25, 26] and *B. napus* [27, 28, 32, 49]. The functions of these protein products in improving cold tolerance are not known, however, the cold-regulated (COR) genes code for boiling-stable, hydrophilic proteins which may function to keep critical proteins hydrated during freezing [see 46 for review]. The possible function of some other cold-induced genes, such as NADPH-aldose reductase, phosphoglucomutase [21], alcohol dehydrogenase [15], and lipid transfer proteins [14] are not readily evident. Other stress treatments, such as desiccation [37], salinity [35], and ABA treatment [2, 16], also can induce freezing tolerance, suggesting that some common mechanism may be induced by these different stresses. ABA-responsive genes have been isolated from cold-acclimated tissues and some cold-induced genes have sequence homology to the dehydrins and late embryogenesis-abundant proteins [3, 5, 8, 19, 48, 50], but it is not clear whether their expression is required for cold tolerance or a response to ABA accumulation caused by freezing-induced dehydration.

In this study, the genetic control of freezing tolerance was investigated in oilseed *Brassica rapa* and *B. napus* using molecular marker and quantitative trait locus (QTL) mapping. In addition, RFLPs detected by cold-induced genes or genes involved in plant stress responses in *B. napus* or *A. thaliana* were mapped in *B. rapa* and QTL analysis was used to determine whether segregation at the candidate loci was associated with variation for freezing tolerance.

Materials and methods

Plant population and RFLP linkage maps

A *B. napus* doubled haploid (DH) population was generated by microspore culture of a single F1 hybrid plant of cv. Major (biennial rapeseed) crossed with a DH line of cv. Stellar (annual canola) [7]. These parents differed in growth habit, freezing tolerance, and winter survival [43]. S2 plants from 105 lines were used to extract DNA for RFLP analysis and for trait measurements. A linkage map of 138 RFLP loci was constructed as reported previously [7].

A *B. rapa* F2 population was generated by self-pollination of a single F1 plant of cv. Per (biennial) crossed with cv. R500 (annual). These parents differed for growth habit, freezing tolerance, and winter survival [43]. F3 families from self-pollination of 85 F2 plants were used for RFLP analysis and trait measurements. A linkage map of 143 loci was constructed using the 85 F2 genotypes which were a subset of 91 F2 genotypes used previously for map construction [44]. The maps had similar distances and identical locus orders, except for linkage group 2 (LG 2) which contained additional loci and was reordered.

Cloned genes which are either cold-induced or stress-related were used as DNA probes to screen for polymorphisms between the *B. rapa* parents, and a subset of these were mapped in the *B. rapa* population [44]. These included 12 DNA clones from *A. thaliana* and 2 cDNAs from *B. napus* (Table 1). Cloned dehydrin genes from barley (*dhn2*, *4*, *5*, *6*) and maize (*dhn1*), kindly provided by T. Close (UC Riverside), were not homologous enough to the *B. rapa* DNA to be mapped. Also screened, but not mapped, were *A. thaliana* dehydrin PAP023 (M. Delseny, personal communication) and *hsp21* [1].

Trait measurements

Self-pollinated progenies of the parents, the F1 hybrids, 85 *B. rapa* F3 families and 105 *B. napus* DH lines were assayed for their *in vitro* freezing tolerance. Twelve plants from each genotype were grown in ComPack six-pack pots filled with autoclaved soil/Jiffy mix/sand (1:1:1 for *B. napus*, 3:2:1 for *B. rapa*) under controlled conditions (22 °C, 250 μE/m^2 light, 14 h daylength) for five weeks. Plants were watered daily with half-strength Hoagland's solution. Six plants were assayed for nonacclimated freezing tolerance, and six were incubated in the cold (4 °C day/2 °C night, 100 μE/m^2 light, 14 h daylength) for an additional three weeks and then assayed for acclimated freezing tolerance. Acclimated and nonacclimated freezing tolerance were estimated by subjecting leaf tissue to a freeze-thaw stress, as described by Teutonico *et al.* [43]. Leaves of similar size and approximate developmental stage were excised from six plants for each genotype. After the midribs were removed, leaf halves were combined and then randomly divided over 18 test tubes per genotype. Each leaf half was subjected to a controlled freeze (1 °C/h for nonacclimated plants, 1 °C/h to −3 °C then 2 °C/h for acclimated plants) in a glycol bath from the initial temperature of 0 °C. There was no difference in relative freezing tolerance calculated for acclimated plants of the parental lines at the two different freezing rates. Tissues were nucleated at −1.0 °C by dropping ice chips into the tubes. Three tubes per family (subsamples) were removed, beginning at a temperature of −2 °C, at specified intervals (every 1 °C to −8 °C for nonacclimated and every 2 °C to −15 °C for acclimated plants). The control consisted of 3 replicates per cultivar that were kept on ice at 0 °C. All samples were thawed slowly on ice overnight. Thawed leaves were then cut into 5 mm strips, wetted with 25 ml of distilled water in the same test tubes in which they were frozen, degassed,

Table 1. Summary of cold-induced or stress-related cloned genes used to map RFLPs in the *B. rapa* 'Per' × 'R500' F2 population.

Probe name	Source	Gene encoded	Reference
Cold-induced			
BN59	*B. napus*	ATPase	[28]
BNC24A	*B. napus*	unknown	[32]
COR6.6a, b	*A. thaliana*	unknown	[10]
COR15a	*A. thaliana*	unknown	[10]
COR47a, b	*A. thaliana*	dehydrin-like	[10]
COR78a, b	*A. thaliana*	unknown	[10]
PEP/4	*B. napus*	phosphoenol-pyruvate-carboxykinase	M. Delseny (pers. commun.)
Stress-related			
pCSODRH	*A. thaliana*	superoxide dismutase	[13]
DHS2	*A. thaliana*	DAHP synthase	[17]
GAP-A	*A. thaliana*	glyceraldehyde-3-phosphate dehydrogenase	[36]
pJ5-3	*A. thaliana*	lipid transfer protein	[45]
PR2	*A. thaliana*	β-glucanase	[47]

and shaken at 200 rpm for 2 h. Freezing damage was assessed by monitoring ion leakage from thawed leaf samples. A freezing curve was constructed for each genotype by plotting temperature vs. % ion leakage. The relative freezing tolerance (nonacclimated FTN; acclimated FTA) for each cultivar was calculated from its respective freezing curve by determining the temperature at which 50% of leakage [(maximum − minimum)/2] occurred. The acclimation ability (FTB) of each family was calculated as the absolute value of the difference in relative freezing tolerance (°C) between nonacclimated and acclimated plants.

The *B. rapa* parents, F1, and F3 families were measured for three growth characteristics: internode length (INT), plant height (HT), and change in leaf number (DLF). Plants of each family were grown in ComPack six-packs in soil mix (3:2:1 soil/Jiffy mix/sand) in the greenhouse (at 22 °C) under supplemental metal halide lamps with a 14 h daylength. Plant height from soil to shoot apical meristem was measured at 28 days after sowing. Internode length was calculated as the height of the plant divided by the number of leaves at 28 days. The change in leaf number was calculated as the difference in leaf number between 28 days and 16 days after sowing.

Data analysis

The SAS 'PROC GLM' procedure [34] was used to test for significant differences among *B. rapa* F3 families or *B. napus* lines. Three ion leakage measurements at six freezing temperatures for each family or line were analyzed using a two factor (% ion leakage and family/line) model with interaction.

Putative QTL controlling six quantitative traits (three freezing tolerance and three growth traits) were identified using the MAPMAKER/QTL v1.1 program [23, 29]. A LOD score of 2.0 was chosen as the threshold for declaring putative QTL and their positions were determined by the peak LOD score. This LOD was chosen to ensure that any QTL with small, but significant, effects contributing to these polygenic traits were detected. The confidence interval for a QTL was determined by the region within one LOD of the peak LOD score. Multiple peaks on the same LG were considered to be a single QTL if fixing the effect of one QTL eliminated the effect of the second. The additive and dominance effects of QTL alleles from the winter parent were calculated for each trait with MAPMAKER/QTL v1.1 [23] and dominance effects were corrected for the use of F3 families for trait measurements. The growth measurements for each family were used as covariates in the QTL analysis, using both MapMaker/QTL and SAS 'PROC GLM' procedures. For FTA and FTB, epistatic interactions among significant QTL were determined using a two-factor model with interaction in the SAS 'PROC GLM' procedure [34]. A multilocus model was developed [51] by including the effects of putative QTL that increased the LOD score for the model by at least 2.0. The percentage of variation explained by each QTL was calculated as the decrease in variation explained when each QTL was removed from the complete model. All QTL in the model were fixed and the genome rescanned for additional QTL using a 2.0 increase in LOD as the threshold [23].

Results and discussion

Freezing tolerance of segregating populations

The relative freezing tolerances of the *B. napus* DH lines ranged between −1.0 and −4.5 °C for nonacclimated plants (FTN) (Fig. 1a) and decreased to a broader range of values (−3.5 to −13.0 °C) for acclimated plants (FTA, Fig. 1c). Acclimation ability (FTB) varied from 0 to 9 °C (Fig. 1e). The F1 was superior to both parents for FTA, FTN, and FTB and 81 of the 105 DH lines (77%) had greater acclimation ability than the biennial parent Major, while only 5 lines had less acclimation ability than the annual parent Stellar. There were significant differences in FTN, FTA, and FTB among *B. napus* lines ($p < 0.01$), among freezing temperatures

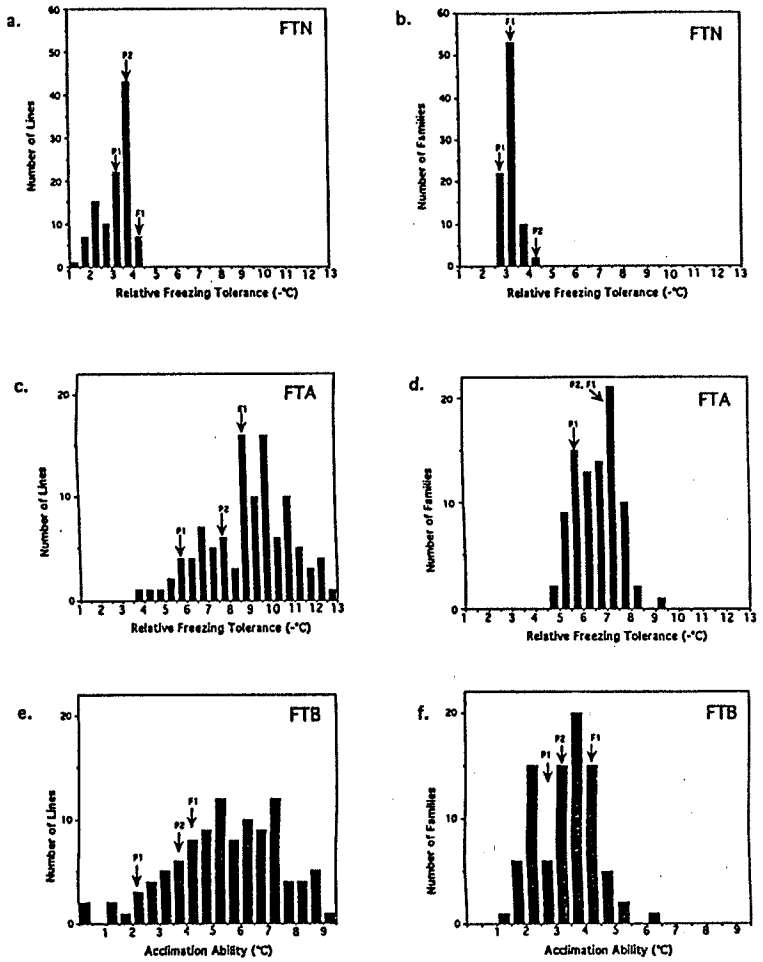

Fig. 1. Relative freezing tolerances of *Brassica napus* doubled haploid lines derived from the F1 hybrid between 'Stellar' and 'Majc (a, c, e); and of *Brassica rapa* F3 families from the F1 hybrid between 'Per' and 'R500' (b, d, f); a and b, nonacclimated relati tolerance (FTN); c and d, acclimated relative freezing tolerance (FTA); e and f, acclimation ability (FTB).

($p < 0.01$), as well as a significant interaction between lines and temperature ($p < 0.01$).

The *B. rapa* F2 population had a narrow range of FTN values between −2.5 and −4.5 °C (Fig. 1b). In a manner similar to *B. napus*, acclimation shifted the FTA of the population to low temperatures and broadened the range of valu (−4.5 to −10 °C, Fig. 1d). The acclimation ab ity of the population ranged from 1.0 to 6.5 ° (Fig. 1f). The biennial parent Per had a high

FTN and FTA than the annual parent R500. The F1 was intermediate between the parents for FTN, but had a higher FTA and FTB than the biennial parent. Of the 86 lines measured, 42 families (41%) were more hardy than Per and 22 families (26%) were less hardy than R500. For each treatment, there was a significant difference in FTN and FTA among families ($p < 0.0001$), among freezing temperatures ($p < 0.0001$), as well as a significant interaction between families and temperature ($p < 0.002$ FTN, $p < 0.0004$ FTA).

The *B. rapa* and *B. napus* populations both displayed transgressive segregation for FTA and acclimation ability (FTB), with the majority of the transgressives achieving greater hardiness. Transgressive segregation may indicate that freezing tolerance is a quantitative trait controlled by many genes and that positive effects come from both parents. There was no significant correlation between FTN and FTA for either the *B. napus* ($r = 0.095$, Fig. 2a) of the *B. rapa* ($r = 0.158$, Fig. 2b) populations, suggesting that different genes may control these two traits.

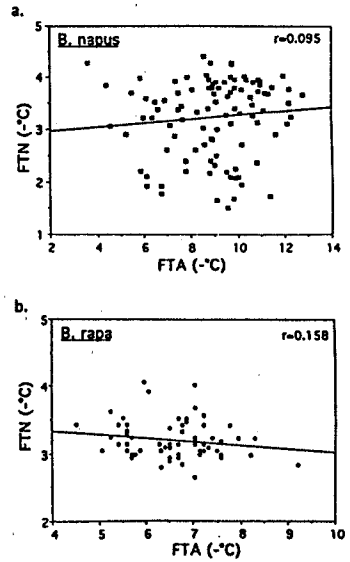

Fig. 2. Correlation between acclimated (FTA) and nonacclimated relative freezing tolerance (FTN) of *B. napus* doubled haploid lines (a) and *B. rapa* F3 families (b).

QTL for freezing tolerance and growth characteristics

For the *B. napus* population, no putative QTL with LOD scores above 2.0 were detected for FTN, FTA, or FTB. This could be due to control by many genes with small effects and/or incomplete coverage of the genome with molecular markers.

For the *B. rapa* population, one putative QTL for FTN was identified on LG 10 (Table 2). When this QTL was fixed and the genome re-scanned, one additional QTL was identified on LG 9. Together these two QTL account for 38% of the variance in nonacclimated freezing tolerance.

Three regions on LGs 2, 5, and 7 (Table 2) of the *B. rapa* genome contained putative QTL with a LOD > 2.0 for FTA and FTB. A multilocus model including the three QTL accounted for 39.7% (FTA) and 45.1% (FTB) of the variation in the traits. When effects of the three QTL were fixed and the genome rescanned, one additional QTL on LG 4 was identified. Addition of this QTL to the multilocus model significantly increased the LOD score and the variance explained increased to 54.3% (FTA) and 55.3% (FTB). This four-QTL model was compared to models containing all possible combinations of subsets of the four different QTL and was found to be the most probable model for FTA/FTB. The QTL on LG 7 explained the most variation (20.5% FTA; 24.7% FTB), which was twice as much as each of the other QTL in the model (Table 2). There were no epistatic interactions detected among any of the four QTL, indicating primarily additive and dominance effects for these QTL. These FTA/FTB QTL were unlinked to the two QTL for FTN, indicating independent genetic control of the traits as expected from the lack of correlation between the two traits (Fig. 2). This also was consistent with the results obtained in *Solanum* species [40].

For FTN, the QTL on LG 9 and LG 10 had

Table 2. Summary of putative QTLs controlling relative freezing tolerance and associated traits in the *B. rapa* 'Per' × 'R500' F2 population.

Trait [a]	LG	Confidence interval [b]	LOD [c]	R^2 [d]	Add [e]	Dom [e]
FTA	2	COR6.6a(+ 6)-off	2.41	12.3	0.11	- 1.20
	4 [f]	wg1g6(+ 0)-GAP-A(+ 2)	2.37	12.5	- 0.04	1.16
	5	PR2a(+ 16)-tg5b2a(+ 14)	1.15	3.0	- 0.05	- 0.84
	7	off-wg8h5(+ 18)	3.76	20.5	- 0.04	- 1.74
FTB	2	COR6.6a(+ 6)-off	1.84	8.6	0.17	- 0.98
	4 [f]	wg1g6(+ 0)-GAP-A(+ 2)	1.86	8.6	- 0.14	0.92
	5	PR2a(+ 14)-tg5b2a(+ 14)	2.08	5.5	- 0.07	- 1.22
	7	ec2e5(+ 0)--wg8h5(+ 18)	4.53	24.7	- 0.07	- 2.0
FTN	9 [f]	wg7h2(+ 0)-COR47a(+ 8)	2.74	21.7	0.10	- 0.24
	10	tg2d6(+ 5)-ec5f11(+ 8)	2.31	16.4	0.09	- 0.21
HT	7	wg1g5a(+ 2)-GS KB6(+ 18)	3.81	17.5	- 1.09	- 2.54
	9	ec5a6b(+ 0)-ec5agb(+ 10)	10.13	51.2	- 2.22	- 3.46
INT	7	wg1g5a(+ 4)-GS KB6(+ 16)	3.56	18.7	- 0.16	- 0.36
	9	ec5a6b(+ 0)-ec5a6b(+ 10)	8.64	45.1	- 0.28	- 0.44
DLF	9	tg5d9(+ 0)-ec4f10(+ 10)	3.54	22.9	0.47	0.60

[a] Trait abbreviations.
[b] One-LOD confidence interval designated by marker locus plus cM (in Haldane map units) to the right of flanking marker; 'off' indicates boundary is beyond the end of LG.
[c] Increase in LOD score provided by addition of each QTL to the model.
[d] Increase in % variation explained by addition of each QTL to the model.
[e] Additive and dominance effects of the allele from the biennial parent 'Per' in units of the trait measurement; calculations based on multiple-QTL model for each trait.
[f] Detected after fixing the effects of other QTL for that trait.

positive additive effects of the Per allele, but negative dominance effects (Table 2), indicating that the Per allele at these loci increased freezing tolerance of the plants and that the heterozygote had a lower tolerance than the R500 homozygote. For FTA/FTB, the QTL on LG 4, LG 5, and LG 7 had negative additive effects indicating that the Per allele at these loci decreased the freezing tolerance of the plants, while the QTL on LG 2 had a positive additive effect. All of these additive effects were quite small, suggesting that the dominance effects were more significant. The dominance effects of the QTL on LG 2, LG 5, and LG 7 were negative since the heterozygote had a reduced tolerance compared with the R500 homozygote, while the heterozygote at the QTL on LG 4 had increased tolerance.

For both the *B. rapa* and *B. napus* populations, the F1 was equally hardy or hardier than the biennial parent (Fig. 1). F1 heterosis for freezing tolerance suggests that some QTL should have dominant or overdominant gene action for freezing tolerance. However, three of the four FTA/FTB QTL identified in *B. rapa* showed a negative (under) dominance effect in which the heterozygote was less tolerant than the R500 homozygote. For the QTL on LG 7, R500 homozygous genotypes had greater FTA than Per homozygous genotypes. These differences between the genotypic class means at these loci were not consistent with the phenotypic differences between the two parents and the hybrid. We repeated the freezing experiment and QTL analysis on a different, overlapping subset of F3 families and found the same

two QTL on LG 5 and LG 7 with similar negative additive and underdominance effects, which suggested that these were not just spurious associations. The higher trait values associated with alleles from the low parent (R500) would be expected for some loci controlling a trait that shows transgressive segregation, and this has been observed in other QTL studies [6, 38]. The underdominance effects at loci controlling a trait that shows F1 heterosis are more difficult to explain. However, we have only identified four QTL which explained a portion of the total genetic variation, and there are probably additional QTL involved which we did not detect and which may show dominant or overdominant gene action for freezing tolerance. In addition, there could have been favorable epistatic combinations of genes in the F1 which we could not detect in the segregating population.

Two *B. rapa* LGs contained significant QTL for the growth characteristics measured. A major region controlling growth was located to LG 9, with a LOD score of 10.13 for height (HT) and 8.64 for internode length (INT) (Table 2). The Per allele in this interval contributed to a shorter plant height, decreased internode length, and a higher rate of leaf production than the R500 allele. The heterozygote had similar characteristics to the Per homozygote at this locus. QTL for plant height (HT) and internode length (INT) also were identified on LG 7 (Table 2).

None of the chromosomal regions associated with growth characteristics corresponded to the FTA/FTB QTL. However, when the growth measurements (HT, INT, or DLF) were used as covariates in the QTL analysis, growth rate differences between the genotypes accounted for a significant portion of the variation in FTA/FTB. The SAS 'PROC GLM' procedure confirmed that growth rate, especially when measured as DLF, was a significant factor in the QTL model for FTA/FTB. Therefore, the most complete model for the genetic control of acclimation ability and acclimated freezing tolerance in *B. rapa* included the four FTA/FTB QTL on LG 2, LG 4, LG 5, and LG 7 (Fig. 3) and growth rate. It is possible that the DLF QTL on LG 9 has a role in FTA and FTB, but was not detected with the freezing tolerance data. The QTL on LG 7 explains the largest portion (26.5%) of the variation in FTA/FTB, accounting for more than twice that of any other followed by LG 2 (10.6%), LG 5 (7.8%), and LG 4 (4.2%). The combination of these five factors explains 62.9% of the varia-

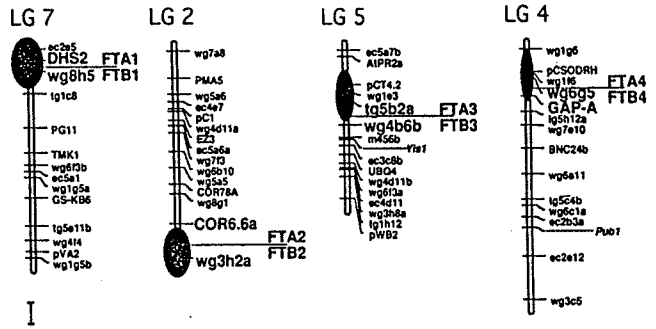

Fig. 3. QTL model for acclimated relative freezing (FTA) and acclimation ability (FTB) of *B. rapa* F3 families. Diameter of each ellipse is proportional to the percent variation explained by the individual QTL in the model and the length of the ellipse is proportional to the confidence interval for the QTL. Locus names are listed on the right of each LG. Linkage group designation and locus order from Teutonico and Osborn [44]. Loci flanking the likelihood peak of each QTL are indicated in large, bold type. The 20 cM linkage distance in Haldane map units is indicated.

tion for this trait in the population, thus, there are probably other FTA/FTB genes that were not detected.

This multi-locus model for FTA/FTB is consistent with studies of winter wheat which concluded there was polygenic inheritance of winter hardiness [11, 20, 33, 41, 42]. Freezing tolerance was reported to be controlled by a few genes in diploid *Solanum* species [40]. In contrast, only one region of the barley genome was associated with cold tolerance [12]; however, additional QTL could have been involved but not identified.

Cold-induced and stress-related genes

The map positions of RFLP loci detected by cDNAs of cold-induced genes or proteins involved in plant stress responses were compared to the map positions of freezing tolerance QTL. An RFLP locus detected by DHS2 mapped very near the FTA/FTB QTL on LG 7. DHS2 encodes 3-deoxy-*D*-arabino-heptulosonate 7-phosphate (DAHP) synthase, the enzyme catalyzing the first committed step in aromatic amino acid synthesis. This enzyme is induced by stresses in *A. thaliana*, including physical wounding and pathogenic attack [17], and may be a member of structurally and functionally related molecules that plants produce in response to various stresses [39]. In addition, cold-induced *COR* 6.6a was just outside of the one-LOD confidence interval for the FTA/FTB QTL on LG 2. The association of RFLP loci detected by these stress-induced cDNAs with FTA/FTB does not imply a function for these genes in freezing tolerance, but suggests a possible involvement that could be studied further.

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