Use of Blueberry to Study Genetic Control of Chilling Requirement and Cold Hardiness in Woody Perennials

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The capacity of woody perennial plants to survive winter freezes is dependent on their entering a state of dormancy and developing cold hardiness (Powell, 1987). Once plants are in this winter dormant or "endodormant" state (Lang et al., 1987), a chilling period is required for vegetative and floral budbreak the following spring. This chilling requirement prevents growth from occurring during transitory periods of warm temperatures throughout a large portion of the winter and helps synchronize plant growth with exposure to favorable environmental conditions. Together, chilling requirement and cold hardiness levels determine to what degree temperate-zone fruit crops will survive the winter and early spring without shoot and flower bud damage.

Chilling requirement and cold hardiness play critical roles in determining winter and early spring survival in woody perennials. Even though these traits are important to fruit crop industries, the inheritance of genes controlling chilling requirement and cold hardiness has not been well studied and little effort has been made to identify and map the genes involved. One of the reasons for this is the difficulty of conducting genetic research in woody perennials. Factors such as long generation times and problems associated with inbreeding depression often render use of recombinant inbred lines and, sometimes, even use of true backcross and F_2 populations impossible for genetic and mapping studies. Another reason for the lack of progress in this area is that the induction of dormancy and the development of cold hardiness occur simultaneously and are triggered by some of the same stimuli, such as increasingly shorter photoperiods and lower temperatures. Thus, discerning if certain physiological changes or changes in gene expression are actually associated with dormancy and/or cold hardiness can be exceedingly difficult.

We are using a combination of molecular, genetic, and physiological approaches to investigate genetic controls of chilling requirement and cold hardiness in blueberry (*Vaccinium* section *Cyanococcus*). In this paper, the term "cold hardiness" specifically refers to flower bud hardiness in the cold-acclimated state. The goals of our research are to: 1) construct a genetic linkage map of blueberry using diploid populations that segregate for chilling requirement and cold hardiness; 2) develop a better understanding of the modes of action of chilling requirement and cold hardiness genes, based on genetic analyses of

gene action and quantitative trait loci (QTL) analyses, and tag genes controlling these traits. Concomitantly, we are trying to identify and isolate chilling/cold-responsive genes from blueberry and determine their relationship to dormancy and/or cold hardiness transitions. Here we describe the system (among blueberry populations) and the strategies used in our research, and discuss our progress in the research areas outlined above.

WHY BLUEBERRY?

There are several advantages to studying chilling requirement and cold hardiness in blueberry. In a recent survey of blueberry research and extension scientists in the United States, lack of cold hardiness and susceptibility to spring frosts were identified as the most important genetic limitations of current cultivars (Moore, 1993). At least eight different state and national breeding programs have as one of their goals either the development of new low-chilling blueberry cultivars for the southern United States or more cold-hardy blueberry cultivars for the northern United States. For the last several decades, one emphasis of the U.S. Dept. of Agriculture (USDA) Fruit Laboratory blueberry breeding program, as well as of similar programs in Florida, Georgia, North Carolina, Mississippi, Arkansas, and Texas, has been to develop hybrid highbush cultivars with low chilling requirements. Most of the cultivars are predominantly *V. corymbosum* L. but with the very low-chilling diploid species *V. darrowi* Camp in their parentage. Such hybrids should be suitable for growing in the southern United States, because they are earlier ripening than the rabbiteye cultivars grown there (Galletta and Ballington, 1996; Hancock and Draper, 1989). Other breeding programs, in Minnesota for example, have been developing more cold-hardy cultivars for the northern United States by hybridizing northern lowbush and highbush species to produce half-high types (Finn et al., 1990; Luby et al., 1986).

In addition to the importance of both chilling requirement and cold hardiness to the blueberry industry, there are other advantages to using blueberry plants for these studies. Blueberry is a small-statured fruit crop amenable to greenhouse and growth chamber experimentation, making it more suitable than most tree fruit species for studies of chilling requirement and cold hardiness. The genome (2C) is small ($\approx 1.2 \times 10^9$ bp) relative to that of plants like maize (*Zea mays* L.) and tomato (*Lycopersicon esculentum* Mill.) (Costich et al., 1993), simplifying any future attempts at marker-based cloning. Genotypes, both wild and cultivated, range in ploidy levels from diploid to hexaploid. There is essentially no sterility barrier between species (Galletta and Ballington, 1996) and ploidy manipulations are well tolerated. The cluster-fruited blueberries appear to be an autopolyploid group displaying bivalent pairing, so homologous chromosomes from different species can pair and segregate normally (Hokanson and Hancock,

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1993; Krebs and Hancock, 1992; Qu and Hancock, 1995, 1997). Consequently, interspecific hybrids can be easily generated and used for developing segregating mapping populations. Production of 2n gametes at a relatively high frequency in some diploid species, such as *V. darrowi* (Draper et al., 1982; Ortiz et al., 1992), has allowed interspecific tetraploid hybrid populations to be generated from diploid x tetraploid F_1 s. We have access to such a diploid *V. darrowi* x tetraploid *V. corymbosum*-derived tetraploid population (Qu and Hancock, 1997) for use in comparative mapping studies with our population of diploid plants derived from *V. darrowi* x *V. caesariense* Mackenz. (also known as diploid *V. corymbosum*).

Furthermore, the feasibility of using *Agrobacterium tumefaciens* to transform blueberry has been demonstrated. We have established the susceptibility of highbush blueberry to infection by *Agrobacterium* (Rowland, 1990), identified a highbush blueberry cultivar particularly amenable to regeneration from leaf disks (Rowland and Ogden, 1992, 1993), and significantly improved efficiency of shoot regeneration from leaf disks (Rowland and Ogden, 1992, 1993). Thus, if genes that control chilling requirement or cold hardiness are isolated, it should be possible to use such gene constructs in transformation experiments to test their effects on chilling requirement and cold hardiness.

CONSTRUCTION OF GENETIC LINKAGE MAPS FOR BLUEBERRY

Description of mapping populations

We previously reported the construction of an initial genetic linkage map for diploid blueberry using a population segregating for chilling requirement, which resulted from a testcross between an F_1 interspecific hybrid (*V. darrowi* x *V. elliotii* Chapm.) and another *V. darrowi* clone (Rowland and Levi, 1994). A testcross was used because diploid blueberry tolerates little inbreeding; therefore, true F_2 or backcrosses cannot be easily generated for mapping. The map currently comprises 72 randomly amplified polymorphic DNA (RAPD) markers mapped to 12 linkage groups (in agreement with the basic blueberry chromosome number), and covers a total genetic distance of over 950 cM, with a range of 3–30 cM between adjacent markers.

Because our interests have expanded to include identification of markers linked to both cold hardiness and chilling requirement, we have recently focused on developing maps for two testcross populations that segregate for both characteristics. These populations were generated by backcrossing *V. darrowi* x *V. caesariense* hybrids (Fla4B

x W85-20) to another *V. darrowi* and another *V. caesariense* (Rowland et al., 1995). Diploid *V. darrowi* is a southern evergreen, lowbush species whose habitat ranges from Florida to Louisiana. *V. darrowi* has been used extensively in blueberry breeding programs to introduce low chilling requirement into the typically high-chilling highbush background (Galletta and Ballington, 1996; Hancock and Draper, 1989; Hancock et al., 1995). The original *V. darrowi* parent plant used in our diploid mapping populations, Fla4B, was, in fact, the primary clone used in the USDA blueberry breeding program to develop low-chilling southern highbush cultivars. Fertile F_1 hybrids (4x) of Fla4B (2x) and *V. corymbosum* (4x) cultivars, such as 'Bluecrop' and 'Bluetta', are relatively easy to produce for breeding purposes, because some *V. darrowi* clones, such as Fla4B, produce a relatively high frequency of unreduced gametes (Draper et al., 1982; Ortiz et al., 1992). Fla4B, collected from Ocala, Fla., has a low chilling requirement and is relatively cold-sensitive (Arora et al., 1998; Rowland et al., 1995). Diploid *V. caesariense* is a highbush, deciduous species that is considered to be either the same species as, or the most closely related species to, the cultivated tetraploid highbush, *V. corymbosum* (Galletta and Ballington, 1996; Luby et al., 1991). The *V. caesariense* clone W85-20, the other original parent plant in our diploid mapping populations, was collected from New Jersey. It has a higher chilling requirement than does Fla4B and is more cold-hardy (Arora et al., 1998; Rowland et al., 1995).

To generate the testcross populations, three F_1 plants were crossed to plants of *V. darrowi* (NJ88-13-15) and *V. caesariense* (W85-23), whose chilling requirements and cold hardiness levels were similar to those of the original parents. Chilling requirements [for the *V. darrowi* parents (Fla4B and NJ88-13-15)], calculated in 1996 and 1997 and averaged over the 2 years, are 386 and 260 chill units (CU), respectively. Here, chill units are defined as the number of hours plants were exposed to temperatures from 0 to 7 °C. Chilling requirements for the *V. caesariense* parents (W85-20 and W85-23) are 1411 and 1188 CU, respectively. Cold hardiness (LT_{50} s) for Fla4B and NJ88-13-15 are -11.3 and -14.3 °C, respectively, whereas cold hardiness for the W85-20 and W85-23 are -21.0 and -21.5 °C, respectively (Arora et al., 1998). *V. darrowi* and *V. caesariense* testcrosses currently comprise ≈150 plants each.

Because of differences in the chilling requirements and levels of cold hardiness of the *V. darrowi* and *V. caesariense* parent plants, the diploid mapping populations were expected to segregate for these traits, and thus would be well suited for identifying markers linked to genes controlling chilling requirement and cold hardiness. The fact

Fig. 1. RAPD-based genetic linkage map of blueberry derived from a cross between diploid *V. darrowi* x *V. caesariense* and *V. darrowi*. Linkage groups are displayed from longest to shortest. Marker names and distances between adjacent markers (in cM) are shown to the right of each linkage group. If no distance is indicated between markers, then no recombination was detected between those markers. Marker names are based on the origin of the primers (either UBC for Univ. of British Columbia or OP for Operon), followed by the primer names (assigned by UBC or Operon), followed by a letter. Letters are assigned in alphabetical order beginning with the highest molecular weight marker generated from a particular primer and ending with the lowest molecular weight marker. Bold lines between markers indicate markers whose order cannot be deduced with good certainty. Symbols (•) indicate markers that were mapped in both the *V. darrowi* and *V. caesariense* testcross populations.

that these populations do segregate for these traits has been confirmed (see following section).

Genetic linkage maps for *V. darrowi* and *V. caesariense* testcrosses

Currently, we have collected segregation data on 89 RAPD markers using an initial 53 plants of the *V. darrowi* testcross and 54 plants of the *V. caesariense* testcross. Of the 89 RAPD markers, 54 could be followed in the *V. darrowi* testcross, 76 in the *V. caesariense* testcross, and 41 in both testcross populations. Using the MAPMAKER program (Lander et al., 1987), 27 of the 54 markers followed in the *V. darrowi* testcross have been assigned to eight linkage groups (Fig. 1) and 37 of the 76 markers followed in the *V. caesariense* testcross to 12 linkage groups (Fig. 2). The number of markers assigned to the individual linkage groups ranges from two to five for the *V. darrowi* testcross and two to seven for the *V. caesariense* testcross. The distance between adjacent markers ranges from 6.3 to 33.7 cM and 5.0 to 43.8 cM, for the *V. darrowi* and *V. caesariense* testcrosses, respectively. Thirteen of the markers have been mapped in both populations. At this stage, the number of shared markers is too few to allow us to superimpose the maps; however, the shared markers appear to group similarly in the two populations.

GENETIC CONTROL OF CHILLING REQUIREMENT AND COLD HARDINESS

Status of QTL and generation means analyses

Although current genetic linkage maps for the *V. darrowi* and *V. caesariense* testcrosses are not saturated enough to map QTLs controlling chilling requirement and cold hardiness, data collected for these traits have been used in generation means analyses (Beaver and Mosjidis, 1988; Mather and Jinks, 1982) to evaluate different additive and epistatic models that may explain the gene action of the traits. In generation means analyses, joint scaling tests (Mather and Jinks, 1982) are used to investigate gene action for a given trait. These tests

use parental, F_1 , and testcross populations' means and variances to estimate values for genetic components and nonallelic (epistatic) interactions of a cross. The estimates are then used to fit genetic models to the data that best explain differences among means for the various populations. The theoretical basis of the joint scaling tests is a linear model. The mean chilling requirement or cold hardiness (X) of a generation can be described by the following linear equation: $X = m + d + h + i + j + l$; where m = means of all possible homozygotes, d = additive component, h = dominance component, i = additive-additive interaction, j = additive-dominance interaction, and l = dominance-dominance interaction. The coefficients of these components for various generations have been described (Mather and Jinks, 1982).

Data for both chilling requirement and cold hardiness have been used to test 18 genetic models that include components md , mh , or mdh in combination with one or more of the epistatic parameters (i , j , or l). Our criteria for accepting a model are that the model must have a chi-square probability of 0.05 or greater and all of its genetic parameters must be significant, as judged by Student's t tests.

Genetic control of cold hardiness

Results from generation means analysis of the cold hardiness data (Arora et al., 1998) are more straightforward than those from the chilling requirement data. Based on the criteria described above, cold hardiness data best fit a simple additive-dominance model (mdh) of gene action (Table 1). Indeed, the observed means for the parental, F_1 , and testcross populations are not significantly different from, and almost identical to, the expected means predicted from the simple additive-dominance model (Table 2). Although both the genetic parameters (d and h) contribute significantly to the additive-dominance model, the h (dominance-gene effect) parameter is only weakly significant ($P \leq 0.10$) relative to the d (additive-gene effect) parameter ($P \leq 0.05$). Similar results have been obtained in other genetic studies. Watkins and Spangelo (1970) concluded that epistasis and dominance are not major factors in the cold tolerance of *Malus* sp., and that low temperature-induced bud damage is controlled by additive variance. In addition, Stone et al. (1993) concluded that cold acclimation ability

Fig. 2. RAPD-based genetic linkage map of blueberry derived from a cross between diploid *V. darrowi* x *V. caesariense* and *V. caesariense*.

in *Solanum* species can be explained by a simple additive-dominance model of gene action, and that the additive component is more highly significant than the dominance component.

Degree of dominance (Falconer, 1989) of cold hardiness has also been evaluated in blueberry (Arora et al., 1998). Because the mean cold hardiness of the F_1 population is -14.7°C —closer to the *V. darrowi* parent than to the *V. caesariense* parent (Table 2)—cold/freezing-sensitivity appears to be partially dominant (or, conversely, cold hardiness is partially recessive). Similar conclusions were drawn by Stone et al. (1993), who noted that cold acclimation ability in *Solanum* is partially recessive. Our results are also analogous to those of Sutka (1981) who reported frost sensitivity in wheat to be partially dominant. Finally, the recovery of parental phenotypes in the relatively small number of testcross individuals analyzed thus far for cold hardiness (40–50 plants of each testcross) suggests that cold hardiness may be controlled by relatively few genes in blueberry (Arora et al., 1998). This, too, has been reported for *Solanum* species by Stone et al. (1993).

Genetic control of chilling requirement

In contrast to the cold hardiness results, generation means analysis of chilling requirement data failed to identify any models that accurately predict the chilling requirement means for the populations analyzed. Simple models lacking epistatic interactions or having only one epistatic component are significant. However, in all cases, one or more of the genetic parameters are not significantly different from zero. Therefore, no models are acceptable.

Several reasons can be offered for the lack of fit. First, it may be partially due to the relatively low chilling requirement mean values for the testcross populations (Table 3). The means of the *V. darrowi* and *V. caesariense* testcross populations are skewed more towards the *V. darrowi* parental and F_1 populations, respectively, suggesting that low chilling requirement is a dominant trait. However, the mean for the F_1 population suggests a low degree of dominance, as the F_1 mean is approximately halfway between the means for the two parental populations. Theoretically, parental and F_1 populations should be uniform and should exhibit less variation than the segregating testcross populations; however, we observed that the parental and F_1 generations exhibit considerable variation. Variances for parental and F_1 populations are approximately equal to or exceed the average variances for the testcross populations. These large variances are due, in part, to a discontinuous distribution of chilling requirement values. For example, the F_1 generation of Fla4B x W85-20, comprised of nine plants, has four plants with chilling requirement values from 530–600 CU and five with values from 800–1010 CU. This discontinuous distribution is indicative of a segregating population. Similarly, the *V. darrowi* parental population, comprised of 14 *V. darrowi* plants, has two plants whose chilling requirements are separated from the remainder of the population by over 600 CU. One of the assumptions of the generation means analysis is that the parents are homozygous for the trait of interest. Heterozygous parents with genes for high and low chilling requirement would result in the segregation of otherwise uniform populations and would invalidate generation means analysis. Hence, we propose that the lack of fit of the models that we tested is due to the segregation and nonuniformity of parental and F_1 generations.

Although we were unable to determine the gene action of chilling requirement from generation means analysis, we attempted to study the inheritance of this trait. Using the distribution of chilling requirement in the F_1 and testcross populations, we have evaluated mono- and digenic models for inheritance of chilling requirement that do not assume that the *V. darrowi* and *V. caesariense* parent plants are each homozygous for low chilling- and high chilling-determining genes. The simplest such model is that chilling requirement is controlled by one gene that is not fixed (i.e., it is heterozygous) in one of the parents. For example, if we assign the *V. darrowi* parent Fla4B the genotype Aa and the *V. caesariense* parent W85-20 the genotype aa, two classes of Fla4B x W85-20 F_1 s would be expected, having the genotypes Aa and aa, which are identical to the parental genotypes. This model can be excluded because, although the F_1 s can be divided into two distinct classes (530–600 and 800–1010 CU), they have chilling requirements

Table 1. Goodness of fit, from joint scaling tests, of various genetic models using cold hardiness data from a cross between *Vaccinium darrowi* and *V. caesariense*.

Model ^a	Chi-square value	P	Component fit
$m [d] [h]$	0.97	0.62	$[d]**[h]^*$
$m [d] [h] [i]$	0.34	0.56	$[d]^*$
$m [d] [h] [j]$	0.59	0.44	
$m [d] [h] [l]$	0.34	0.56	$[d]^*$
$m [d] [i]$	4.38	0.11	$[d]^*$
$m [d] [l]$	0.36	0.84	$[d]^*$
$m [d] [i] [l]$	0.34	0.56	$[d]^*$
$m [d] [j] [l]$	0.02	0.89	

^a m = estimate of the mean of all possible homozygotes, d = additive component, h = dominance component, i = additive-additive interaction, j = additive-dominance interaction, and l = dominance-dominance interaction. Models shown are those in which chi-square probability (P) is greater than or equal to 0.05. Models in which $P \geq 0.05$ and each individual component of the model is significant as judged by t tests were accepted. Only the simple additive-dominance model (mdh) of gene action fits these criteria (Arora et al., 1998).

* **Component significant at $P \leq 0.10$ or 0.05, respectively.

Table 2. Observed (determined by freeze-thaw tests) and expected (based on simple additive-dominance model of gene action) means for cold hardiness (LT_{50} s, $^\circ\text{C}$) for parental (P), F_1 , and testcross (TC) generations of a cross between *V. darrowi* and *V. caesariense* ^a.

Generation	Observed mean $\pm$ SE	Expected mean
P_1 (<i>V. darrowi</i>)	-13.0 ± 1.00	-13.1
TC_1 (F_1 x <i>V. darrowi</i>)	-14.0 ± 0.43	-13.9
F_1	-14.7 ± 0.33	-14.8
TC_2 (F_1 x <i>V. caesariense</i>)	-18.0 ± 0.30	-17.8
P_2 (<i>V. caesariense</i>)	-20.0 ± 1.00	-20.8

^aAdapted from Arora et al. (1998).

Table 3. Observed mean chilling requirements (CU), measured in 1997, for parental (P), F_1 , and testcross (TC) generations of a cross between *V. darrowi* and *V. caesariense*.

Generation	N ^a	Observed mean
P_1 (<i>V. darrowi</i>)	14	425
TC_1 (F_1 x <i>V. darrowi</i>)	54	375
F_1	9	751
TC_2 (F_1 x <i>V. caesariense</i>)	49	817
P_2 (<i>V. caesariense</i>)	12	1005

^aN = number of plants evaluated in each population.

that fall between those of the parents (Table 3).

The next simplest model is that chilling requirement is controlled by two genes (A and B) with equal effects and that A is fixed in the parent populations and B is not fixed. Based on the range of values for chilling requirement, we assigned each allele, a or b, as contributing 300 CU toward the chilling requirement, and proposed that Fla4B and W85-20 have the genotypes AABb and aabb, respectively. In this case, Fla4B would have an expected chilling requirement of 300 CU and W85-20 1200 CU. Two classes of Fla4B x W85-20 F_1 s would result, having the genotypes AaBb and Aabb and chilling requirements of 600 and 900 CU, respectively, at a segregation ratio of 1:1. The theoretical mean for the F_1 population would be 750 CU. The observed segregation ratio for the nine F_1 s is 4 (530–600 CU):5 (800–1010 CU) and fits a 1:1 segregation ratio. The observed mean is 750.7 CU. If we continue on with this model (Table 4), we find that it fairly accurately predicts the segregation ratios observed in the testcross populations, as well, with one exception. In crosses involving the high chilling F_1 s and NJ88-13-15 as the *V. darrowi* testcross parent, the chilling requirements of the progeny are skewed lower than predicted by the model, with a segregation ratio of about 3:1 (≤ 450 CU: 451–750 CU) rather than the predicted 1:1 ratio. This could possibly be explained by the presence of additional modifier genes in NJ88-13-15 that tend to reduce the chilling requirements of progeny. However, this phenomenon is not observed in crosses involving the low chilling F_1 class and NJ88-13-15. The discrepancies may also simply be due to the small population size. More accurate segregation ratios will await the

Table 4. Expected and observed genotypes, chilling requirements, segregation ratios, and means for chilling requirement model that assumes that chilling requirement is controlled by two genes (A and B) with equal and additive effects, and each allele (a or b) is assumed to contribute 300 CU toward the chilling requirement.

Populations	Expected genotypes and segregation ratios	Expected CRs ^z , segregation ratios, and means	Observed CRs, segregation ratios, and means	(χ^2 , P) ^y
P₁				
Fla4B	AABb	300	386	
NJ88-13-15	AABB ^x	minimum, 260	260	
P₂				
W85-20	aabb	1200	1411	
W85-23	aaBb	900	1188	
F₁				
Fla4B x W85-20	1 (AaBb): 1 (Aabb)	1 (600): 1 (900) Mean = 750	4 (530-600): 5 (800-1010) Mean = 750.7	(0.11, 0.74)
TC₁				
F ₁ AaBb x NJ88-13-15	1 (AABB): 1 (AaBB): 1 (AABb): 1 (AaBb)	3 (260 or 300): 1 (600) Mean = 365	9 ($\leq$ 450): 5 (451-750) Mean = 418.1	(0.86, 0.35)
F ₁ Aabb x NJ88-13-15	1 (AABb): 1 (AaBb)	1 (300): 1 (600) Mean = 450	31 ($\leq$ 450): 9 (451-750) Mean = 360.2	(10.1, 0.0015)
TC₂				
F ₁ AaBb x W85-23	1 (AaBB): 1 (aaBB): 2 (AaBb): 2 (aaBb):	1 (300): 3 (600): 3 (900): 1 (1200)	3 ($\leq$ 450): 9 (451-750): 9 (751-1050): 4 (1051-1350)	(0.28, 0.96)
F ₁ Aabb x W85-23	1 (AaBb): 1 (aaBb): 1 (Aabb): 1 (aabb)	1 (600): 2 (900): 1 (1200) Mean = 900	3 (451-750): 16 (751-1050): 5 (1051-1350) Mean = 879.3	(3.00, 0.22)

^zCR = chilling requirement, expressed in chill units (CU).

^yA chi-square test was used to determine the goodness of fit of the observed segregation ratios to the expected ones. $P \leq 0.05$ was considered a significant deviation from the expected values.

^xFor genotype AABB, a minimum chilling requirement of 260 CU is assumed.

maturation of more progeny plants and determinations of their chilling requirements. We do not presently exclude this model, however, to explain chilling requirement gene action in blueberry.

CHILLING/COLD-RESPONSIVE GENES IN BLUEBERRY

Identification of chilling-responsive proteins

To identify proteins associated with low-temperature exposure or "chilling," we have examined changes in protein levels associated with CU accumulation in blueberry floral buds (Muthalif and Rowland, 1994a, 1994b). From profiles of soluble proteins, levels of three proteins of 65, 60, and 14 kD were observed to increase with CU accumulation such that they become the predominant proteins visible on SDS gels. Further characterization by partial sequence analysis and immunoblot analysis has revealed that they belong to the dehydrin family of proteins (Muthalif and Rowland, 1994a, 1994b). Since that time, additional dehydrin proteins have been identified in blueberry, with molecular weights of $\approx$ 40 kD (Arora et al., 1997a), 22 kD (Muthalif and Rowland, 1995), and 10 kD (Muthalif and Rowland, 1994a). Presence and sizes of these additional dehydrins, however, vary depending on species and cultivar.

Dehydrins are a group of heat-stable, glycine-rich plant proteins that are induced by environmental stimuli that result in dehydration, including drought, low temperature, salinity, and seed maturation (Close, 1996; Close et al., 1993). Another characteristic of dehydrins is the presence of a highly conserved, lysine-rich, 15-amino-acid sequence (consensus sequence EKKGIMDKIKEKLP), referred to as the K box, which is often repeated several times throughout the protein (Close, 1996; Close et al., 1993). A number of physiological studies have demonstrated a correlation between accumulation of dehydrins and tolerance to environmental stresses leading to dehydration. The first evidence for a causal relationship between such genes and freezing tolerance was reported earlier this year from work on *Arabidopsis* (Jaglo-Ottosen et al., 1998).

Cloning and sequencing members of the dehydrin gene family

Peptide sequence information from the 65 and 60 kD dehydrins of blueberry has been used to synthesize degenerate DNA primers for PCR amplification of a part of the gene(s) encoding these or other

dehydrins. One pair of primers amplified a 174 bp fragment; sequence analysis confirmed that the fragment was derived from a dehydrin gene (Rowland et al., 1996). The 174 bp fragment has been used to screen a cDNA library (prepared from RNA from cold-acclimated blueberry floral buds) and has resulted, to date, in the isolation of one cDNA clone with a 2.0 kb insert (Rowland and Arora, 1997). The cDNA has been sequenced and found to be a full length clone encoding a K_s type of dehydrin (contains 5 K boxes). From the deduced protein sequence and coupled in vitro transcription/translation reactions, the clone appears to encode a dehydrin with a molecular weight of $\approx$ 40 kD. The dehydrin cDNA hybridizes on RNA blots to two chilling-responsive messages of 2.0 and 0.5 kb (Fig. 3), the 2.0 kb message being the same size as the clone and the 0.5 kb message being of an appropriate

Fig. 3. Northern blot of total RNA extracted from blueberry cultivars 'Tifblue' and 'Bluecrop' and hybridized with the 2.0 kb dehydrin cDNA. RNA was extracted from floral buds collected from field plants after different lengths of chilling (from 0 to 1500 CU) and equal amounts were loaded in each lane.

size to possibly encode the 14 kD dehydrin. An examination of dehydrin mRNA expression patterns during the winter in two blueberry cultivars, 'Bluecrop' and 'Tifblue', with different freezing tolerances revealed that both the 2.0 and 0.5 kb messages increase more rapidly and to higher levels in the more cold-hardy 'Bluecrop' than in the cold-sensitive 'Tifblue'; in addition, the 0.5 kb message declines relatively faster in 'Tifblue' than in 'Bluecrop'. Also, the dehydrin cDNA hybridizes on DNA blots to an RFLP marker suitable for mapping in our diploid blueberry populations, which segregate for chilling requirement and cold hardiness. We are currently attempting to use this clone as a probe to isolate and characterize additional members of the dehydrin gene family.

Relationship of dehydrins to dormancy or cold hardiness in woody perennials

Because cold acclimation and development of dormancy, as well as deacclimation and release from dormancy, occur simultaneously in woody perennials, it is impossible from the work described above (Muthalif and Rowland, 1994a, 1994b) to discern whether the major dehydrins of blueberry are more closely associated with cold acclimation or with the development or maintenance of dormancy. In a recent study, we have carried out a series of physiological experiments to better make this distinction (Arora et al., 1997a). These experiments utilized a temperature treatment of 15 °C day/12 °C night for 2 weeks to trigger deacclimation in dormant, cold-acclimated plants (plants that had only 50% of their chilling requirements satisfied). This treatment had no effect on dormancy status (negation of CU) while resulting in loss of cold hardiness (deacclimation). Moreover, for blueberry cultivars that have undergone this deacclimation treatment, levels of the dehydrins decrease to about the levels in nonacclimated plants (Fig. 4). Thus, we conclude from these experiments that dehydrin levels are more closely associated with cold hardiness transitions than with changes in dormancy status per se.

CONCLUSIONS

Because of limited understanding concerning chilling requirement and cold hardiness in woody perennials, we are using a combination of genetic, molecular, and physiological approaches to investigate genetic control and regulation of these traits in blueberry. Genetic and

molecular approaches are being used to map QTLs controlling chilling requirement and cold hardiness and to determine their mode(s) of action. To achieve the mapping goal, RAPD-based genetic linkage maps are being constructed using two diploid testcross populations, segregating for chilling requirement and cold hardiness. To date, 27 RAPD markers have been assigned to eight linkage groups in the *V. darrowi* testcross and 37 RAPD markers have been assigned to 12 linkage groups in the *V. caesariense* testcross population. Chilling requirement and cold hardiness data obtained from the mapping populations have been used in generation means analyses to evaluate different models of gene action. Data for cold hardiness best fit a simple additive-dominance model of gene action, whereas data for chilling requirement do not. The lack of fit of the chilling requirement data is apparently due to at least one of the parental populations not being homozygous for one or more genes controlling chilling requirement. By examining segregation ratios of chilling requirement in the F_1 and testcross populations, as well as the goodness of fit to some relatively simple genetic models that do not assume homozygosity of the parental populations for chilling requirement-determining genes, we have developed a two-gene model that fairly accurately predicts the observed segregation ratios. In its simplest form, this model assumes that two genes with equal and additive effects control chilling requirement.

Concomitantly, molecular and physiological approaches are being used to identify and isolate chilling-responsive genes from blueberry and determine their relationship to chilling requirement or cold acclimation. Major chilling-responsive proteins found in blueberry flower buds have been identified as dehydrins, and one of the members of the dehydrin gene family has been cloned and sequenced. Using an experimental approach that triggers deacclimation without affecting dormancy status of buds, we have also established that the dehydrins are associated with changes in cold hardiness rather than in dormancy status.

Finally, once chilling-responsive genes are cloned, they can be mapped to determine if they are associated with loci identified from QTL analyses that control chilling requirement and cold hardiness. In addition, gene constructs can be used in transformation experiments to determine their effect(s) on these characteristics. These experiments will allow us to more definitively establish the relationship between chilling-responsive genes and the determination of chilling requirement and cold hardiness.

Fig. 4. (A) SDS-PAGE protein profiles of 'Tifblue' blueberry buds at various levels of cold hardiness and CU accumulation. Protein samples (three lanes) correspond to various treatments as follows: *lane 1*, nonacclimated/0 CU; *lane 2*, cold acclimated/50% of chilling requirement satisfied; *lane 3*, deacclimated/50% of chilling requirement satisfied. Thirty micrograms of protein was loaded in each lane. To the left, molecular weight markers are shown. Arrows to the right mark the 65, 60, 14, and 10 kD dehydrins. (B) Western blot analysis of proteins in (A), using anti-dehydrin antiserum. Five micrograms of protein was loaded in each lane (Arora et al., 1997b).

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