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Systems and Approaches to Studying Dormancy: Introduction to the Workshop

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Dormancy has significant practical and economic impacts on the control, maintenance, and production of herbaceous and woody plants. As the demand for quantity and diversity of food and forestry crops continues to expand, extending the production ranges of temperate and tropical crops into marginal environments, controlling weed species, and precisely timing cultural practices for production and storage have intensified the need for a greater understanding of plant dormancy. Phenological, physiological, biochemical, and biophysical descriptions of this progressive phase of seed and plant development have been reported for a wide variety of species. In the last 30 years, this research has improved quantification and modification of dormancy in some production systems. However, the interactions of genotype with environmental factors and cultural practices continue to limit our ability to consistently predict and regulate dormancy induction, maintenance, and release. The objective of the "Systems and Approaches to Studying Dormancy Workshop" was to present and discuss genetic systems and molecular approaches to studying endodormancy in seeds and buds.

In the previous workshop on quantification of plant dormancy, Fuchigami and Wisniewski (1997) emphasized the need to expand existing models by precisely correlating underlying physiological and molecular events with phenological events to help identify specific phases and transitions in the dormancy process. Khan (1997) and Seeley (1997) indicated that the facility to quantify dormancy at the

biochemical and molecular level requires separation and direct measurement of the induction, maintenance, and release processes in dormancy. Faust et al. (1997) suggested that in buds of temperate plants, quantification could be approached by considering two major components of dormancy research, the hormonal component that normally regulates growth, and a second system, superimposed over the hormonal system, that is regulated by environmental factors and stresses.

These proposals all emphasize the importance of identifying systems and approaches that can be used to separate dormancy, growth, and acclimation processes. In the last 10 to 15 years, considerable progress has been made in developing genetic systems to study the inheritance and molecular basis of dormancy induction, maintenance, and release. Dormancy has been shown to be controlled by a single gene or a few genes in some seed and bud systems: seed dormancy in wild oats (*Avena fatua* L.), bud dormancy in filbert (*Corylus avellana* L.) and lack of terminal bud dormancy in evergreen peach [*Prunus persica* (L.) Batsch, 'Sempervirens'] (Li and Foley, 1997; Rodriguez et al., 1994; Thompson et al., 1985). However, physiological and modeling research in other species indicate that, in most genetic systems, dormancy is a more complex trait. Therefore, a combined physiological, molecular, and genetic approach is needed to provide an understanding of dormancy induction, maintenance, and release in plant systems.

Extensive studies and reviews have addressed the phenological, physiological, biochemical, and ultrastructural approaches to studying dormancy. Typically, temporal studies under natural conditions, or studies using imposed environmental or chemical stimuli, are conducted to correlate factors with depth of dormancy. These studies have

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shown that dormancy is genetically controlled and induced by plant hormones, photoperiod, temperature, and abiotic stresses. This has provided a rough road map of the dormancy cycle and indicated areas of research that can be studied using molecular approaches.

Molecular studies can be roughly grouped into two approaches: 1) analysis of differential gene expression in search of unidentified genes or cDNAs that are correlated with dormancy processes, and 2) searching for specific gene expression based on ultrastructural, physiological, or biochemical information or genes identified in other systems.

Analysis of differential gene expression using either differential display of mRNA or differential screening of cDNA libraries has produced many cDNAs related to dormancy in seeds, buds, and other storage organs (Johnson et al., 1995; Smart, 1996; Stafstrom et al., 1998; Walker-Simmons and Goldmark, 1996). Characterization of cDNAs in response to natural cycles and artificial stimuli that induce or break dormancy provides clues as to their role in dormancy. Sequence analysis and comparison of this information with known gene sequences can provide information on the potential identity and function of the genes. Further studies on transcriptional and posttranscriptional control provide information on the regulation of gene expression during dormancy. These genes can then be used for in situ hybridization and tissue printing to identify tissue and cellular specificity. Additionally, transgenic plants can be produced with chimeric genes, to study expression in vivo, to help verify function in the dormancy processes (Rohde et al., 1997).

Targeted analysis of genes and cDNAs identified in other dormancy mutants or expression systems can be used to test or verify functional relationships with dormancy processes. Similarly, use of antibodies for specific proteins, identified in physiological/biochemical studies, can be used to isolate cDNAs from expression libraries. Degenerate primers can be constructed based on sequence information and used to isolate genes for testing. Characterization and analysis of these genes proceed in steps very similar to those used to study differentially expressed cDNAs. Cell cycle, phytochrome, kinases, peroxidase, and dehydrin and storage protein genes are a few examples that are being used to study dormancy processes. Both the targeted gene and the differential expression investigative approaches can provide markers to identify specific stages or transitions in the dormancy cycle. These markers can also be used as probes for investigation of signal transduction from stimuli to response and marker-assisted genetic analysis and breeding programs.

The identification of molecular markers associated with dormancy has facilitated the genetic analysis of this complex trait. Random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP), or amplified restriction fragment length polymorphisms (AFLP) analysis are being coupled with mapping and inheritance studies to provide greater understanding of the regulation of dormancy. Coupling segregating genetic systems with molecular and genetic analysis in the identification and functional characterization of dormancy-related genes and subsequent molecular processes will increase our understanding of, and potential to manipulate, the dormancy cycle in cropping systems.

The papers presented in this workshop focused on three different genetic model systems that have been developed to study molecular and genetic regulation of dormancy in buds and seeds. Bud dormancy in a low-chill vs. high-chill blueberry (*Vaccinium* sp.) model system

was presented by Rowland et al. (1999), who focused on the genetic control of dormancy, acclimation, and fulfillment of chilling requirements. Bulk segregant analysis and RAPD approaches to identify markers coupled with quantitative trait loci for dormancy in wild oat were presented by Foley (1999). Physiological and genetic relationships in poplar (*Populus* sp.) bud dormancy were presented by Howe et al. (1999), who are using a combined physiological and quantitative trait loci (QTL) strategy to map and study the complex phenotypic and molecular traits associated with the dormancy phenomena in *Populus*. This workshop was the first dedicated to the discussion of development and use of genetic systems to investigate the inheritance and regulation of dormancy in seeds and buds. It provided an excellent opportunity to discuss the strengths and limitations of molecular approaches and genetic systems being used to study bud and seed dormancy phenomena.

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Genetic Basis for Dormancy in Wild Oat

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Wild oat (*Avena fatua* L.) is a serious weed because it mimics the life cycle of small-grain crops and displays seed dormancy. Wild oat is used as an experimental system to investigate dormancy and afterripening under warm-dry conditions. Dormancy in wild oat is controlled by the action and interaction of genetic and environmental factors. In the 1970s, Canadian researchers made reciprocal crosses of some highly inbred dormant and nondormant lines of wild oat and developed a preliminary genetic model for dormancy (Jana et al., 1979). To further investigate the inheritance of dormancy, we crossed a well-characterized and highly inbred dormant (M73) with a nondormant (SH430) line of wild oat. Germinability was evaluated for seeds in an F_2 , backcross, and a recombinant inbred (RI) line population segregating for dormancy. The data were used to develop a genetic model for dormancy, identify molecular markers associated with quantitative trait loci (QTL) for dormancy, and investigate a genotype $\times$ environment interaction on germinability. Germination phenotypes were determined by imbibing all populations at 15 °C for 6 weeks and recording the relative number of days to germination. Progeny testing was used to verify the germination phenotypes. The most dormant caryopses did not germinate. Dormancy is recessive and at least three genes regulate dormancy in wild oat. One or more of these genes is responsive to germination temperature. Genes at the G_1 and G_2 loci promote early germination, while the third gene (D) delays germination.

According to our model, if at least two copies of the dominant G_1 or G_2 allele are present (i.e., two at the G_1 locus, or two at the G_2 locus), regardless of the genotype at the D locus, then the seed will be nondormant. If the genotype is $G_1g_1G_2g_2$, then the seed will be nondormant, provided that the genotype at the D locus is Dd or dd . If only one allele of either G_1 or G_2 , or none, is present and the genotype is dd , then the seed will also be nondormant. The $G_1g_1G_2g_2DD$, $G_1g_1g_2g_2DD$, $G_1g_1g_2g_2Dd$, and $g_1g_1G_2g_2Dd$ individuals will have a phenotype intermediate between nondormant and dormant. If the genotype is $g_1g_1G_2g_2DD$ or $g_1g_1g_2g_2D$, then the phenotype will be dormant. Using bulked segregant analysis and random amplified polymorphic DNA (RAPD) techniques, we identified two independently segregating markers that are linked in coupling with QTL for dormancy in wild oat. Further research is needed to identify additional and tightly linked molecular markers for dormancy QTL. Molecular markers will facilitate improvements in our model and genotypic classification of RI lines. Investigation of allelic interactions, epistatic interactions, and genotype $\times$ environment interactions will be essential for understanding the fundamental basis for dormancy.

An abstract only is presented here because a detailed paper was previously published (Foley and Fennimore, 1998).

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Physiological and Genetic Approaches to Studying Endodormancy-related Traits in *Populus*

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Plants native to boreal, temperate, and subtropical regions are subjected to large seasonal variations in temperature. In these regions, woody perennial plants have evolved annual growth cycles that promote long-term survival and growth. These annual growth cycles involve alternations between active shoot growth and vegetative dormancy (endodormancy) that are closely timed with seasonal changes in the local climate. Dormancy, in general, is defined as the temporary suspension of visible growth of any plant structure containing a meristem (Lang, 1987). Endodormancy, which develops in the fall, is characterized by a requirement for sustained exposure to low, near-

freezing temperatures before active shoot growth can resume in the spring. This need for exposure to low temperatures is called a chilling requirement. Endodormancy is also referred to as "winter dormancy" or "rest" (Fuchigami and Nee, 1987).

The development of endodormancy is an important adaptive strategy in woody perennial plants and greatly influences their use in both horticulture and forestry. Endodormancy is adaptive because plants are more resistant to freezing and dehydration stress during these periods of relative inactivity. On the other hand, biomass production may be substantially reduced by relatively long periods of dormancy when growth does not occur. Therefore, the length of the dormant period represents a trade-off between winter survival and biomass yield. A greater understanding of endodormancy-related traits would allow us to better match horticultural varieties or seed sources with zones of plant hardiness (Cathey, 1990), breed trees that are more cold hardy, or develop higher-yielding genotypes for use in reforestation (reviewed in Howe et al., 1995).

We define endodormancy-related traits in the broad sense, as traits associated with any aspect of endodormancy induction, maintenance, or release. In some cases, endodormancy-related traits may be associated with endodormancy per se, but in other cases, the association may be indirect. For example, short-day-induced traits are endodormancy-related because short days (SDs) can induce endodormancy, and because these traits are temporally associated with the induction of endodormancy in nature. Quantitative or semi-quantitative measurements have been made on a number of endodormancy-related traits. These include the timing of spring bud flush (Bradshaw and Stettler, 1995), timing of fall bud set (Riemenschneider et al., 1994), degree of frost hardiness in the spring or fall (Aitken and Adams, 1995), degree of endodormancy (Farmer and Reinhold, 1986), and chilling requirement (Sorensen, 1983). These traits have been well studied because they are clearly adaptive and because they shed light on what is happening at the whole-plant level. In addition, the number of investigations of endodormancy-related biochemical traits has increased in recent years. A number of these biochemical traits will be discussed.

The objectives of this paper are threefold. First, we will introduce the reader to the use of *Populus* species and hybrids as models for studying the physiology and genetics of endodormancy-related traits in trees. Second, we will discuss approaches for studying endodormancy-related processes and genes, focusing on approaches that will shed light on genetic variation in these traits. We will use examples of our research on endodormancy-related traits in *Populus* to illustrate these approaches. Finally, we will discuss priorities for future research.

POPULUS SPECIES AND INTERSPECIFIC HYBRIDS ARE GOOD MODELS FOR STUDYING ENDODORMANCY-RELATED TRAITS IN TREES

In addition to their economic importance, the poplars (poplars, cottonwoods, and aspens) have a number of ecological, physiological, and molecular genetic traits that make them excellent models for studying endodormancy-related traits in trees. First, a number of poplar species, including black cottonwood (*Populus trichocarpa* Torr. & Gray) and eastern cottonwood (*P. deltoides* Bartr.), have extensive latitudinal distributions and contain northern and southern ecotypes that have dramatically different endodormancy-related characteristics (Howe et al., 1995; Pauley and Perry, 1954). Second, the poplars have seasonally indeterminate growth—that is, they will grow continuously under long days (LDs), but will stop growing, form a terminal bud, and become endodormant under SDs (Howe et al., 1995; Jian et al., 1997). In both eastern and black cottonwood, for example, SDs alone are capable of inducing a high degree of bud endodormancy, comparable to that observed under natural outdoor conditions (Jian et al., 1997; G.T. Howe, unpublished results). In addition, because photoperiodic responses in poplar are mediated by photoreceptor proteins called phytochromes (Howe et al., 1996; Olsen et al., 1997b), the poplars are excellent models for studying both the effects of SDs on endodormancy-related traits in general, and the roles of the phytochromes, in particular. Third, the poplars are the fastest-growing trees in the temperate zone and are easy to propagate by root or stem

cuttings. The availability of clonal material facilitates destructive sampling and the testing of identical genotypes in multiple environments. Fourth, the poplars are ideal for molecular genetic studies of trees because of their small genome size (Bradshaw and Stettler, 1993), the availability of genetic linkage maps (Bradshaw et al., 1994; Liu and Furnier, 1993), and their ability to be transformed using either biolistics or *Agrobacterium*-mediated transformation (Charest et al., 1997; Fillatti et al., 1987). Transgenic technology is more advanced in the poplars than in any other forest tree genus. Fifth, interspecific hybridization is relatively easy and useful for capturing hybrid vigor (heterosis) for commercial use, or for producing pedigrees that are desirable for mapping quantitative trait loci (QTLs). Interspecific hybrid pedigrees, for example, have already been used to map QTLs affecting stem growth, spring bud flush, and disease resistance (Bradshaw and Stettler, 1995; Newcombe and Bradshaw, 1996; Newcombe et al., 1996). Finally, the taxonomic relationship between *Populus* and *Arabidopsis thaliana* (L.) Heynh. is close enough that information on the molecular genetics of *Arabidopsis* should be useful for gaining a better understanding of endodormancy-related traits in trees. In fact, this has already been done for a number of putative endodormancy-related genes, including genes encoding the phytochromes and those involved in abscisic acid (ABA) perception (Howe et al., 1998; Rohde et al., 1998, 1999).

PHYSIOLOGICAL VS. GENETIC APPROACHES FOR STUDYING ENDODORMANCY

Many morphological, physiological, and biochemical changes are associated with endodormancy, including those responsible for the expression of the endodormancy-related traits described above. Although substantial research has been devoted to characterizing many of these changes, we remain largely ignorant of the events that occur at the biochemical level, including changes in gene expression. The general biochemical changes that are associated with endodormancy, including changes in phytohormone levels, have been reviewed (Faust et al., 1997; Lang, 1994; Powell, 1987; Rowland and Arora, 1997). We will focus our discussion on characterizing changes in gene expression and identifying endodormancy-related genes.

Approaches to detecting changes in gene expression and identifying the corresponding genes can be divided into two classes, physiological and genetic. Most of our understanding about endodormancy is derived from physiological approaches that focus on characterizing the morphological, physiological, and biochemical differences between plants that are in different physiological states (e.g., dormant vs. nondormant). Generally, these approaches are based on the hypothesis that the differences that are observed between physiologically different plants are functionally important. Trees with contrasting physiological characteristics can be achieved by exposing the plants to contrasting environmental conditions (Coleman et al., 1991; Jian et al., 1997), sampling the plants at different times in a changing environment (Coleman et al., 1991), or exposing different genotypes to an environment that elicits a different physiological response in each genotype (Arora et al., 1992).

Physiological approaches aimed at identifying endodormancy-related genes include general analyses of differential gene expression and expression analyses of specific candidate genes. In contrast to the physiological approaches, genetic approaches seek to identify endodormancy-related genes based on their genetic cosegregation with endodormancy-related traits. Genetic approaches include Mendelian genetic analyses and mapping of quantitative trait loci (QTL mapping).

PHYSIOLOGICAL APPROACHES

The physiology of endodormancy induction, maintenance, and release in *Populus*

Because SDs can induce endodormancy (discussed below), photoperiodic responses are important endodormancy-related traits in many tree species. The timing of SD-induced growth cessation and bud set varies among latitudinal ecotypes of *Populus*. We investigated the

Fig. 1. Photoperiodic responses of a northern (—●—) and a southern (---○---) ecotype of black cottonwood (*Populus trichocarpa*) growing in a greenhouse. For terminal bud set (A), the numerical values are the percentages of trees that formed a permanent or temporary bud during the experiment. Treatments in which all of the trees formed a terminal bud are connected by solid lines and treatments in which some (or all) of the trees were actively growing on the last day of the experiment are connected by dashed lines. The number of new leaves (B) represents the number of newly visible leaves that appeared from the time that the photoperiodic treatments were initiated until the end of the 53-d experiment (Adapted from Howe et al., 1995).

Fig. 2. Growth curves for a northern (—) and a southern (-----) ecotype of black cottonwood (*Populus trichocarpa*) growing under either a 13- or 21-h photoperiod in a greenhouse.

photoperiodic responses of two northern (53°35' and 53°50'N) and two southern (34°10' and 40°32'N) genotypes of black cottonwood by growing trees under a range of photoperiods in the greenhouse (Howe et al., 1995). Although SDs induced bud set in both the northern and southern ecotypes, the northern ecotype had a longer critical photoperiod (i.e., the longest photoperiod that elicited a short-day response) and greater photoperiodic sensitivity (i.e., the change in response per unit change in photoperiod) (Fig. 1 A and B) (Howe et al., 1995). Because of these differences, the southern trees exhibited a significant delay in the timing of bud set compared with the northern trees, even under a 9-h photoperiod (Fig. 1A). We also found that a 13-h photoperiod could be used to obtain maximum discrimination between the photoperiodic responses of the northern and southern ecotypes. Under the 13-h photoperiod, the northern genotypes set bud and stopped growing in ≈ 17 d (on average), whereas this daylength had little effect on the growth of the southern genotypes (Fig. 2). Therefore, the 13-h photoperiod provides a good experimental system for comparing the physiological genetics of photoperiodism under uniform environmental conditions.

In addition to inducing growth cessation and bud set, SDs eventually induce endodormancy in both eastern and black cottonwood (Jian et al., 1997; G.T. Howe, unpublished results). Nonetheless, other changes, such as leaf senescence and abscission, are not induced by SDs alone, but require exposure to low temperatures (≈ 5 °C; T.H.H. Chen, unpublished results; G.T. Howe, unpublished results). Therefore, one set of endodormancy-related responses appears to be induced by SDs, and another set by low temperatures. These low-temperature responses, however, may require prior exposure to SDs. Analogous results are observed for SD-induced cold acclimation. In a number of

Table 1. Geographic origins of eastern cottonwood (*Populus deltoides*) genotypes.

Clone	State	Latitude (°N)
S7C4	Texas	30°30'
108	Oklahoma	33°30'
117	Oklahoma	33°50'
118	Oklahoma	33°50'
251-3	Missouri	38°45'
235-2	Missouri	38°50'
217-3	Ohio	38°55'
243-1	Missouri	39°00'
52-2	Ohio	40°30'
273-1	Illinois	41°45'
286-1	Illinois	41°55'
284-3	Illinois	41°55'
172-2	Minnesota	44°20'
172-4	Minnesota	44°20'

Fig. 3. The relationship between latitudinal origin and the timing of terminal bud set for eastern cottonwood (*Populus deltoides*) trees growing in the field in Corvallis, Ore.

species, a modest degree of cold hardiness is induced by SDs alone, but a second stage of acclimation, which leads to significant increases in cold hardiness, is induced by subsequent exposure to low temperatures (reviewed in Fuchigami et al., 1971b).

We also investigated ecotypic variation in eastern cottonwood growing under natural conditions. Genotypes from a range of latitudinal origins (Table 1) were grown in Corvallis, Ore. (44°34'N), and the timing of bud set was recorded. The number of days to bud set was negatively correlated with latitudinal origin, demonstrating that pronounced ecotypic variation can be detected for this species under natural conditions (Fig. 3) (Pauley and Perry, 1954). The ecotypic variation that is present in both eastern and black cottonwood provides us with useful systems for studying genetic variation in a host of endodormancy-related traits.

We characterized endodormancy induction and release in eastern cottonwood (clone 172-2) by growing trees under natural conditions, and under SDs in a controlled environment (8-h day/16-h night) (Fig. 4). On each sampling date, trees from each environment were moved to LD conditions (16-h day/8-h night) in the greenhouse to determine the degree of endodormancy. After these trees were manually defoliated and the apical buds were removed, the degree of endodormancy was measured as the number of days to axillary bud flush. After 10 weeks, the trees growing under SDs were transferred to a cold room to fulfill their chilling requirement, and endodormancy was measured every 2 weeks thereafter. Although the trees growing under SDs formed a terminal bud in ≈ 3 weeks, there was no change in endodormancy during this time. There was a slight change in endodormancy by week 5, and after 6 weeks, endodormancy increased rapidly. After 10 weeks of SDs, the trees required >250 d to break bud under LD conditions. At this time, the trees were transferred to a cold room. After 10 weeks of chilling (i.e., by week 20), the endodormancy of these trees had declined significantly, almost to the level of the controls growing under LDs (<20 d to bud flush). Thus, we can reproduce the annual endodormancy cycle in ≈ 20 weeks, and can complete almost three endodormancy cycles per year. Under natural conditions, the degree of endodormancy varies among years because of temperature fluctuations and variability in other environmental factors. Because we can use daylength and temperature treatments to induce and release endodormancy in controlled environments, this system is both reproducible and reliable.

We used this system to investigate changes in apical bud ultrastructure in eastern cottonwood (Jian et al., 1997). As endodormancy increased, we observed increases in the thickness of cell walls, in the number of starch granules, and in storage proteins in the vacuoles of the apical bud cells. The most striking change was the constriction and blockage of the plasmodesmata. Cell division in the apical cells

seemed to be completely arrested after 2 to 3 weeks of SDs. The constriction and blockage of plasmodesmata may cause discontinuities in symplastic transport and may limit cellular communication and signal transduction between adjacent cells. This, in turn, may lead to events associated with growth cessation and bud endodormancy. The potential roles of plasmodesmata in bud endodormancy were recently reviewed by van der Schoot (1996).

We also investigated calcium distribution within the apical bud during the induction of endodormancy (Jian et al., 1997). Calcium antimonate precipitates form when apical buds are fixed with potassium antimonate. Under LDs, calcium precipitates were found mainly in the vacuoles, intercellular spaces, and plastids. Some Ca^{2+} deposits were also found in the cell walls and at the orifice of the plasmodesmata, but there were few Ca^{2+} deposits in either the cytosol or nucleus. After exposure to 20 SDs, when cell division had stopped and the plants were beginning to become endodormant, fewer Ca^{2+} deposits occurred in the intercellular spaces, and deposits were now found in both the cytosol and nucleus. A large number of Ca^{2+} deposits remained in the cytosol and nucleus between days 28 and 49 of SD exposure. When deep endodormancy was reached after 77 d of SD exposure, Ca^{2+} deposits were less pronounced in both the cytosol and nucleus, whereas numerous deposits were again observed in the cell walls and intercellular spaces. These results indicate that changes in subcellular Ca^{2+} localization and apical bud ultrastructure occur during the induction of endodormancy. Alterations in Ca^{2+} levels regulate many cellular processes in plants, including ion transport and gene expression (Bush, 1995). In addition, environmental stimuli such as temperature and light can alter cellular Ca^{2+} distribution (Bush, 1995). Our results suggest that calcium may be an important regulator of SD-induced endodormancy.

Analyses of differential gene expression

Biochemical changes are associated with bud endodormancy in woody plants, including changes in enzymatic activity and nucleic acid synthesis (Häggman et al., 1985; Li et al., 1989; Nir et al., 1986; Wang et al., 1991). Significantly, Zimmerman and Faust (1969) observed substantial incorporation of uracil and valine into the nucleic acid and protein fractions of pear (*Pyrus calleryana* Dcne.) buds during the winter, indicating that RNA and protein metabolism are occurring in endodormant buds. These results suggest that changes in gene expression are important during dormancy induction, maintenance, and release.

Analyses of differential gene expression are essentially physiological approaches that seek to identify genes whose patterns of expression are closely associated with the trait of interest. Differences in gene expression are usually evaluated by measuring differences in the steady-state levels of either proteins or mRNAs within tissues, organs, or whole plants. Direct evidence for dormancy-associated changes in gene expression was obtained by *in vitro* translation of poly(A)⁺ RNA isolated from dormant buds of Scots pine (*Pinus sylvestris* L.) (Nuotio et al., 1990). In imbibed seeds of oat (*Avena fatua* L.) and wheat (*Triticum aestivum* L.), the levels of individual polypeptides and/or mRNAs differ between dormant and nondormant embryos (Dyer, 1993; Li and Foley, 1994; Reid and Walker-Simmons, 1990). The cDNAs corresponding to differentially expressed genes were isolated using differential screening (Li and Foley, 1995) and differential display (Johnson et al., 1995). Sequence analyses of these cDNAs suggest that some of the differentially expressed genes encode late embryogenesis-abundant (LEA) proteins, aldose reductase, and glutathione peroxidase, whereas others have no significant similarities to any known genes (Johnson et al., 1995; Li and Foley, 1995). Overall, these observations suggest that differential gene expression probably plays an important role in both seed and bud dormancy.

Differential expression at the protein level

Techniques based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) are commonly used to examine differential gene expression at the protein level. In one-dimensional SDS-PAGE, proteins are isolated from the tissue of interest, dissociated into

Fig. 4. Degree of endodormancy of eastern cottonwood trees (*Populus deltoides* clone 172-2) sampled bi-weekly from controlled environments. Degree of endodormancy was measured as both the number of days to bud flush (—●—) and percentage bud flush (—□—) on decapitated and defoliated trees as described in the text.

individual polypeptide subunits using SDS, and the resulting SDS-polypeptide complexes are separated on a polyacrylamide gel. Using this technique, one can determine the relative abundance of polypeptides that differ in mass. Using one-dimensional SDS-PAGE, two classes of highly abundant proteins have been discovered that are associated with endodormancy in a wide array of woody plants. These are the vegetative storage proteins and the dehydrins (reviewed in Rowland and Arora, 1997). We will discuss one class of vegetative storage proteins, the bark storage proteins, as an example of how analyses of differential expression at the protein level can lead to important insights about endodormancy-related processes.

Bark storage proteins (BSPs). A class of vegetative storage proteins (VSPs) that accumulate mostly in bark tissue and are, therefore, called bark storage proteins (BSPs) has been studied in *Populus* (reviewed in Coleman, 1997). The BSPs belong to a larger group of VSPs that function as important nitrogen reserves in many plant species (reviewed in Stepien et al., 1994). In deciduous trees, leaf proteins are hydrolyzed in the autumn and the amino acids are translocated to perennial organs where they are incorporated into VSPs (Coleman, 1997; Stepien et al., 1994; Titus and Kang, 1982). During the fall and winter, VSPs accumulate in the bark and wood of both stems and roots (Stepien et al., 1994). In the spring, the VSPs are broken down and their products are translocated to the sites of active spring growth (Coleman, 1997; Stepien et al., 1994; Titus and Kang, 1982).

A temporal association between BSP accumulation and endodormancy was discovered by analyzing seasonal changes in protein levels using one-dimensional SDS-PAGE and other techniques (Nsimba-Lubaki and Peumans, 1986; O'Kennedy and Titus, 1979; van Cleve et al., 1988; Wetzel et al., 1989). In poplar stem tissues, for example, proteins of 32, 36, and/or 38 kDa were found to accumulate in the fall and decline in the spring (Langheinrich and Tischner, 1991; Stepien and Martin, 1992; van Cleve et al., 1988). Therefore, the accumulation and decline of BSPs is correlated with endodormancy induction and release. Detailed analyses of these proteins in hybrid poplar suggest that the 32, 36, and 38 kDa proteins are glycosylated isoforms of the same polypeptide, and that native BSP occurs as a heterodimer of two isoforms (Langheinrich and Tischner, 1991; Stepien and Martin, 1992; Stepien et al., 1992). BSPs are also associated with endodormancy for another reason; both BSP accumulation and endodormancy are induced by SDs (Coleman et al., 1991, 1992). Despite these associations, there is no good evidence that BSPs play a direct role in endodormancy per se (Coleman et al., 1993, 1994).

Purified poplar BSPs were used to produce antibodies, and the resulting BSP antisera were used for isolating the corresponding genes (Clausen and Apel, 1991; Coleman et al., 1992). For example, both cDNA and genomic clones corresponding to the 32 kDa BSP from eastern cottonwood were isolated and sequenced, and the associated gene was designated *bspa* (Coleman and Chen, 1993; Coleman et al., 1992). Analyses of gene expression indicated that BSP accumulation is influenced by daylength, temperature, nitrogen availability, and wounding, and that there is an interaction between these factors (Coleman et al., 1991, 1992, 1994; Davis et al., 1993; Langheinrich and Tischner, 1991; Stepien and Sauter, 1994; van Cleve and Apel, 1992). For example, SD-induced BSP accumulation is delayed in plants that are provided with low levels of nitrogen (Bañados, 1992; Coleman et al., 1994). Conversely, BSP accumulation can be induced under LDs by providing excess nitrogen (Coleman et al., 1994). Results from nuclear runoff transcription assays and promoter analyses indicate that the effects of SDs and nitrogen on BSP accumulation involve different mechanisms (G.D. Coleman and T.H.H. Chen, unpublished results). Therefore, BSP may be regulated at both the transcriptional and translational levels. The induction of BSP accumulation by SDs and nitrogen is tissue-specific; BSP and BSP mRNA are mainly detected in the bark tissues of the stem, and in lateral and terminal buds (Bañados, 1992; Coleman et al., 1994).

Results from Southern blot analyses indicate that BSPs are encoded by a small, clustered, multigene family in poplars (Coleman et al., 1992; Davis et al., 1993). Although poplars have been particularly important for studying BSPs in woody plants, BSPs have been found in a diverse array of angiosperm and coniferous species (Coleman,

1997). Whether BSPs have any direct role in bud endodormancy is still unclear.

Dormancy-related changes in gene expression have also been detected using two-dimensional SDS-PAGE. In pea (*Pisum sativum* L.), for example, lateral buds remain paradormant until they are stimulated to develop by decapitating the stem. Stafstrom and Sussex (1988) compared the protein patterns of lateral buds collected from intact plants with those collected from plants after decapitation. They demonstrated that unique sets of proteins were expressed in the nondormant and paradormant buds. One drawback to analyses of gene expression at the protein level is the relative difficulty of identifying the genes that encode the differentially expressed proteins. Devitt and Stafstrom (1995), for example, subsequently used both differential screening and candidate gene approaches (discussed below) to identify genes that were up-regulated in the nondormant pea buds. These paradormancy-related genes encode histones, ribosomal proteins, MAP kinase, cdc2 kinase, and cyclin B (Devitt and Stafstrom, 1995; Stafstrom and Sussex, 1992; Stafstrom et al., 1993).

Analyses of protein profiles of endodormant poplar buds. We analyzed the protein profiles of terminal and lateral buds of eastern cottonwood during endodormancy development (10 weeks of SDs) using two-dimensional SDS-PAGE (Jeknić and Chen, 1999). The majority of the polypeptides present in the LD controls were also present in the trees grown under SDs. Nonetheless, SD treatment resulted in the appearance of new polypeptides and the down-regulation of other polypeptides (Table 2). In terminal buds, for example, eight polypeptides appeared or increased in abundance, whereas 11 polypeptides disappeared by week 10. The SD-induced changes in polypeptides that occurred in lateral buds were similar to those found in terminal buds. In lateral buds, six polypeptides appeared or increased in abundance by week 6, whereas eight polypeptides disappeared during the 10-week experiment. Four of the eight polypeptides that appeared in terminal buds seem to be identical to those that also appeared in lateral buds. Similarly, five of the 10 polypeptides that disappeared in terminal buds seem to be identical to those that also disappeared in lateral buds.

Because the SD treatment resulted in the appearance of new polypeptides and the disappearance of others, specific changes in gene expression appear to be associated with the induction of endodormancy. The next step will be to identify these polypeptides and their corresponding genes. In particular, we will focus on those polypeptides that either appeared or disappeared in both terminal and lateral buds during SD treatment. The identification and molecular characterization of these polypeptides should contribute to a better understanding of the molecular mechanisms controlling endodormancy induction, maintenance, and release.

Although changes in proteins may be more biologically meaningful than changes in mRNAs, protein-based methods are not sensitive enough to detect genes that are expressed at very low levels, and are not suitable for easily identifying the differentially expressed proteins. For these reasons, analyses of differential gene expression at the RNA level are important adjuncts or alternatives to the analyses described above.

Differential expression at the RNA level

Analyses of differential expression at the RNA level have some advantages over analyses at the protein level. For example, most RNA-based techniques facilitate the identification and cloning of genes that are differentially expressed and permit the detection of genes that are expressed at very low levels. Approaches that have been used to study

Table 2. Number of proteins (polypeptides) that either appeared (+) or disappeared (–) in poplar buds during 10 weeks of short-day (SD) treatment.

Bud position	SD treatment (weeks)										Total
	2		4		6		8		10		
	+	-	+	-	+	-	+	-	+	-	
Terminal	2	4	3	1	1	3	2	2	0	1	8
Lateral	3	2	1	1	2	3	0	1	0	1	6

endodormancy-related genes include in vitro translation, differential screening, differential display, and analyses of cDNA amplified restriction fragment polymorphisms (cDNA-AFLPs). In vitro translation, for example, was used to identify genes that were differentially expressed in Scots pine buds during the winter (Nuotio et al., 1990). Based on differences in the polypeptide patterns between buds collected in January and February, gene expression appeared to change during periods of very low temperatures (Nuotio et al., 1990). With the advent of more powerful techniques, however, in vitro translation is no longer widely used for analyses of differential gene expression.

Differential screening. In most differential screening protocols, a cDNA library is screened using two probes representing mRNA populations from two tissues that differ in the trait of interest. For example, cDNA libraries constructed from dormant embryos of rye brome (*Bromus secalinus* L.) and wild oat (*Avena fatua* L.) were screened using probes representing mRNAs from dormant and nondormant embryos (Goldmark et al., 1992; Li and Foley, 1995). By comparing the levels of plaque hybridization using these two different probes, cDNAs encoding genes that were preferentially expressed in dormant embryos were identified (Goldmark et al., 1992; Li and Foley, 1995). There are many variants to this basic approach, including the screening of a single (e.g., dormant) cDNA library (Goldmark et al., 1992; Li and Foley, 1995), or the screening of cDNA libraries from each tissue of interest (e.g., dormant and nondormant). This latter approach would facilitate the detection of both up-regulated and down-regulated genes in dormant tissues. In other cases, the libraries or probes may be derived from differentially expressed mRNAs. Li and Foley (1995), for example, screened a "subtracted" cDNA library that was enriched for genes preferentially expressed in dormant embryos. Compared with protein-based methods and in vitro translation, the main advantages of differential screening (and the other RNA-based techniques discussed below) are that they facilitate the identification and cloning of the differentially expressed genes and the detection of genes that are expressed at relatively low levels.

In the examples cited above, the genes that were up-regulated in dormant embryos appear to encode LEA proteins, aldose reductase, and other unidentified proteins (Goldmark et al., 1992; Li and Foley, 1995). In addition, genes that were up-regulated in dormant embryos were also up-regulated by ABA in nondormant embryos (Goldmark et al., 1992; Li and Foley, 1995).

Differential display and analyses of cDNA-AFLPs. Differential display and cDNA-AFLPs are related PCR-based techniques that can be used to identify differentially expressed mRNAs (T.H.H. Chen, unpublished data; Johnson et al., 1995). In both techniques, the differentially expressed messages can be cloned and identified relatively easily. Compared with differential screening, differential display and cDNA-AFLPs are less biased against rare messages, have a lower requirement for starting material (because of the PCR amplification step), and are relatively simple (Johnson et al., 1995). The main disadvantages are that differences in gene expression at the protein level will not be detected, there is often a large number of false positives, and important genes could be missed because only a subset of the genes are amplified in each reaction (Johnson et al., 1995).

Differential display was used to identify and clone cDNAs for several genes that are differentially expressed in dormant and nondormant oat embryos (Johnson et al., 1995). Five genes were cloned, two that were preferentially expressed in dormant embryos, and three that were preferentially expressed in nondormant embryos. One of these latter genes may encode a glutathione peroxidase (Johnson et al., 1995).

Using the cDNA-AFLP procedure, we identified and cloned several differentially expressed genes from terminal buds of eastern cottonwood trees growing under SDs. The AFLP-cDNA fragments were generated using PCR, then visualized on polyacrylamide gels. The fragments that appeared to correspond to differentially expressed genes were re-amplified and cloned into plasmids. To verify the differential expression of the cloned genes, they were hybridized to slot blots containing total RNA isolated from buds that had been collected after 0, 1, 2, 4, 6, and 8 weeks of exposure to SDs.

Using these procedures, we detected 20 to 50 cDNA-AFLP bands per primer combination, many of which appeared to represent differ-

Fig. 5. cDNA-AFLP fragments synthesized from eastern cottonwood trees (*Populus deltoides* clone 172-2) growing under SD conditions (8-h day/16-h night) for 0 to 8 weeks. cDNA-AFLPs were generated using the Perkin-Elmer AFLP Plant Mapping Kit according to the protocol provided by the manufacturer (Perkin-Elmer Corp., Norwalk, Conn.). Selectively amplified cDNA-AFLP fragments were subjected to electrophoresis on 6% polyacrylamide denaturing gels, stained with silver, then air-dried overnight. Arrows indicate cDNA fragments that appear to correspond to differentially expressed mRNAs.

entially expressed genes. Some fragments appeared only after 4 weeks of SD treatment, whereas others appeared shortly after the SD treatments were initiated, but later disappeared (Fig. 5). From the first 32 primer combinations, 76 cDNA-AFLP bands were chosen for re-amplification because they appeared to represent differentially expressed genes. Sixty-four of these were successfully re-amplified and are currently being studied. To date, 24 of these cDNAs have been cloned and sequenced, 11 have been checked for similarity to sequences in the GenBank and EMBL databases (Table 3), and four were used to probe the RNA slot blots. Although signals were not detected for two of these four clones, the other two appeared to be differentially expressed (Fig. 6). We are continuing to generate additional cDNA fragments for cloning and sequencing, and expect to have ≈ 100 differentially expressed cDNA clones for study.

Genetic approaches

Genetic approaches are those aimed at identifying endodormancy-related genes by comparing the inheritance patterns of endodormancy-

Table 3. Sequence analyses of cDNA-AFLP clones.

Clone	Size (bp)	Match (bp/bp)	Best match ¹	
			Species or hybrid	Putative gene product or genomic sequence ²
A1-7	128	101/114	<i>Populus tremula</i> L. x <i>P. tremuloides</i> Michx.	Cytosolic phosphoglucosyltransferase (AF097938)
A2-2	203	33/44	<i>Arabidopsis thaliana</i> (L.) Heynh.	Genomic sequence from BAC clone F26F24 (AC005292)
A2-4	200	42/50	<i>Arabidopsis thaliana</i>	ATAF1 mRNA (X74755)
A3-1	237	68/108	<i>Phaseolus vulgaris</i> L.	Peroxidase 5 precursor (AF149280)
A3-3	210	30/37	<i>Saccharomyces cerevisiae</i> Meyen	Subunit of cAMP-dependent protein kinase (X05051)
B1-3	371	37/49	<i>S. cerevisiae</i>	Bifunctional anthranilate synthase:indole-3 glycerol phosphate synthase
B3-3	358	128/170	<i>A. thaliana</i>	Genomic sequence from BAC clone F9L1 (AC007951)
B3-5	304	154/221	<i>Sinapis alba</i> L.	Small subunit of ribulose biphosphate carboxylase (X16435)
B4-3	390	130/177	<i>A. thaliana</i>	Genomic sequence from BAC clone F18F4 (AL021637)
B6-4	364	29/34	<i>Williopsis saturnus</i> (Klöcker) Zender var. <i>saturnus</i>	Killer toxin (D13446)
C1-5	267	119/157	<i>A. thaliana</i>	Genomic sequence from PAC clone MRG7 (AB012246)

¹Best matches were determined in June 1999 using the Basic Local Alignment Search Tool (BLAST).

²From the GenBank *Definition* field. Designations in parentheses are GenBank Accession numbers, BAC denotes bacterial artificial chromosome, PAC denotes P1 artificial chromosome.

related traits with the inheritance patterns of specific chromosomal regions defined by molecular markers (i.e., cosegregation analyses). Genetic approaches include classic Mendelian genetic analyses and analyses of quantitative trait loci. Because genetic approaches are fundamentally different from the physiological approaches described above, they are useful alternatives for detecting and identifying endodormancy-related genes. Genetic approaches can also be used to test whether specific candidate genes (including those identified using the physiological approaches described above) are associated with endodormancy-related traits.

Mendelian genetic analyses

If the trait of interest varies in a qualitative fashion, simple Mendelian genetic analyses may be useful for detecting endodormancy-related genes. Mendelian genetic analyses were used, for example, to infer that "nondormant" genotypes of both peach [*Prunus persica* (L.) Batsch] and hazelnut (*Corylus avellana* L.) result from mutations in single genes (Rodriguez et al., 1994; Thompson et al., 1985). Chilling requirement was inherited in a semi-qualitative fashion in both open-pollinated and control-cross progeny of the apple (*Malus × domestica* Borkh.) cultivar Anna (Hauage and Cummins, 1991). Therefore, the low chilling requirement of this cultivar appears to be controlled by at least one major dominant gene. Dominance of low chilling requirement has also been observed in other apple crosses (Oppenheimer and Slor, 1968). Ultimately, it may be possible to identify these major genes by determining which specific loci cosegregate with the mutant phenotypes.

In contrast to the examples cited above, most endodormancy-related traits appear to vary in a quantitative manner. Results from

progeny tests indicate that phenotypes for a number of endodormancy-related traits exhibit continuous distributions, presumably resulting from multigenic control plus environmental effects (Aitken and Adams, 1995; Billington and Pelham, 1991; Ekberg et al., 1985; Farmer and Reinholt, 1986). Results from controlled breeding experiments also suggest that endodormancy-related traits are influenced by multiple genes (Eriksson et al., 1978; Pauley and Perry, 1954). For traits that exhibit quantitative variation, Mendelian genetic analyses might be possible by first generating mutants (i.e., by creating new qualitative genetic variation). Although this approach is commonly used in model plants such as *Arabidopsis*, it is impractical for most tree species because they are difficult to self (due to substantial inbreeding depression or dioecy) and have generation times of 5 to 40 years (Kramer and Kozlowski, 1979).

QTL analyses

If Mendelian genetic analyses are not possible using either existing genetic variation or mutants, QTL analyses can be used to dissect quantitative traits into their Mendelian components (Tanksley et al., 1989). A QTL is a locus or region of the chromosome that has a significant effect on a quantitatively inherited trait (Tanksley, 1993). The first step in QTL analysis usually involves the development of a genetic linkage map using DNA-based markers such as RFLPs, RAPDs, AFLPs, or microsatellites, followed by a search for those genetic markers that cosegregate with the trait of interest (Lander and Botstein, 1989). A number of different designs have been used to map QTLs in trees, including inbred-like F_2 and backcross pedigrees, F_1 pedigrees, and three-generation full-sib pedigrees (Williams, 1998). Simplified alternatives such as bulk segregant analysis or selective genotyping may also be useful for detecting QTLs (Liu, 1998).

Molecular markers have been used to detect endodormancy-related QTLs in trees. In apple, the timing of reproductive bud flush was associated with a single molecular marker, and the timing of vegetative bud flush was associated with two molecular markers and one morphological marker (Lawson et al., 1995). Because these traits were studied using simple cosegregation analyses, other important QTLs may have been missed. In *Populus*, growth and phenological traits were studied using a three-generation inbred-like pedigree derived from a cross between black cottonwood and eastern cottonwood (Bradshaw and Stettler, 1995). Based on measurements made at a single field site, five QTLs with major effects on the timing of spring bud flush were identified. A model that included information on all five QTLs accounted for 85% of the genetic variation. In addition, the heritability of clonal means was 0.98, higher than that for any other trait. These results indicate that the timing of bud flush is controlled by a modest number of major genes, and that environmental variation is low.

We are currently mapping endodormancy-related QTLs in *Populus* using the same pedigree design, but with a larger number of environments and F_2 progeny. Preliminary QTL analyses have been conducted using as many as 337 F_2 progeny and 278 AFLP and microsatellite

Fig. 6. Slot blots showing differential expression of two cDNA-AFLP clones isolated from eastern cottonwood trees (*Populus deltoides* clone 172-2). Total RNAs isolated from buds which had been collected after 0 to 8 weeks of exposure to SDs (8-h day/16-h night) were blotted, then hybridized to one of two cDNA-AFLP clones (B3-3 or B3-5).

markers. These markers are distributed among 26 maternal and 24 paternal linkage groups, and cover 68% to 77% of the estimated 2600 cM length of the *Populus* genome. Because poplars can be vegetatively propagated, these experiments include clonally replicated field plantations in both Oregon and Minnesota, as well as replicated experiments in controlled environments. In Oregon, we measured fall bud set and spring bud flush, whereas in Minnesota, we measured fall bud set, fall frost damage, and winter survival.

Preliminary quantitative genetic analyses indicate that bud set and bud flush are highly variable and under strong genetic control. Based on combined analyses of bud set in Oregon and Minnesota, for example, mean bud set dates ranged from 27 Aug. to 23 Nov., with a clonal mean heritability of 0.81. Because the genetic correlation between bud set in Oregon and Minnesota was 0.81, there appears to be a modest genotype $\times$ environmental interaction for this trait. These results indicate that mapping bud set QTLs that are applicable to multiple environments should be possible. Based on the Oregon data, mean bud flush dates ranged from 4 Mar. to 13 Apr., with a clonal mean heritability of 0.94. Comparable analyses of the timing of bud flush in Minnesota were not possible because many of the trees did not survive the harsh winters. The trees in Minnesota were severely damaged by frosts at the end of October and beginning of November. Therefore, we also obtained information on genetic differences in freeze damage. Some of the F_2 genotypes were killed by these early fall frosts, whereas others exhibited no damage. The heritability of freeze damage was lower than the heritabilities of the other endodormancy-related traits. The genetic correlation between bud set in Oregon and freeze damage in Minnesota was moderately positive, suggesting that genotypes that set bud earlier in the season are more resistant to freeze damage.

The timing of SD-induced bud set was also measured in controlled environments under warm temperatures and an 8-h photoperiod. In the greenhouse, SD-induced bud set was highly variable among F_2 progeny and under strong genetic control. The earliest F_2 progeny set bud 16 d after the start of the 8-h photoperiod, whereas other progeny set bud nearly 2 months later. The heritability of clonal means was 0.80. The genetic correlation between the timing of bud set under an 8-h photoperiod (warm temperatures) and the timing of bud set in Oregon and Minnesota was relatively modest ($r_g = 0.60$). This result suggests that environmental factors other than daylength significantly influence genetic differences in the timing of bud set in the field. By combining both field and controlled-environment experiments it may be possible to dissect a complex collection of QTLs that control bud set in the field into sets of QTLs that regulate the timing of bud set via distinct environmental signals.

Results from preliminary QTL analyses using information from the Oregon field experiments indicate that multiple QTLs with modest effects on both the timing of bud set and bud flush can be detected. We are currently testing whether endodormancy candidate genes *PHYA*, *PHYB1*, *PHYB2*, *ABI1B*, *ABI1D*, and *ABI3* are located near any of these QTLs (discussed below); QTL and candidate gene analyses of other endodormancy-related traits are underway. Although quantitative genetic analyses and QTL mapping are continuing, the results cited above clearly indicate that endodormancy induction and release are under strong genetic control, and that mapping QTLs for endodormancy-related traits is possible using this hybrid poplar pedigree.

CANDIDATE GENE APPROACHES

Candidate genes

"Candidate gene" approaches may be useful for identifying endodormancy-related genes. A candidate gene is a gene thought to be involved in some endodormancy-related process based on the gene's probable function. For example, the phytochrome, gibberellin (GA), and ABA signaling pathways appear to be important regulators of endodormancy-related traits (reviewed in Rohde et al., 1999). Therefore, genes encoding biosynthetic enzymes, receptors, or other components of these pathways should be investigated.

Phytochrome genes (*PHY*) encode photoreceptors involved in a diverse array of light-mediated responses, and one or more of the

phytochromes are known to control photoperiodic responses in *Populus* (Howe et al., 1996; Olsen et al., 1997b). Therefore, phytochrome genes are logical candidates for playing important roles in a number of endodormancy-related traits. In addition, there are reasons to suspect that variation in the early steps in the phytochrome signaling pathway(s) may contribute to genetic variation in photoperiodic responses. In red-osier dogwood [*Cornus sericea* L. (= *C. stolonifera* Michx.)], for example, ecotypic differences in cold-hardiness are largely the result of differences in the timing of cold-acclimation; acclimation began early in a cold-hardy genotype from North Dakota, but was delayed in a less-hardy genotype from Seattle, Wash. (Fuchigami et al., 1971a). When these genotypes were grafted and acclimated under SDs and low temperatures, the leaves of the North Dakota genotype increased the cold-hardiness of the Washington genotype. These results suggest that signaling events that occur in the leaves (i.e., at the sites of photoperiodic perception) differ between these genotypes. In addition, naturally occurring mutations in peach and hazelnut trees inhibit their ability to respond to SDs, resulting in "nondormant" phenotypes (Rodriguez et al., 1994; Thompson et al., 1985). Taken together, these results suggest that genetic variation in photoperiodic responses is associated with variation in relatively few genes that control groups of endodormancy-related traits, rather than variation in a large number of individual response genes. Therefore, genes that encode the phytochromes and other members of the phytochrome signaling pathway(s) should be investigated to determine their roles in endodormancy-related traits in general, and whether they contribute to genetic variation in photoperiodic responses in particular.

The phytochromes consist of dimers of two apoproteins, each of which is covalently attached to a tetrapyrrole chromophore (Furuya and Song, 1994). Genes (*PHY*) which encode these apoproteins exist as a small gene family in most plants, including black cottonwood (Howe et al., 1998; Mathews and Sharrock, 1997). Black cottonwood contains three phytochrome genes, *PHYA*, *PHYB1*, and *PHYB2*, but apparently lacks members of the *PHYC/F* and *PHYE* subfamilies found in other angiosperms (Howe et al., 1998). Based on overexpression in transgenic plants, oat *PHYA* is capable of affecting photoperiodic responses in hybrid aspen (*P. tremula* L. $\times$ *P. tremuloides* Michx.), including SD-induced growth cessation and cold acclimation (Olsen et al., 1997b). However, whether or not the endogenous aspen *PHYA* exerts substantial control over these traits remains unclear. Current experiments designed to inhibit endogenous *PHYA* expression using antisense techniques should shed light on this question (T. Moritz, personal communication). In contrast, results from other species implicate *PHYB* in the photoperiodic control of flowering and tuberization (Childs et al., 1997; Jackson et al., 1998). Analyses of the functional roles of poplar *PHYB1* vs. *PHYB2*, however, may be difficult because the protein-coding regions of these genes are very closely related (Howe et al., 1998). Nonetheless, these genes differ substantially in their putative 5' and 3' untranslated regions (P. Sarul and G.T. Howe, unpublished results). Of course, both *PHYA* and *PHYB* may be important photoreceptors for controlling photoperiodic responses in trees. In fact, models for phytochrome control of flowering that include roles for multiple phytochromes have been proposed (Thomas, 1991).

Because poplars contain at least three *PHY*, each can be considered a candidate gene for endodormancy-related traits. To evaluate the roles of these genes, two questions need to be answered. First, which *PHY* play important functional roles in photoperiodic responses in trees? Second, are these genes responsible for genetic variation in photoperiodic responses? Answers to these questions will probably come from physiological approaches, such as modification of *PHY* expression in transgenic plants, and genetic approaches, such as gene mapping and QTL analyses. Limited research on genetic variability among black cottonwood genotypes suggests that substantial variability exists for at least one of the two *PHYB*, although no variability was detected for *PHYA* (Howe et al., 1998). In addition, sequence variation has been detected among black cottonwood genotypes in the putative 5' untranslated leaders of both *PHYB1* and *PHYB2* (P. Sarul and G.T. Howe, unpublished results).

We are using polymorphisms between black and eastern cottonwood to map poplar *PHY* using a three-generation hybrid poplar

pedigree (unpublished results). The map locations of these genes are being compared with the locations of QTLs affecting the timing of bud set in the field and SD-induced bud set under controlled conditions (discussed above).

The GAs are a large group of tetracyclic diterpenes that regulate diverse aspects of plant growth and development, including photoperiodic control of growth cessation in woody perennial plants (Olsen et al., 1995; 1997b). Genes encoding GA biosynthetic enzymes have been isolated from a number of plant species (reviewed in Hedden and Kamiya, 1997), and genes encoding three key enzymes are being studied in hybrid aspen (D. Mozley, M.E. Eriksson, and T. Moritz, personal communication).

A number of genes involved in ABA signaling have been identified in *Arabidopsis* (reviewed in Merlot and Giraudat, 1997). Mutations in the *ABI1* and *ABI3* genes, for example, confer insensitivity to ABA. *ABI1* encodes a protein phosphatase and *ABI3* encodes a transcription factor (Merlot and Giraudat, 1997). Analyses of the expression of *ABI1* and *ABI3* homologues in *Populus* suggest roles for these genes in bud endodormancy (reviewed in Rohde et al., 1999). Other potential candidate genes include those with secondary regulatory functions, such as cell cycle genes, and genes with direct (i.e., nonregulatory) roles in endodormancy-related adaptations, such as genes encoding bark storage proteins and dehydrins (Coleman 1997; Rohde et al., 1997; Rowland and Arora, 1997).

We are mapping *PHYA*, *PHYB1*, *PHYB2*, *ABI1B*, *ABI1D*, and *ABI3* in the hybrid poplar pedigree described above. This information, plus the results from QTL analyses, will allow us to test the hypothesis that these candidate genes are major QTLs for endodormancy-related traits. Because auxin, cytokinins, and ethylene may also regulate dormancy-related traits in seeds and/or buds (Kępczyński and Kępczyńska, 1997; Moritz, 1995; Olsen et al., 1997a, 1997b), genes associated with these pathways may be important as well. Genes encoding components of signaling pathways are particularly interesting because they are potentially involved in coordinated regulation of multiple endodormancy-related traits.

CONCLUSIONS AND PROSPECTS

Species and hybrids of *Populus* are good models for studying the physiology and genetics of endodormancy-related traits in trees and other perennial plants. Physiological and genetic approaches are being used to identify "new" endodormancy-related genes in poplars, and to examine the roles of specific candidate genes in endodormancy-related traits. A major challenge will be to develop information that contributes to the applied use of woody crops. Research aimed at identifying and characterizing endodormancy-related genes, for example, may contribute to the development of 1) better tree crops via conventional breeding or genetic engineering, 2) improved cultural practices for controlling endodormancy-related traits, and/or 3) additional tools for studying the physiological genetics of endodormancy.

Much of the research on poplars has focused on the induction of endodormancy in general, and the regulatory role of photoperiod, in particular. These topics are being addressed using a range of approaches, including analyses of differential gene expression, candidate gene evaluation, and QTL mapping. Research on both the maintenance and release of endodormancy, however, is lagging. In addition, we know little about how temperature affects endodormancy-related traits at the molecular genetic level, yet temperature influences all aspects of endodormancy. Temperature interacts with daylength to control the timing of growth cessation and bud set (Downs and Bevington, 1981; Junttila, 1980), and a number of other endodormancy-related traits are induced by low temperatures, rather than daylength alone. Although SDs induce bud set and endodormancy, poplar trees do not shed their leaves unless they are exposed to low, above-freezing temperatures (T.H.H. Chen unpublished data; G.T. Howe, unpublished data). In other species, near-freezing temperatures are needed to induce the so-called second stage of cold acclimation (reviewed in Fuchigami et al., 1971a). These responses, however, seem to require prior exposure to SDs.

Temperature is also the major environmental factor controlling endodormancy release via chilling, and the timing of bud flush after

the chilling requirement has been satisfied. Although work on fruit trees has addressed the physiological genetics of chilling requirements and bud flush (reviewed in Rowland and Arora, 1997), more work is needed. The influence of temperature on endodormancy-related traits has often been viewed as occurring through "global," nonspecific processes. For example, low temperatures influence enzyme activities and membrane properties, which could influence a large number of endodormancy-related traits. Nonetheless, we should entertain the hypothesis that specific temperature signaling mechanisms exist. Analogies can be made between the effects of temperature on SD-induced growth cessation and the influence of day/night temperature differentials (DIFs) on internode elongation (Downs and Bevington, 1981; Jensen et al., 1996; Junttila, 1980). In addition, the influence of chilling on bud endodormancy may be analogous to effects of chilling and vernalization on seeds and seedlings of annual plants. These analogies may point to other approaches and candidate genes that should be investigated in relation to bud endodormancy. Vernalization-related genes, for example, have been detected in wheat (*Triticum aestivum* L.) and *Arabidopsis thaliana* (Chong et al., 1994; Clarke and Dean, 1994), and similar endodormancy-related genes may exist in trees.

In addition to daylength and temperature, other environmental signals such as drought and nutrient availability influence endodormancy-related traits (Junttila 1989; Perry 1971). Eventually, information on these factors should be integrated into an overall picture of endodormancy. Much of our discussion has focused on the environmental control of endodormancy because genes involved in these processes probably have substantial impacts on entire groups of endodormancy-related traits, and may be responsible for much of the genetic variation in these traits. Nonetheless, the individual "response" genes and processes that are directly responsible for the adaptations that allow plants to survive harsh winter conditions deserve attention. In contrast with the seasonally indeterminate growth exhibited by young poplar trees, older trees and trees of other species often stop growing in early summer and become dormant in response to unknown "endogenous" factors. A complete understanding of endodormancy will ultimately require that we understand the physiological genetics of this process, as well.

Finally, to merely describe endodormancy-related genes and processes in individual plants is insufficient. The physiological genetics of endodormancy-related traits must be studied at the population level. Genetic variation in endodormancy-related traits has had an enormous impact on plant micro- and macroevolution. In addition, an understanding of this genetic variation is important for designing breeding strategies and for evaluating the potential impact of global climate change. Unlike mobile animal species and annual plants, populations of long-lived perennial plants such as trees may have difficulty adapting to changing environments, particularly if these changes occur rapidly (on an evolutionary time scale). Therefore, information on the levels and distributions of genetic variation in endodormancy-related traits will be important for predicting the long-term survival of natural populations of trees.

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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.

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/freeze-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 ($F_1 \times V. darrowi$)	-14.0 ± 0.43	-13.9
F_1	-14.7 ± 0.33	-14.8
TC_2 ($F_1 \times V. caesariense$)	-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 ($F_1 \times V. darrowi$)	54	375
F_1	9	751
TC_2 ($F_1 \times V. caesariense$)	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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