EXPLORATION OF THE FEASIBILITY OF DEVELOPING A WINTER HARDY 'MARION' BLACKBERRY THROUGH GENETIC ENGINEERING

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AN ABSTRACT OF THE DISSERTATION OF

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Experiments focusing on plant growth regulators' concentrations and combination, mineral salt formulations and TDZ pretreatment formations were conducted to optimize *in vitro* shoot regeneration from leaf and petiole explants of 'Marion' blackberry. Optimum shoot formation was obtained when stock plants were incubated in TDZ pretreatment medium for three weeks before culturing leaf explants on regeneration medium in darkness for 1 week before transfer to light photoperiod at 23 °C ± 2 °C for 4 weeks**.** Under these conditions, about 70% of leaf explants formed approximately forty shoots that could be harvested from each petri dish and rooted to form plantlets.

The expression of an intron-containing *uidA* gene coding for β-glucuronidase was used as an indicator of successful T-DNA delivery and GUS gene expression in 'Marion' leaf explants. Using the optimized protocol, about 20% of the leaf explants showed blue staining 7 d after cocultivation. Putative transgenic shoots obtained

during the optimization experiments, however, were all GUS negative, indicating the necessity of further evaluation of factors that lead to the recovery of real transgenic plants.

Two transgenic lines of 35S::AtCBF1 increased freezing tolerance 3.1°C and 2.2°C in leaves and stems respectively, when compared to wild type (WT) plants grown under non-acclimation conditions, and a 3.3°C and 3.8°C increase in the freezing tolerance of leaves and stems respectively after 4 weeks of cold acclimation at 2°C. After acclimation, there was no increase in freezing tolerance found in the rd29A::AtCBF1 line compared to the WT. The three 35S::AtCBF2 lines grown under acclimation condition had a decreased freezing tolerance, compared to the WT. All four tested transgenic 35S::AtCBF3 lines gave the most significant increases in freezing tolerance, with an average of 5.3°C increase in leaf tissue tolerance when grown under non-acclimating conditions and 6.1°C after being grown under acclimating conditions, and for stem tissues a 3.0°C and 6.3°C increase in freezing tolerance when grown under non-acclimating and acclimating conditions, respectively. Three of the six rd29A::AtCBF3 lines, after cold acclimation, were significantly more tolerant to freezing than the WT, with an average increase of 2.5°C in leaf tissue tolerance and 3.3°C in stem tissues. Level of cryoprotectant such as soluble sugars (sucrose, fructose, and glucose) and proline in leaf tissue of transgenic lines were increased with a manner along with the increase of freezing tolerance.

Exploration of the feasibility of developing a winter hardy 'Marion' blackberry through genetic engineering

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CONTRIBUTION OF AUTHORS

Yonghai Li assisted with statistical analysis of Chapter 2. Dr. Tony H.H. Chen was involved with the design and writing of Chapter 2, 3 and 4.

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CHAPTER 1

Introduction

1. 1. Blackberry

1.1.1. Taxonomy, growth habit and morphology

Blackberries, along with red and black raspberries and their hybrids, are fruiting plants from the Rosaceae and the *Rubus* genus (Tourn.) L. *Rubus* is one of the most diverse genera in the plant kingdom with approximately 740 species (Gu et al., 1993). *Rubus* is distributed widely in all ecosystems of the world except desert regions (Daubeny, 1996). Blackberries and raspberries are often called caneberries in western North America or brambles in eastern North America. Blackberries, most of which are native to the northern hemisphere, are in the subgenus *Eubatus* (Jennings et al., 1988). Blackberries are mostly from subgenus *Eubatus,* with chromosome number ranging from 2n=2x=14 to 2n=14x=98 (Darrow, 1937; Moore, 1984; Hall, 1990). In the Pacific Northwest (PNW), *R. ursinus* Cham. et Schlecht has been the major source of germplasm for trailing blackberry breeding (Hall, 1990; Finn, 2001). Cultivars or selections have ploidy levels ranging from 2x-9x as well as aneuploids (Hall, 1990; Finn, 2001; Meng and Finn, 2002). Ploidy level of 2n=13x=91 was found in *R. ursinus* germplasm by Meng and Finn (2002) using flow cytometry.

Plants of the various blackberry species range from tiny to very large bushes over 6 m tall. Most have herbaceous biennial canes emerging from perennial crowns although some have perennial canes. The biennial canes require a dormant period before

flowering. Most species are spiny although spineless types from a few different genetic sources have been selected and developed through breeding programs.

Blackberries produce typical Rosaceae flowers that are white or pink. Some species such as *R. ursinus* are dioecious, although breeding programs have selected for hermaphroditic types. The flowering laterals break in the spring and several flowers are borne on each lateral. The flowers are insect pollinated and if pollination and fertilization are successful an aggregate fruit that is a collection of drupelets attached to the central receptacle develops. Blackberries are differentiated from raspberries as they have drupelets that adhere to the central receptacle when picked and raspberry drupelets separate from the receptacle (held together by fine hairs).

Present blackberry cultivars differ greatly in fruit and plant habit because they originated from the interbreeding of many genetically heterogeneous and morphologically variable species (Moore and Skirvin, 1990). Based on gross morphology, blackberries are categorized into three major types, including the trailing, erect, and semi-erect type.

The primary commercial trailing cultivars (e.g. ‘Marion’, ‘Black Diamond’, ‘Black Pearl’, ‘Kotata’, ‘Thornless Evergreen’, ‘Waldo’ ‘Olallie’, ‘Silvan’, ‘Siskiyou’, etc.) are largely derived from *R. ursinus* although ‘Thornless Evergreen’/'Everthornless' is derived from *R. laciniatus*. ‘Boysen’ and ‘Logan’ are important trailing cultivars that

are hybrids between red raspberry and blackberry and have a typical trailing growth habit (Hall et al., 2002). Derivatives of *R. trivialis*, such as 'Flordagrand' and 'Oklawaha' are grown on small acreages in the southeastern U.S. primarily for the fresh market. These cultivars produce vigorous trailing canes that grow across the ground unless trained up to a trellis system. While there is a great deal of variability, the fruit of the trailing blackberries generally are characterized as being somewhat soft, but with less noticeable seeds and excellent flavor, and they are adapted for machine harvest. Trailing blackberries require support in the field (Moore and Skirvin, 1990). While the original species were all spiny, spineless cultivars have been developed in breeding programs, primarily using spinelessness from the 'Austin Thornless' source of thornlessness. Not only is the gene in 'Austin Thornless' dominant but the genotype is 8x thus it is used more easily in breeding with the higher ploidy western trailing blackberries. Trailing blackberries generally are grown commercially in mild climates on the Pacific Coast of North and South America and in the Mediterranean region of Europe primarily due to their lack of winter hardiness. Due to their growth habit and the adherence of receptacles to the fruit when picked, several raspberry/trailing blackberry hybrids such as 'Boysen', 'Logan', and 'Tayberry' are considered trailing blackberries.

Erect cultivars (e.g. 'Brazos', 'Cherokee', 'Shawnee', 'Choctaw', 'Kiowa' and 'Navaho', etc.) most likely are derived from hybridization of the erect species such as *R. allegheniensis* Porter, *R. argutus* Link, and *R. frondosus* Bigelow. These species

have erect and spiny canes and produce primocanes from adventitious buds on roots and crowns. Erect blackberry fruit are large, somewhat lumpy and have large seeds. They tend to be firm with good skin toughness making them well suited for fresh market shipping. The flavor can be very good but is not as aromatic as that of trailing blackberries. Erect blackberries don't need support in the field in Arkansas but they are always grown with support in the western USA. The erect blackberries are the most cold hardy of the commercial blackberries.

Semi-erect cultivars (e.g. 'Hull Thornless', 'Chester Thornless', 'Triple Crown' and 'Loch Ness', etc.) were derived primarily from the same species as erect blackberries but were selected for a crown forming habit and for very vigorous spineless canes. The semi-erect cultivars are all spineless as the 'Merton Thornless' source of spinelessness was used early in their development. The 'Merton Thornless' source of thornlessness is tetraploid and was also used in the development of the spineless erect cultivars. The semi-erect cultivars have very vigorous canes that are termed semi-erect because they begin by growing erect but since they are vigorous and will grow 4-6 m, they eventually arch over. Semi-erect blackberries must be trellised in the field. The fruit of the semi-erect blackberries are very similar to those of the erect types and are used primarily for fresh market sales. The semi-erect cultivars are noted for their extremely high yield potential.

1.1.2. Consumption and production distribution

While many parts of the blackberry plant have historically been used for dyes, silkworm forage, and pharmaceuticals, they are cultivated almost exclusively for their fruit. (Roach, 1985; Hall, 1990). Archeological studies found that blackberries had been consumed since as early as Neolithic and Bronze age in Britain and documented in Greek and Roman literatures (Roach, 1985; Hall, 1990).

The commercial blackberry industry can be traced back to the late 1800's. Today, while blackberry production regions are found around the world including North America (United States and Mexico), Central America (Costa Rica and Guatemala), South America (Ecuador, Chile, and Brazil), Oceanic (Australia and New Zealand), Europe (Serbia, Hungary, Croatia, Germany, United Kingdom, Romania, Poland), and South Africa (Strik, 2006), the most important production region is North America. In North America, the erect and semi-erect types predominate in the eastern and southern regions, and the trailing blackberries are predominant in the Pacific Northwest (PNW; Oregon and Washington) and California (Strik, 1992). In the PNW, the commercial blackberry industry is concentrated in Oregon's Willamette Valley, which has many more hectares than production areas in California or the eastern United States (Finn, 2001). While a large amount of fruit is sold in the fresh market, most blackberries from this region are first processed into either individually quick frozen (IQF) fruit, puree, or juice that in turn are used as ingredients in processed products such as preserves, jam, jelly, baked goods, jam, dried products, ice cream and yogurt (Hall, 1990).

‘Marion’, ‘Olallie’, ‘Waldo’, ‘Black Butte’, ‘Black Diamond’, ‘Black Pearl’, ‘Obsidian’, and ‘Siskiyou’, etc. are representative trailing cultivars developed by the U.S.D.A.- Agricultural Research Service in cooperation with Oregon State University in Corvallis, Oregon (Finn, 2001). ‘Marion’ is the most widely planted blackberry cultivar in the world as well as in the PNW, accounting for more than 50% of the world’s hectarage (Strik, 1992). ‘Marion’, a hexaploid, was from a cross between ‘Chehalem’ and ‘Olallie’ (Waldo, 1957; Finn et al., 1997); and captured the best attributes of both berries and produces fruit that are aromatic, have an ideal balance of tart and sweet, and an intense blackberry flavor. This premium fruit quality makes ‘Marion’ a superb choice for processing and has earned ‘Marion’ an outstanding reputation worldwide and it is typically marketed as “marionberry” (Finn et al., 1997). The berries ripen in July and are medium sized and bright black with relatively small seeds. The plant has vigorous but thorny canes.

1.2. Blackberry breeding objectives

The breeding objectives in blackberry breeding programs have included environmental adaptation, resistance to biotic stress, desirable plant characters (upright growth, thornlessness, greater number of fruits per lateral, vigor, suckering growth habit, and greater strength and pliability of canes and fruiting laterals), higher yield and desirable fruit characteristics (large fruit size, firmness, high sugar content, medium acidity, desirable flavor and aroma, suitable color, intensity of color and pigment stability,

resistance to sunburn, etc.) (Jennings, 1980, 1986, 1988; Moore, 1984; Perry and Moore 1985; Lawrence, 1986; Hall, 1990). Having winter hardy cultivars in this region is important in order to have a reliable production. In years like 1991, when there was a great deal of winter injury (Strik, 1992), yields for the region dropped and prices for the fruit rose dramatically. This has negative effect on the end users of the product as they need a relatively stable price and fruit supply in order for their companies to supply products to consumers nationally and internationally. In the last 10 years, thornlessness has become a critical trait as well. Thorns that are dislodged during machine harvest and that end up in the final product can be a legal liability problem (Finn et al., 1997, Strik and Buller, 2002). Neither 'Thornless Evergreen', derived from *R. laciniatus*, nor 'Waldo', both of which are thornless, has good enough fruit quality to displace 'Marion'. Hopefully the new spineless releases 'Black Diamond' (Finn et al., 2005a), 'Black Pearl' (Finn et al., 2005b), or 'Nightfall' (Finn et al., 2005c) will help solve this problem. Finally, in general, the erect and semi-erect cultivars have firmer fruit than the trailing cultivars making them better suited to the fresh market. Plant breeders and horticulturists are working together towards the goal of having winter hardy, thornless trailing cultivars with firm fruit (Strik, 1992; Hall et al., 2002).

1.2.1. Freezing injury

Freezing injury is a common cause of economic loss in many fruit crops. Fruit plants can suffer freezing injury when temperature suddenly decreases in late fall or early winter, when plants are not fully hardened. Extreme low temperatures in winter can cause injury in cambial tissue and vegetative and flowering buds, which may not be obvious until the following spring, when the buds fail to open, or open but then immediately die. Some injured fruit buds may abscise before spring (Harvey and Stushnoff, 1983).

Spring frosts and freezes are also an annual threat to the buds of many fruit crops. As the weather warms up, the buds begin to come out of dormancy and lose their hardiness. The further developed the buds are, the more susceptible they are to frost. The critical temperature at which injury occurs depends on the stage of bud development, as well as the length of time the temperature stays at or below the critical temperature (Harvey and Stushnoff, 1983).

Both types of injury, i.e. winter kill and spring frost, occur when temperatures drop below certain threshold levels. The injury threshold temperature is lower for the hardened than non-hardened tissues, and varies for different tissues/organs, different species, genotypes, and stages of development.

1.2.2. Impact of freezing injury on blackberry industry

In the blackberry industry, temperature limitations are the major factors limiting successful production in much of North America. Low temperature stresses such as severe winter conditions (in Canada, the northern and inland United States and even areas with relatively more moderate climate such as Oregon's Willamette Valley) or spring frost can cause severely reduced yield due to bud injury, cane injury , flower damage, poor fruit set, or even whole plant death in susceptible cultivars. Periodic, severe freeze damage remains a characteristic of the blackberry industry in the Pacific Northwest. For example, in Oregon, 50% to 60% of the trailing blackberry crop was lost to winter injury in 1989 and 1991 (Strik, 1992).

'Marion' trailing blackberry is the dominant commercial cultivar (Bell et al., 1995; Finn et al., 1997). However, due to the lack of cold tolerance (injury often exhibited at temperatures at -5 °C to -22 °C) (Bell et al., 1995; Cortell and Strik, 1997), its production can be seriously reduced as a result of 1) damage to or complete kill of overwintering canes (Warmund and George, 1990; Bell and Strik, 1995; Cortell and Strik, 1997) or 2) damage that kills floral buds, causes erratic budbreak along the cane, reduces the percentage of nodes that produce fruitful laterals, or stunts fruiting lateral growth (Bell et al., 1992; 1995; Cortell and Strik, 1997). After a severe winter in 1990-1991, 'Marion' production was down about 30% from the previous year in parts of the Willamette Valley, tremendously reducing the economic return to the growers and the whole industry (Bell et al., 1992; Bell and Strik, 1995; Cortell and Strik, 1997).

1.3. Plant cold hardiness and freezing injury

1.3.1. Plants under low temperature stress

Low temperature is one of the most important factors that determine the normal distribution of natural plant communities and defines the range of distribution and growth of important agricultural crops, many of which are grown at or near the temperature boundary of their genetically determined survival abilities. Therefore, low temperature has an immense impact on world agriculture production. There was an annual expenditure of $100 million to minimize frost damage to crops and annual losses of $10-100 million or higher from freezing damage (Steponkus, 1992). A dramatic example of the impact injury can have on a single crop is the estimated $60 million loss when unseasonably low temperatures damaged cotton seedlings in 1980 (Wilson, 1984).

Plants injury at temperatures above the freezing point (10-15°C) is “chilling injury” and the plants are under “chilling stress”, during which there is no ice-formation in plant tissues. When plants are killed or injured by temperatures below freezing and with ice formation in the tissues, “freezing injury” occurs and the plants are under “freezing stress” (Raison and Lyons, 1986).

1.3.2. Freezing process and injury in plants

Freezing injury is primarily due to the formation of ice in plant tissues at subzero temperatures (Chen et al., 1995). Ice formation is initiated by internal or external ice nucleators. Heterogeneous and homogenous are the two types of ice nucleation. Heterogeneous ice nucleation is caused by ice nucleators including inorganic materials such as dust, wind, snow, agitation, and ice itself, and biological factors that are present in some species of bacteria, fungi, and insects. Ice formed on the plant surface causes plant interior ice formation through stomata, lenticels, and any physical lesion on the plant surface. Homogeneous ice nucleation refers to the temperature that pure water or a solution freezes without the help of heterogeneous nucleator. Consequently, this type of ice nucleation usually involves supercooling of liquid or solution to temperatures around or below -40 ºC.

Ice normally forms first in the large vessels of the xylem in leaves and stems, in sub-stomatal cavities, and intercellular spaces (Levitt, 1980). This process is called "extracellular ice formation", due to the fact that the extracellular fluid has a lower solute concentration therefore a higher freezing point and more active heterogeneous ice nucleators than the intracellular fluid. The large diameter of the xylem vessels favors ice formation, and once ice forms it will rapidly propagate throughout the vascular systems and into the extracellular spaces of other tissues (Kitaura, 1967). If the ice crystal cannot penetrate through cell walls and intact plasma membranes, the cytoplasmic water will remain unfrozen (Ashworth and Abeles, 1984). At a given

subzero temperature, the chemical potential of ice is less than that of liquid water. Thus, ice formation causes the extracellular solution to become more concentrated, a gradient in the chemical potential of water (water potential) is created between the intracellular and extracellular solutions. Consequently, there is a movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces. The net amount of water movement required to bring the system into chemical equilibrium depends on the initial solute concentration of the intracellular fluid and the subzero temperature, which directly determines the chemical potential of the ice. At -10°C, more than 90% of the osmotically active water will generally move out of the cells to the intercellular spaces, and the osmolality (Osm) of the remaining unfrozen intracellular water can be in excess of 5 Osm (Thomashow, 1998). Under these conditions, water keeps moving out of the cell and results in the cytoplasm becoming severely dehydrated.

Freeze-induced dehydration has a number of effects that result in cellular damage, such as the denaturation of proteins and precipitation of various molecules. However, the best documented injury occurs at the membrane level (Steponkus and Webb, 1992). Detailed analyses have demonstrated that freeze-induced dehydration can cause multiple forms of membrane lesions (Steponkus et al., 1993).

The membrane system has a critical function in the freezing process. Plasmalemma plays the role as a physical barrier preventing ice nucleation of the cytoplasm from

extracellular sites, preserving cellular integrity as the cell contracts and expands during freezing and thawing, and maintaining proper permeability to keep normal flux of water, ions, and solutes between the cytoplasm and the apoplasm.

At relatively high freezing temperatures, between about -2°C and -4°C, the predominant injury in nonacclimated plants appears to be "expansion-induced lysis" which is caused by the osmotic contraction and expansion cycle that occurs with freezing and thawing. As water is moved outward from the cell, ice forms extracellularly and the cytoplasm shrinks. While the plasma membrane is shrinking, the cell wall is rigid and gradually develops resistance against further shrinkage. Finally, the membrane will tear and detach from the cell wall, causing mechanical damage to the plasma membrane and cell death. Bartolo et al. (1987) found that cold acclimation resulted in increased rigidity of plant cell walls. Rajashekar and Lafta (1996) showed that the cell wall rigidity affected certain freezing characteristics of plant tissues, such as freezing-induced cell dehydration and the temperature of ice nucleation. The rapid freezing and thawing are responsible for most of the injury that plants sustain in environments prone to freezing (Nilsen and Orcutt, 1996b).

At lower temperatures, between about -4°C and -10°C, the predominant form of injury in nonacclimated plants is freeze-induced "lamellar-to-hexagonal II phase transitions" of cellular membranes, an interbilayer event involving the fusion of cellular membranes (Fujikawa, 1991). At temperatures below -10°C, with the consequent

lower water potentials and more severe dehydration, other forms of membrane damage can occur, including fracture-jump lesions (Uemura et al., 1995).

1.3.3. Avoidance and tolerance of freezing

Plants survive freezing temperatures by avoidance and/or tolerance of freezing. Avoidance of freezing is mainly achieved by supercooling, production of metabolic heat, or storing absorbed heat energy (Bowers, 1994; Nilsen and Orcutt 1996b). Intracellular freezing (ice formation within the cell) is usually lethal. This type of freezing occurs commonly in non-cold acclimated tissues. In acclimated tissue, plants may tolerate freezing by allowing ice to form in extracellular space, such as wall space and intercellular space among cells, and the xylem, without lethal consequences because dehydration is reversible to a characteristic threshold.

In supercooling, cellular solutes accumulate in cells and lower the freezing temperature of the cytoplasm to well below the freezing point of pure water. The accumulation of solutes is accomplished in one of two ways, either solutes (sugar, polyols, and other osmotic molecules) are synthesized through metabolism or water is removed from one tissue to another area that is less sensitive to freezing. Supercooling mainly occurs in special organs such as seeds, overwintering buds or ray parenchyma cells (Sakai and Larcher, 1987). Fruit trees can avoid freezing by deep supercooling (Quamme, 1991) in certain organs such as flower buds, vegetative buds, and portions

of their bark and woody tissue (Sakai and Larcher, 1987). In peach, at approximately -5 °C, ice forms first in the scales surrounding the flower buds and then in the bud axis (Ashworth, 1982; Ashworth et al., 1989). But when temperature is further lowered and the supercooling limit is exceeded, the supercooled water finally freezes, with ice forming either inside the cell, which is lethal because ice crystals rupture the cells' membranes, or in extracellular space, which will cause injury by cell dehydration and may kill nonacclimated plants.

Tolerance to freezing through cold acclimation is the dominant mechanism by which woody plants survive freezing. The ability of hardy plants to survive freezing is dependent on many factors, such as whether supercooling freezing occurs, site of ice nucleation, rate of cooling during crystallization, whether there is a barrier to ice propagation through the plant, rate of ice growth, minimum temperature of exposure, duration of exposure to freezing condition, the extent of cellular dehydration, how dehydration-tolerant the cells are, etc. (Levitt, 1980; Pearce, 1999),

The membrane systems of the cell have been shown to be the primary site of freezing injury in plants (Levitt, 1980; Steponkus, 1984). The freezing induced cellular dehydration can cause the expansion-induced-cell-lysis, lamellar-to-hexagonal-II phase transitions, fracture jump lesions (Steponkus et al., 1993), as mentioned above. Thus, a key function of any freezing tolerance strategy must be the ability to stabilize membranes against freezing injury. Cold acclimation prevents expansion-induced-

lysis and the formation of hexagonal II phase lipids in many plants and has been shown to be the most important feature in plants for freezing tolerance (Levitt, 1980; Steponkus, 1984).

1.3.4. Plant cold acclimation to freezing stress

Many temperate and boreal plants have the ability to increase their degree of freezing tolerance in response to low, non-freezing temperatures (below approximately 10°C), a phenomenon known as cold acclimation (Levitt, 1980; Sakai and Larcher, 1987; Thomashow, 1999). Woody perennials can also acclimate in the fall in response to shortening days in addition to low, non-freezing temperatures. These plants are the most cold hardy in mid-winter when they are fully acclimated and dormant. In response to warming temperatures in the spring, the plants begin to deacclimate when bud break occurs.

The inherent ability of temperate plants to acclimate to cold and the timing and rate of cold acclimation are the major factors that determine whether they will survive winter freezing. Mechanisms that could potentially contribute to freezing tolerance would include helping to prevent or reverse freeze-induced denaturation of proteins, preventing molecules from precipitating, and lessening direct physical damage caused by the accumulation of intercellular ice.

Acclimation promotes water loss from the tissue and accumulation of osmotically inactive molecules such as starch and proteins. The loss of water has obvious adaptive advantages because there would be less water to freeze, less ice to accommodate, and physically less expansion in the intercellular spaces. During cold acclimation, there is commonly an increase in ABA whose quiescence or dormancy effect is positively associated with cold tolerance, and a decrease in GA whose growth promoting effect is negatively associated with tolerance. A decrease in respiration and increase in photosynthesis have been observed, but the relationship with freezing tolerance is not straight forward.

What is certain, however, is that cold acclimation involves the stabilization of membranes against freeze-induced damage (Thomashow, 1998). Plasma membranes from nonacclimated plants suffer expansion-induced lysis and formation of hexagonal II phase lipids upon freezing, while membranes from cold-acclimated plants do not (Steponkus et al., 1993). The stabilization of membranes against freeze-induced injury appears to involve multiple mechanisms. Steponkus et al. (1993) provided compelling evidence that the increase in membrane-freezing tolerance that occurs with cold acclimation involves changes in membrane lipid composition. Alterations that can contribute to increased freezing tolerance include increased levels of fatty acid desaturation in membrane phospholipids and changes in levels and types of membrane sterols and cerebrosides. In addition, the accumulation of sucrose and other simple sugars that typically occurs with cold acclimation seems likely to contribute to the

stabilization of membranes, since these molecules can protect membranes against freeze-induced damage in vitro (Anchordoguy et al., 1987). Finally, there is emerging evidence that certain hydrophilic polypeptides help to stabilize membranes against freeze-induced injury (Thomashow, 1998).

1.3.4.1. Cold acclimation response

During cold acclimation, plants undergo a series of morphological, cellular, biochemical, and membrane system changes. Chen et al. (1995) summarized these changes during plant cold acclimation. Changes in soluble extracellular polysaccharides were found to accompany cold acclimation in suspension-cultured pear cells (Wallner et al., 1986). Cell wall augmentation and compositional increase were documented by Weiser et al. (1990) in pea. Chen and Li (1980) showed the dry matter accumulation such as the increase of carbohydrates in *Solanum* species. Johnson-Flanagan and Singh (1986) observed that the number of contacts between plasma membrane and cell wall increased, during cold acclimation in alfalfa cell cultures. The development of freezing tolerance as plants acclimate is associated with changes in the physical properties and composition of the plasma membrane (Chen et al. 1995). In winter rye (Lynch and Steponkus, 1987) and *Arabidopsis thaliana* (Uemura et al., 1995), dramatic changes in the lipid composition of the plasma membrane were observed as the plants acclimated. Steponkus et al. (1990) reported the altered biophysical properties of membranes in rye protoplasts. Changes in the

degree of fatty acid unsaturation, proportion of phospholipids, and sterol composition, increase in the phospholipid/protein ratio, and decrease in the sterol/phospholipid ratio were observed by Uemura and Yoshida (1984). Changes in plasma membrane protein pattern and altered glycoprotein fraction during acclimation (Yosida, 1984), the appearance of proteins in hardened tissue (Uemura and Yoshida, 1984), and the association of a membrane protein with the induction of freezing tolerance have been reported. In cytoplasm, an increase in soluble sugars (Levitt, 1980; Sasaki et al., 1996) and an accumulation of proteins (both total content and specific polypeptide species) (Chen and Li, 1980; Gilmour et al., 1988; Guy, 1990; Lee et al., 1992, etc.) have been observed to change significantly in the hardening response of plants.

During cold acclimation, changes in gene expression were reported (Guy et al., 1985). The expression of many genes are found to be up-regulated during cold acclimation, a number of which encode proteins with known enzyme activities that potentially contribute to freezing tolerance, such as the *Arabidopsis FAD8* gene (Gibson et al., 1994) encoding a fatty acid desaturase that might contribute to freezing tolerance by altering lipid composition, spinach cold-responsive *hsp70* genes encoding molecular chaperones (Anderson et al., 1994), *Brassica napus hsp90* gene (Krishna, et al., 1995) likely contributing to freezing tolerance by stabilizing proteins against freeze-induced denaturation, etc. Many cold-responsive genes encoding various signal transduction and regulatory proteins have been identified as well (Jarillo et al., 1994; Mizoguchi, et al., 1993; Mizoguchi, et al., 1996; Polisensky and Braam, 1996). With the hope that

some of these genes are involved in freezing tolerance and that studies of their regulation and function would provide new insights into the cold acclimation process, among numerous studies, the research on cold-regulated gene expression in *Arabidopsis* led to the discovery of a family of transcriptional activators, the CBF/DREB1 proteins, that have a key role in cold acclimation (Thomashow, 2001).

1.3.4.2. Cold-regulated (cor) genes and CBF cold response pathway

In *Arabidopsis*, many cold regulated (cor) genes have been identified and cloned (Thomashow, 1994). Artus et al. (1996) indicated that *COR15a* acted in concert with other cor genes to enhance freezing tolerance by an unknown mechanism. Although constitutive expression of *COR15a* clearly enhances freezing tolerance at the organelle (chloroplast) and cellular (protoplast) levels, the effects are modest (Artus et al., 1996). Moreover, unlike cold acclimation, *COR15a* expression alone does not result in a detectable increase in freezing survival of whole plants (Jaglo-Ottosen et al., 1998). These findings are not surprising given the results of genetic analyses indicating that freezing tolerance is a multigenic trait involving genes with additive effects (Thomashow, 1990). Indeed, multiple genes are activated by cold acclimation in many plant species studied (Thomashow, 1994; Hughes and Dunn, 1996).

Expression of the entire battery of COR genes was accomplished by overexpressing the *Arabidopsis* transcriptional factor CBF1 (C-repeat binding factor) (Stockinger et

al., 1997). CBF1 binds to a DNA *cis*-acting regulatory element, the C-repeat (CRT) dehydration responsive element (CRT/DRE), which activates transcription in response to low temperature and water deficit (Yamaguchi-Shinozaki and Shinozaki, 1994). The element is present in the promoters of COR15a, COR78, COR6.6, COR47, and presumably other yet-to-be-identified *COR* genes. Jaglo-Ottosen et al. (1998) found that constitutive overexpression of CBF1 induces expression of *COR6.6*, *COR15a*, *COR47*, and *COR78* in nonacclimated *Arabidopsis* plants. Moreover, it results in an increase in freezing tolerance that is greater than when COR15a is expressed alone. Overexpression of CBF1 increased freezing tolerance at the whole-plant level.

The identification of a DNA regulatory element, the C-repeat (CRT) dehydration responsive element (DRE), that imparts responsiveness to low temperature and dehydration (Yamaguchi-Shinozaki and Shinozaki, 1994), led to the subsequent identification of the transcriptional activators that bind to the CRT/DRE, named CBF1, CBF2, and CBF3 by Stockinger et al. (1997) and Gilmour et al. (1998), or DREB1b, DREB1c, and DREB1a by Liu et al. (1998), respectively. Under normal growing conditions, constitutive overexpression of the *CBF1/DREB1b* (Jaglo-Ottosen et al., 1998) or *CBF3/DREB1a* (Kasuga et al., 1999; Liu, et al., 1998) genes in transgenic *Arabidopsis* plants induces the expression of multiple cold-responsive CRT/DRE-containing genes. Jaglo-Ottosen et al. (1998) found that the nonacclimated transgenic plants overexpressing *CBF1/DREB1b* are more freezing tolerant than nonacclimated control plants. Similar results were found for overexpressing *CBF3/DREB1a* (Kasuga

et al., 1999; Liu, et al., 1998). These results lead to the conclusion that CRT/DRE-contained genes which are induced by the CBF/DREB1 transcription factors, called the CBF regulon, includes genes with roles in cold acclimation. The fact that CRT/DRE-regulated genes are not expressed at warm temperatures indicates that the CBF/DREB1 genes themselves are cold regulated (Gilmour et al., 1998; Liu, et al., 1998). Furthermore, *Arabidopsis* plants overexpressing *CBF3/DREB1a*, can increase tolerance of dehydration stress caused by either drought or high salinity (Kasuga et al., 1999; Liu et al., 1998). The functions of the CBF regulon appear to be protection of cells from freezing and other stresses involving dehydration (Thomashow, 2001).

1.3.5. Freezing injury evaluation

Artificial freezing under controlled conditions is the most common method used to study cold injury. While there are many tests that may be used to evaluate cold injury, the most commonly used include (1) regrowth test and visual rating of oxidative browning tissue (Bell et al., 1992; Cortell et al., 1997; Hummer et al., 1986 and 1995; Sakai et al., 1986; Pearce et al., 1996 and 1998; Warmund et al., 1988), (2) color reaction tests (e.g. tetrazolium chloride and neutral red) (Malone and Ashworth, 1991; Takeda et al., 1993), (3) electrical conductivity of diffused electrolytes (Stuart, 1939; Khanizadeh et al., 1989; Ryyppö et al., 1998), (4) exotherm analysis (e.g. daifferential thermal analysis) (Montano et al., 1987; Quamme, 1991; Flinn and Ashworth, 1994;

Warmund et al., 1988 and 1992; Warmund and George, 1989 and 1990; Bourne and Moore, 1992).

The effectiveness and reliability of these methods depend on factors such as plant species and experimental conditions. In the case of evaluating freezing tolerance in blackberry, as well as many woody plant tissues, visual rating of oxidative tissue browning and electrical conductivity of diffused electrolytes are the most frequently used methods. These two methods will be used to assess cold hardiness of *Populus* and 'Marion' blackberry in this research.

1.4. Cold hardiness in blackberry

1.4.1. Inheritance studies

There are few studies on the genetics of cold hardiness in blackberry. Bourne and Moore (1992) evaluated 12 seedling blackberry populations of complex hybrids of tetraploid parents for cold hardiness of stem and bud tissues. They found that there were significant population effects for xylem, phloem, and bud cold hardiness. Seedling populations having 'Darrow' as a parent were generally hardy, those having 'Brison' as a parent were less hardy, and those from a cross between 'Darrow' and 'Brison' had consistently good hardiness. They concluded that 1) the cold hardiness of blackberries was a quantitatively inherited characteristic, which was supported by the

fact that the cold injury in plant tissues of the progenies were generally predictable from the parent response, i.e. there is a consistent level of hardiness between parents and progenies; 2) a dominance effect may also play a role in determining cold hardiness of the progenies.

1.4.2. Variability in Cold hardiness of blackberry cultivars

There is considerable variation in cold hardiness among blackberries, even among supposedly hardy genotypes. While eastern erect and semi-erect species are more hardy than trailing species, neither have adequate hardiness (Jennings, 1988). Developing cold hardy cultivars has been an important objective for many breeding programs. The ideal genotypes are expected to retain cold hardiness for a long period, to have a sufficiently long rest period, not respond to fluctuating temperatures, and are capable of re-hardening if their initial cold hardiness is lost (Ourecky, 1975). Among 11 blackberry cultivars, the floral buds of an erect cultivar Darrow were found to be the hardiest, with 45% of the primordia in primary buds surviving -33 °C in January (Warmund and George, 1990). While useful information, they did not evaluate trailing blackberries rather they evaluated the more cold hardy eastern blackberries. Hummer et al. (1995) evaluated many more *Rubus* species and genotypes but their evaluation of each genotype was less intensive and they were evaluating material grown in Oregon where the acclimating conditions are milder than much of the eastern U.S. They evaluated cultivated and wild genotypes from the USDA-ARS National Clonal

Germplasm Repository (NCGR) by contrasting survivability between and within crop groupings (including 42 blackberry genotypes) using controlled laboratory freezing in January (Hummer et al., 1995). The result showed that, with LT_{50} values of -24 °C (canes) and -20 °C (buds), ‘Black Satin’ was the most hardy blackberry cultivar, followed by 'Smoothstem', 'Dirksen', 'Bailey', 'Womack', and ‘Darrow’. 'Chester Thornless', 'Comanche', 'Hull Thornless', 'Cherokee', 'Cheyenne', Chehalem', 'Logan Thornless', and 'Marion', had moderate hardiness. In general, erect and semi-erect cultivars were more hardy than the trailing cultivars, and among trailing cultivars, ‘Marion’ with T_{50} value of -17 ºC, -17 ºC, and -15 ºC for stem, bud and bud base, respectively, was one of the most hardy cultivars in the group (Hummer et al., 1995). In this study, the bud base of 'Darrow' only survived -13 C°, which was much less than the hardiness shown in Warmund and George's (1990) study. The difference was supposed to be due to the different hardening regimes experienced in each study, with the latter having possible insufficient low temperature (-2 °C) for plants grown in the Willamette Valley and the former storing the materials at -7 °C before testing.

1.4.3. Cold hardiness of different blackberry tissues

At very low temperatures, some tissues are hardier than others. Warmund et al. (1986) evaluated midwinter hardiness of buds and phloem tissues of six erect-growing blackberries (‘Cherokee’, Comanche’, ‘Cheyenne’, and ‘Shawnee’, ‘Darrow’, and ‘A-1172’) and found that ‘Darrow’ had the greatest bud hardiness but at temperatures

where the buds survived, there was severe phloem injury. The occurrence of supercooling of the cellular water in the bud tissue may be the reason for this difference (Warmund et al., 1988). Artificial freezing test of eastern thornless blackberries showed that bark tissues were hardier than bud tissues (Kraut, 1986). Warmund et al. (1989a) assessed the freezing injury of tissue cultured 'Shawnee' blackberry by visually rating tissue browning and reported that the apical meristems were more cold hardy (surviving at least -8 °C) than the roots and the leaves; and the younger leaves were more cold hardy than more mature leaves. 'Shawnee' blackberry was assessed for freezing avoidance of canes by Warmund and George (1989b), and they found that phloem tissue was injured at a higher temperature than was xylem tissue. In Hummer's (1995) comprehensive screening for cold hardiness of *Rubus* genotypes, the relative cold hardiness of the cambial region of canes, dormant buds, and the axillary attachment point of the dormant bud (bud base) was determined. Buds were generally found to be less hardy than canes, and the bud tissues, enclosed in the bud scales, were usually more hardy than bud bases (Hummer et al., 1995).

1.4.4. Supercooling in blackberries

Warmund and George (1989) found that xylem tissue of 'Shawnee' can avoid freezing by supercooling. The floral primordia in primary buds of 'Smoothstem' and 'Dirksen' (Kraut, 1986), 'Darrow' (Warmund et al., 1988), 'Shawnee' (Warmund and George, 1989a and 1990), 'Dirksen' (Warmund and George, 1990), and 'Black Satin' and 'Hull

Thornless' (Warmund et al., 1992), and secondary buds of ‘Dirksen’ (Warmund and George, 1990), were all found to be able to avoid freezing by supercooling. In the differentiating buds of eastern thornless blackberries ‘Hull Thornless’ and ‘Black Satin’, the extracellular ice formation was found to occur in the scales surrounding inflorescence and in the bud axis, but not in the inflorescence axis and the developing floral primordia (Warmund et al., 1992).

Bud hardiness and bud hydration were found to be closely related in *Rhododendron* and fruit trees that display supercooling (Graham and Mullin, 1976; Hewett et al., 1978; Ishikawa and Sakai, 1981). However, there was no relationship between whole bud moisture content and supercooling capacity in a freezing survival test of 11 blackberry cultivars (Warmund and George, 1990). This was probably due to the smaller primordia size of blackberries and raspberries than those of the previously studied species. Small primordia only contain small amounts of water and, therefore, whole bud hydration level was not a reflection of the amount of water in the primordia or of the primordia supercooling capacity. Viability tests revealed that secondary buds generally had a lower survival temperature than primary buds (Warmund and George, 1990).

Differential thermal analysis experiments were conducted by Warmund and George (1990) on primary and secondary buds of ‘Dirksen’ and ‘Shawnee’, and the results were contrasted with the LT_{50} values. They found that the low temperature exotherms

detected were correlated with freezing injury in primary buds, but their number seldom corresponded to the number of differentiated floral primordia.

1.5. Cultural practices to prevent cold injury

1.5.1. Cultural practices to prevent cold injury in fruit crops

Cold hardiness is determined primarily by genotype, but may be enhanced by different cultural practices including: site selection, soil management, rootstock choice in grafted fruit, use of covers or tunnels, adjust plant spacing, management of crop loads, nutrients, irrigation, growth regulators, and pest and disease control, etc. (Bell et al., 1995; Gubbels, 1969; Krezdorn and Martsolf, 1984; Palonen and Buszard, 1997; Westwood, 1978).

To minimize cold injury, commercial growers avoid low spots where cold air can pool and cause greater mid-winter or spring frost injury (Krezdorn and Martsolf, 1984). Keeping topsoil moist, compact, smooth, and free from weeds and litter is important to protect crops from radiation frost because upward flow of heat is assured (Westwood, 1978; Krezdorn and Martsolf, 1988). In grape and berry crops, using taller plants and trellis reduces frost damage because of temperature inversion (Blanc et al., 1963). In grafted tree fruits, choosing the cold hardy rootstocks can improve cold hardiness of scions. For example, in apples rootstocks ‘M.9’ and ‘M.26’ have been found to impart

greater hardiness than ‘MM.106’ in terms of tip die-back of branches caused by frost damage (Callesen, 1994). Similar examples can be found in peach (Brown and Cummins, 1988). Using tunnels (Finn and Knight, 2001) or covering low-growing strawberries, dwarf apples and grapes, and caneberries, etc. with straw, row cover, artificial stable foams, water, muslin, or plastic have been shown to protect against freezing (Westwood, 1978). Planting fruit trees at a high density means there is more canopy to intercept radiant heat from the soil and trees transfer more radiant heat to each other thereby decreasing injuries (Flore et at., 1987; Krezdorn and Martsolf, 1984). Chemical primocane suppression early in the season improved winter survival of several raspberry cultivars and cane thinning treatments had a similar effect (Buszard, 1986). Reducing excessive vegetative growth by limiting late application of nitrogen is considered an effective cultural practice to improve fruit tree hardiness (Krezdorn and Martsolf, 1984), because the development of dormancy and acclimation will not be delayed. Reducing irrigation in the fall and early winter has been shown to induce dormancy and cold hardiness (Krezdorn and Martsolf, 1984). While not commercially reliable, application of growth regulators such as Trinexapac-ethyl and paclobutrazol tried to enhance cold hardiness in fruit crops (Blanco, 1990; Coleman, 1992; Durner and Gianfagna, 1991; Gusta et al., 1988; Seeley et al., 1992). The effects of growth regulators vary between plant species and even between cultivars. A better understanding of how growth regulators affect the plant cold hardiness would be important for the application of these techniques to the

commercial industry (Palonen and Buszard, 1997). Several cryoprotectant chemicals had a positive effect on grape cold hardiness (Himelrick et al., 1991).

1.5.2. Cultural practices to prevent cold injury in blackberry

While cultural practices in general have been shown to have an impact through years of experience or through research discussed previously, several cultural practices have been specifically addressed in caneberries. Covering canes with soil or straw when they are dormant in the fall is one technique that can be used in areas with severe winter but it is not commercially viable. Slowing down vegetative growth late in the growing season helps encourage canes to harden (Weiser, 1970). Therefore, besides reducing application of irrigation and fertilizer, using natural grasses or cover crops as competition for water, nutrients, and light can be effective in improving cold hardiness of blackberry (Moore and Skirvin, 1990).

For ‘Marion’, winter training tends to lead to less cold injury than summer training (Bell et al., 1992). Bell et al. (1995) studied the effect of primocane suppression at different dates over 4-month period on the hardiness of 'Marion' primocanes. Cutting off the primocanes of 'Marion' plants at ground level in April, May, June, or July increased cold hardiness of the next flush of primocanes. The latest suppressed (July) primocanes were generally hardiest for all tissues. The June-suppressed plants were less hardy than July-suppressed plants and April- and May- suppressed plants were

least hardy. The level of soluble carbohydrates in cane tissues of July-suppressed and unsuppressed plants were higher than those of the other suppression dates. Cortell and Strik (1997) reported the effect of floricane number on primocane growth and subsequent cold hardiness of 'Marion'. ‘Marion’ plants were pruned to 0 to 12 floricanes /plant in the spring and primocane hardiness was measured the next winter. They found that plants without floricanes produced primocanes (“alternate year production system”) that were significantly hardier than plants with floricanes.

Primocane fruiting occurs in some blackberries (Ourecky, 1975; Moore, 1984) and Hall (1990) pointed out that this character could be utilized in breeding to improve winter hardiness. The University of Arkansas released two primocane fruiting cultivars in 2004 and they are trademarked as Prime-Jim™ and Prime-Jan™ (Clark et al., 2005).

Since genotype is the most critical factor in determining hardiness, developing cold hardy cultivars for different climatic conditions through the power of the combination of classical breeding, molecular breeding and genetic engineering is essential.

1.6. Improving cold tolerance of ‘Marion’

1.6.1. Traditional breeding methods

Reciprocal recurrent selection where the hardiest selections are crossed to produce the next generation of seedlings has been the predominant breeding strategy for improving winter tolerance in blackberry. However, since test winters are needed in order to screen the parents and progeny, the process can be very slow (Hall, 1990). Also, it is impossible to introduce only the trait of improved cold hardiness, which is a complex trait controlled by a multigene family, without simultaneously disrupting other existing good traits. Finally, artificial freezing runs to identify tolerant genotypes may not reliably reflect actual field performance.

1.6.2. Genetic engineering

Since ‘Marion’ is considered to have ideal fruit quality by the processing industry, in a perfect world, ‘Marion’s cold hardiness could be increased without altering these fruit quality characteristics. This is not possible by traditional breeding but may very well be possible through genetic engineering where exogenous DNA is transferred into a crop genome of interest. This procedure can break down the sexual barriers, so that, in theory, any gene from any organism can be introduced into a plant. Genetic transformation allows the introduction of new traits into proven cultivars without disrupting their otherwise desirable genetic constitutions. This advantage is exactly what is needed for the improvement of an excellent cultivar like 'Marion'.

The rapid advances in plant molecular genetics has led to the cloning of many cold regulated genes from plants, and their regulation mechanisms has been elucidated. Foreign genes are efficiently being introduced into an expanding array of valuable plants and the techniques are being improved in recalcitrant crops. This progress has made genetic engineering a valuable complementation to classical plant breeding in developing crops with increased low temperature stress tolerance and a tool for studying the mechanisms of low temperature adaptation. Examples of improved freezing tolerance in plants through genetic engineering are given in Table 1.1.

Table 1.1. Example of transgenic plants with improved freezing tolerance

Gene function	**Gene**	**Gene product**	**Transformed species**	**Reference**
Encoding enzymes that synthesize osmotic and other protectants	*codA*	choline oxidase (glycine betaine synthesis)	tobacco	Parvanova et al., 2004
	COR15a	cold induced gene	*Arabidopsis*	Steponkus et al., 1998
	P5CS	pyrroline carboxylate synthetase (proline synthesis)	tobacco	Parvanova et al., 2004

	SPE	spermidine synthase	*Arabidopsis*	Kasukabe et al., 2004
Regulatory genes	*CBF1*	transcription factor	*Arabidopsis* tomato, canola, strawberry, aspen	Jaglo-Ottosen et al., 1998; Kasuga et al. 1999; Jaglo et al., 2001; Owens et al. 2002; Novillo et al., 2004; Pino et al. 2005; Benedict et al. 2006
	CBF3	transcription factor	*Arabidopsis* rice	Dubouzet et al., 2003; Novillo et al., 2004; Amundsen 2004
	CBF4	transcription factor	*Arabidopsis*	Haake et al., 2002
	CBL1	Ca sensing protein	*Arabidopsis*	Cheong et al., 2003
	desC	acyl-lipid 9-desaturase	tobacco	Orlova et al., 2003
	DREB	transcription factor	*Arabidopsis* rice	Kasuga et al., 1999; Ito et al., 2006

	GPAT	glycerol-3-phosphate acyltransferase of chloroplasts	Rice	Ariizumi et al., 2002
	OsCDPK7	transcription factor	Rice	Saijo et al., 2000
	SCOF-1	transcription factor	*Arabidopsis* tobacco	Kim et al., 2001
	SacB	fructan synthesis	tobacco	Parvanova et al., 2004
	wft1, wft2	fructan synthesis	ryegrass	Hisanoa et al., 2004
	SPS	sucrose phosphate synthase	*Arabidopsis*	Strand et al., 2003
	OsDREB1A	transcription factor	*Arabidopsis*	Dubouzet et al., 2003
	Gal	Raffinose hydrolysis	petunia	Pennycooke et al., 2003
Hormone regulating genes	*AtPP2CA*	protein phosphatase	*Arabidopsis*	Tahtiharju and Palva, 2001

Oxidative stress related genes	*ALR*	aldose/aldehyde reductase	tobacco	Hegedus et al., 2004
	Apx	ascorbate peroxidase	cotton	Kornyeyev et al., 2003
	CAT	wheat catalase	Rice	Matsumura et al., 2002
	GPX	glutathione peroxidase	tobacco	Yoshimura et al., 2004
	SOD	Mn superoxide dismutase	Alfalfa	McKersie et al., 1993
Encoding proton pumps, antiporters and ion transporters	*CAX1*	vacuolar Ca2+/H+ antiporter	*Arabidopsis*	Catalá et al., 2003
Encoding for molecular chaperones	*pBE2113 /hiC6*	cryoprotective protein	tobacco	Honjoh et al., 2001

1.7. Using *Populus* as a model to see the effect of CBF genes on cold hardiness of woody plants

1.7.1. A model system for woody perennials

While *Arabidopsis* has been used as a model to study plants because of its small genome, its a rapid life cycle, its easy transformation, its wide natural distribution, and since its genome has been sequenced, it has some drawbacks as a model when studying characteristics unique to woody perennials (Tayor, 2002).

Woody plants exhibit complex patterns of activity. For example, acclimation, dormancy, and bud break involve the complex interactions between environmental signals, with day-length and temperature being the most important and signal transduction pathways in plants. While the homologous genes may be conserved among plants including *Arabidopsis*, but the regulatory mechanism and the control of their expression could be hard to study because *Arabidopsis* never needs to go through the same molecular, morphological, physiological and biochemical processes in its life cycle (Tayor, 2002). For these reasons, *Populus* has been increasingly used as a model for woody perennials.

1.7.2. *Populus* as a model for woody perennials

Populus, a commercially important timber species, is an ideal model species for molecular study of woody perennials for several reasons that are summarized by Brunner et al. (2004) and include: 1) it grows rapidly, achieving tree phenotypes in 1-

3 years in the greenhouse or field, 2) it is precocious and can flower as soon as 1 year after seed germination, 3) it is easy to propagate (cutting or root suckering) or micropropagate (with little maturation-related somatic variations), 4) it has considerable genetic variation, 5) it is easily transformed, with many genotypes having high transformation frequency, stable transgene expression, and minor somaclonal variation problems, which make it ideal for analyzing hundreds of transgenes within a reasonable time, 6) it has a small genome size (~500-550 Mb) compared to other trees and a similar genetic-physical map distance as *Arabidopsis* between linkage groups (Bradshaw et al., 2000), which make it relatively easy for molecular characterization or manipulation.

More than 200,000 ESTs have been identified from a wide range of tissues and laboratories, which are being used to produce a range of microarray chips for expression analysis (Brunner et al., 2004). A sequencing project has been announced by the Joint Genome Institute (JGI) that the genome of black cottonwood (*Populus trichocarpa*) was being sequenced, for the first time in woody plants (Wullschleger et al., 2002).

We used a hybrid aspen (*P. tremula* × *P. alba*) clone to express three CBF genes (CBF1, CBF2 and CBF3) constitutively by 35S promoter or by a cold-inducible rd29 promoter to study the gene expression and freezing tolerance in the woody plant model. Upon successful transformation of ‘Marion’ using reporter genes, we will also

introduce the CBF genes into ‘Marion’, to explore the possibility to increase the cold hardiness of ‘Marion’.

1.8. Strategy and objectives

This project was carried out with the following stages: 1) establish an efficient regeneration system for ‘Marion’ blackberry, 2) establish a genetic transformation system for ‘Marion’ blackberry, 3) develop transgenic poplar lines whose CBF genes overexpress to see if this trait can increase freezing tolerance in a perennial plant, and if so, 4) transform ‘Marion’ blackberry with CBF genes and characterize transgenic plants for freezing tolerance.

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CHAPTER 2

Improving *In vitro* Shoot Regeneration from Leaf and Petiole Explants of 'Marion' Blackberry

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Improving *In vitro* Shoot Regeneration from Leaf and Petiole Explants of 'Marion' Blackberry

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2.1. Abstract

Experiments focusing on plant growth regulators' concentrations and combination, mineral salt formulations and TDZ pretreatment formations were conducted to optimize *in vitro* shoot regeneration from leaf and petiole explants of 'Marion' blackberry. Optimum shoot formation was obtained when stock plants were incubated in TDZ pretreatment medium for three weeks before culturing leaf explants on regeneration medium (WPM with 5 µM BA and 0.5 µM IBA) in darkness for 1 week and they were transferred to light photoperiod (16-hour photoperiod at photon flux of ≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 23 °C $\pm$ 2 °C for 4 weeks**.** Under these conditions about 70% of leaf explants formed approximately five shoots per petri dish that could be harvested and rooted to form plantlets.

2.2. Abbreviations

ANOVA: Analysis of Variance; BA: N^6-benzyladenin; BMM: Blackberry Multiplication Medium; 2,4-D: 2,4-dichlorophenoxyacetic acid; GA_3: Gibberellic acid; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; LSD: Least Significant Difference; MBMM: Modified Blackberry Multiplication Medium; MS: Murashige & Skoog (1962) Medium; NAA: α-naphthaleneacetic acid; TDZ: Thidiazuron (N-

phenyl-N'-1,2,3-thidiazol-5-ylurea); WPM: Lloyd and McCown Woody Plant Medium.

2.3. Introduction

'Marion' blackberry (*Rubus* sp. L.) is a hexaploid cultivar derived from a cross between 'Chehalem' and 'Olallie'. It was released in 1956 by the cooperative breeding program of the USDA-ARS and the Oregon Agricultural Experiment Station (Finn et al., 1997), and is the most widely planted blackberry cultivar in the world and is the predominant cultivar grown primarily in Oregon's Willamette Valley (Strik, 1992). 'Marion' has become the dominant blackberry with worldwide appeal because of its processing quality including superior texture, color, and flavor. However, the lack of sufficient cold tolerance seriously affects its yield and may lead to an erratic supply from year to year (Bell et al**.,** 1992**;** Bell and Strik, 1995; Cortell and Strik, 1997). One way to improve 'Marion's cold hardiness is through the introduction of hardiness genes by traditional breeding. However, this is a slow process and may result in the loss of many of the favorable traits that make up this elite cultivar.

Incorporating cold tolerance genes through recombinant DNA technology is a viable alternative to the use of traditional breeding techniques. Improvement of freezing tolerance traits by genetic engineering was recently demonstrated for *Arabidopsis thaliana* (L.) Hey n.h. (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Sakamoto et al.,

2000). In these cases, genes that confer freezing tolerance were introduced without disrupting the otherwise desirable genetic constitutions. This might be an ideal approach to improve cold hardiness of ‘Marion’ while maintaining favorable processing characteristics. A prerequisite to achieving successful genetic transformation in 'Marion' is the development of an efficient regeneration system that facilitates the regeneration of whole plants from cultured cells, tissues and/or organs. Such a system is currently not available for 'Marion' blackberry.

Protocols for *in vitro* plant regeneration from a number of *Rubus* genotypes, including blackberries, raspberries (*R. idaeus* L.) and their hybrids, are available (Cousineau and Donelly, 1991; Fiola and Swartz, 1986; Graham and McNicol, 1990; Mathews et al., 1995; Mezzetti et al., 1997; McNicol and Graham, 1990; Swartz et al., 1990; Tsao, 1999; Turk et al., 1994). In these studies, shoot regeneration competence varied widely among genotypes dictating that experiments be conducted to develop an optimized protocol for individual genotypes. Factors studied include plant growth regulator compositions (type and concentration), medium salt formulation, special treatment of explant (chemical or physical), explant types (leaf, stem), incubation conditions (time, light intensity, and temperature), gelling agent and firmness, age of source plant, and explant orientation during regeneration. Among these studies, only one reported *in vitro* plant regeneration of 'Marion' (Tsao, 1999). In that study, Tsao (1999) obtained about 40% plant regeneration from whole leaf explants. However, the number of shoots obtained from responding explant per vessel was not reported. As a

highly efficient regeneration method is one of the most important factors in determining the success of a genetic transformation protocol, the major objective of this study was to establish an efficient *in vitro* shoot regeneration system for 'Marion' blackberry.

2.4. Materials and Methods

Tissue culture maintenance and explant preparation. In vitro cultures of 'Marion' were multiplied and maintained on a Modified Blackberry Multiplication Medium (MBMM, Reed, 1990), which consists of MS salts and vitamins (Murashige and Skoog, 1962), 1x MS iron plus $0.2 g \cdot L^{-1}$ of Sequestrene Fe 138 (Novartis, Greensboro, NC) with 4.4 µM N^6-benzyladenine (BA), 0.5 µM indole-3-butyric acid (IBA) and 2.9 µM gibberellic acid (GA_3). Two- to three-month old *in-vitro* micropropagated stock plants were cut into stem segments, each with one to two leaf buds and the leaf blades removed. Stock plants and stem segments were cultured in GA7 Magenta® vessels (Magenta Corp., Chicago, IL), each with 40 ml MBMM or TDZ pretreatment medium (MBMM with 1 µM TDZ), respectively.

General culture methods and experimental design Unless otherwise described, healthy and fully expanded leaves and petioles collected from 3-week-old plantlets initiated on TDZ pretreatment medium were used for all experiments. Hyperhydric leaves were discarded. Petioles were excised and the distal 1/3 to 1/4 of leaves were cut

transversely through the mid-vein and placed on medium abaxial side down. For petiole explants, petioles were cut into 5 mm segments and placed abaxial side on the surface of regeneration medium. Newly cut explants on regeneration medium were incubated in darkness at 23°C $\pm$ 2°C for 1 week before transfer to 16-h photoperiod (PPF≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 23 °C $\pm$ 2 °C for 4 weeks. Each treatment consisted of four replicate plastic petri dishes (100 × 15 mm) containing 25 ml of WPM (Lloyd and McCown, 1980) medium and 12 explants. Replicate dishes were arranged in a completely randomized design with subsampling. All experiments were conducted three times. Data collected in all experiments included the number of regenerating explants and number of shoots per petri dish.

Data collection and statistical analysis. Percent regeneration was defined as the percentage of explants that produced at least one shoot. Mean number of shoots per plate was obtained by counting the total number of shoots from regenerating explants in a plate. We recorded percent regeneration and number of shoots/explant weekly from 1-4 weeks after the plates were placed in light. However, we only reported the data from the fourth week because differences among treatments were greatest. Statistical analysis was conducted using the Logistic Regression Procedure for the regeneration efficiency (%) and Poisson Procedure for number of shoots per petri dish of SAS Version 8.02 (SAS Inst. Cary, N.C., 1999). Regression analysis was used to determine the dosage effect of BA and IBA levels on each explant and time effect of TDZ pretreatment.

Effect of BA and IBA combinations on shoot organogenesis. Thirty factorial combinations of BA (0, 1, 2, 5, 10 µM) and IBA (0, 0.5, 1, 2, 5, 10 µM) were tested for their effect on shoot regeneration. Leaf and petiole explants were prepared and cultivated as stated above.

Effect of cytokinin type on shoot organogenesis. Using the best BA/IBA concentrations from the experiment described above, we compared the effect of equal molar concentration of BA, kinetin, and zeatin on shoot regeneration. Leaf explants were placed abaxial side down on WPM with 5 µM of individual cytokinin and 0.5 µM IBA.

Effect of auxin type on shoot organogenesis. Using the best result from the cytokinin experiments, we compared the effect of equal molar concentration (0.5 µM) of IBA, indole-3-butyric acid (IAA), α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) combined with the best cytokinin and its concentration on shoot regeneration. Leaf explants were prepared as described above.

Effect of medium type on shoot organogenesis. Once the best plant growth regulator and concentrations were known, the effect of three medium formulations WPM, MS, and BMM (Blackberry Multiplication Medium) on shoot organogenesis was compared. Leaf explants were prepared as described above.

Effect of TDZ pretreatment duration on shoot regeneration. We compared the effect of 0, 3, 4, 5, and 6 weeks of culture on TDZ (1 µM) containing medium on shoot regeneration, using the optimum conditions identified in the previous experiments. Leaf explants were prepared and placed on WPM with 5 µM BA and 0.5 µM IBA.

Effect of leaf integrity on shoot organogenesis. Shoot regeneration efficiency from entire leaves, the basal 2/3-3/4 and the upper 1/3-1/4 of the leaf blade was compared. Leaf explants collected from TDZ pretreated plantlets were placed on WPM with 5 µM BA and 0.5 µM IBA.

Effect of leaf explant orientation on shoot organogenesis. The top 1/3 to 1/4 of each leaf, collected from TDZ pretreated plantlet, was cut transversely through the mid-vein and placed on WPM with 5 µM BA and 0.5 µM IBA with either abaxial or adaxial surface in contact with medium.

Effect of wounding on shoot organogenesis. Leaf explants collected from TDZ pretreated plantlets were either wounded with a sharp point dissecting needle or transferred intact to WPM with 5 µM BA and 0.5 µM IBA with the abaxial surface in contact with medium. About 1/3 to 1/4 of wounded leaves was cut transversely through the mid-vein and then stabbed about 10 times with a needle.

2.5. Results and discussion

Our goal was to develop an efficient plant regeneration system for 'Marion' blackberry in which shoots are directly regenerated from leaf and petiole explants with minimal callusing to minimize the potential of somaclonal variation. Generally, shoots appeared on the explant surface within 10 days of transfer to light. Examination using a dissecting microscope revealed that shoots originated directly from explants without visible callus. The number of shoots from regenerating explants peaked at around the third week and then only increased in size but not number (data not shown).

Explant type, BA and IBA combinations on shoot organogenesis. There was significant main effects of explant, BA, and IBA and significant interaction effects between each of them on shoot regeneration rate and number of shoots per petri dish (Table 1, Table 2). No regeneration from leaves occurs when IBA increased to 5 and 10 µM (Figure 1A) regardless of BA concentration. At such IBA levels, low rate of shoot regeneration was obtained from petioles only when IBA 5 µM was combined with BA 2 µM (Figure 1B). Since explant responded differently to BA and IBA (Table 1, Table 2), dosage effect of BA and IBA was analyzed using polynomial regression for leaf and petiole separately [Regeneration efficiency (%) $_{leaf}$ = 13.21 + 12.26*BA – 16.29*IBA – 0.93*BA^2 – 1.83*BA*IBA + 3.56*IBA^2 + 0.17*BA^2*IBA - 0.20*IBA^3 (R^2 = 0.65, P < 0.0001); Regeneration efficiency (%) $_{petiole}$ = 27.13 – 16.42*IBA – 0.51*BP*IBA + 0.38*IBA^2 + 0.04*BA*IBA^2 – 0.2*IBA^3 (R^2 = 0.65, P< 0.0001); Log

(No. shoots/petri dish) $_{leaf}$ = 1.24 + 0.69*BA - 0.21*IBA – 0.07*BA^2 ; Log (No. shoots/petri dish) $_{petiole}$ = 0.77 + 0.69*BA - 0.48*IBA – 0.07*BA^2]. Among the BA and IBA combinations tested, leaf explants incubated in WPM with 5 µM BA and 0.5 µM IBA produced more regenerating leaf explants and shoots per petri dish than did other combinations (Fig. 1, A and B). For petiole explants, we found that no single plant growth regulator alone stimulated high frequency shoot regeneration or a large number of shoots per petri dish (Fig. 1, C and D). Tsao (1999) reported that 5 or 10 µM BA combined with zero or 0.5 µM IBA was best for stimulating regeneration of 'Marion' leaf explants. Plant regeneration

percentage and the number of shoots per petri dish were significantly higher from leaf (Fig. 1, A and B) than petiole (Fig. 1, C and D) explants. Explants on WPM with 5 or 10 µM IBA combined with any concentration of BA produced callus but failed to produce shoots, although some explants developed roots (data not shown). Our results differ from that reported by Cousineau and Donnelly (1991) who did not pretreat 'Comet' red raspberry stock material with TDZ but added TDZ to their regeneration medium and found no difference in adventitious shoot regeneration efficiency among leaf blade explants, detached petioles and leaf explants with petioles attached. We observed that shoots grew from either or both of two cut ends of petioles and very seldom from the center, resulting in fewer shoots per petri dish. In our experiments, three-week-old plantlets produced thin (≈1 mm thick) and short (≈0.5 cm long) petioles, which usually made it impossible for them to be cut into sections. In contrast,

shoots emerged from the cut surface at the base of the leaf blades and from the surface of the leaf blade, resulting in more shoots per explant for leaf explants.

Effect of cytokinin type on shoot organogenesis. In our research, BA (5 µM) combined with 0.5 µM IBA was found to be significantly more effective in inducing shoot regeneration than either kinetin or zeatin, at equal concentrations when shoot cultures were pretreated with TDZ (Fig. 2, A and B). BA and TDZ are the most commonly used cytokinins in successful regeneration of *Rubus* genotypes (Cousineau and Donnelly, 1991; Fiola and Swartz, 1986; Fiola et al.,
1990; Gingas and Stokes, 1993; Graham and McNicol, 1990; McNicol and Graham, 1990). The effect of zeatin on organogenesis appears to be less efficient than TDZ in raspberry, blackberry and hybrid cultivars (Graham et al. 1997).

Effect of auxin type on shoot organogenesis. We found that, when combined with 5 µM BA, 0.5 µM IBA was significantly better than either IAA, NAA, or 2,4-D for shoot regeneration of 'Marion' blackberry at the same concentration (Fig. 2, C and D). The auxins 2,4-D and IBA are commonly used in *Rubus* regeneration protocols. Fiola and Swartz (1986) reported that 2,4-D level at 1.0 mg/L was ideal for promoting somatic embryogenesis from mature and immature red raspberry ovules. Graham et al. (1997) reported that NAA was the most effective auxin for initiating somatic embryogenesis in a wide range of *Rubus* genotypes.

Effect of medium type on shoot organogenesis. Medium formulation affects regeneration efficiency for *Rubus* genotypes (Turk et al, 1994). While MS medium is used for a wide range of plant species, including *Rubus*, BMM was developed specifically for blackberry (Reed, 1990), and WPM, which was developed for Ericaceous plants, has also been used for some non-Ericaceous fruit crops (Zimmerman and Swartz, 1994). We found that regeneration percentage and the number of shoots per petri dish were significantly better on WPM medium than BMM or MS medium (Fig. 3, A and B).

Effect of TDZ pretreatment on shoot regeneration. 'Marion' explants were unable to regenerate shoots without TDZ pretreatment under our conditions (Fig. 4, A and B). The regeneration percentage was significantly improved when a 3 week TDZ pretreatment was used. However, the regeneration percentage declined when longer pretreatments were used (Fig. 4A). The number of shoots per petri dish was reduced when explants were taken from shoots pretreated 4 weeks or longer. When testing the effect of the length of time in the pretreatment, we found following polynomial formula: Regeneration efficiency (%) = $36.41*\text{Week} - 5.76*\text{Week}^2$ ($R^2 = 0.80$, $P<0.0001$) and No. shoots/petri dish = $22.82*\text{Week} - 3.88*\text{Week}^2$ ($R^2 = 0.77$, $P< 0.0001$). Although TDZ is a very effective cytokinin for many woody plant species, Tsao (1999) found that BA was better than TDZ for inducing shoots when it was included in the pretreatment and regeneration medium for a number of blackberry genotypes, including ‘Marion’. However, TDZ induced shoot formation in other

Rubus species when added directly to regeneration medium (Hassan et al., 1993; Turk et al, 1994) and when pretreating stock plants (Swartz et al., 1990; Tsao 1999). Swartz et al. (1990) reported that TDZ concentrations greater than 1 μM excessively inhibited leaf size and their ability to be manipulated *in vitro*. We also observed that TDZ concentrations higher than 1 μM stimulated hyperhydricity. Hence, we selected 1 μM TDZ for optimizing stock plant pretreatment conditions. Although hyperhydricity and abnormal leaves were found in all stock plants with various durations of TDZ pretreatment, prolonged TDZ treatment significantly increased the frequency of abnormalities.

Effect of leaf integrity on shoot organogenesis. Whole leaves and the basal parts of the leaf explants had equally competent shoot regeneration efficiencies and shoots per petri dish (Fig. 5). However, the distal part of the leaf explants did not produce shoots. While Tsao (1999) found whole leaves were better than cut leaves, in our experiment there was no advantage to cutting leaf explants transversely as they did not produce more shoots than the intact leaf explants. Shoots did not form on the cut surface despite a significant amount of callus formation but were commonly observed at, and around, the cut surface at the base of the leaf blade (Data not shown).

Effect of leaf explant orientation on shoot organogenesis. It was reported that *Rubus* leaves responding similarly regardless of which epidermal layer was in contact with the medium surface (Mathews et al., 1995; Turk, et al., 1994). However, we found that

regeneration percentage was higher and there were more shoots per petri dish when the abaxial side of the leaf was in contact with the medium (64.3 ± 8.9 % and 4.6 ± 1.0 shoots/explant vs. 40. 4 ± 11.5 % and 2.8 ± 0.6 shoots/explant). Regardless of their orientation, all shoots were regenerated from the adaxial side of leaf explants.

All the parameters we examined had significant effects on *in vitro* shoot regeneration of 'Marion'. Our results confirmed those reported by Tsao (1999) that TDZ pretreatment and high concentration of BA, in combination with low concentration of IBA, induced shoot regeneration of 'Marion'. By optimizing all parameters described above, we were able to improve the regeneration efficiency to about 70% compared to that reported previously (about 40%). We are currently applying this improved *in vitro* plant regeneration system toward the development of a transformation protocol for 'Marion'.

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Table 2.1. Logistic procedure (forward selection) summary table for the percentage of explants that produced shoots from 3-week old TDZ pretreated 'Marion' leaf and petiole explants on WPM supplemented with BA and IBA in a $2 \times 5 \times 6$ factorial experiment. Whole experiment was conducted three times. Non-significant effects were rejected by forward selection.

Logistic Procedure Statistics

	Score Chi-Square	DF	Pr > ChiSq
Likelihood Ratio	977.1	6	<0.0001
Effect entered			
Explant	7.2	1	0.0071
BA	22.01	1	<0.0001
IBA	818.1	1	<0.0001
Explant*BA	20.0	1	<0.0001
Explant*IBA	4.0	1	0.0447
BA*IBA	6.0	1	0.0143
Residual	2.8	9	0.9707

Maximum Likelihood Estimates

DF	Estimate	SE	Wald Chi-Square	Pr > ChiSq

Intercept	1	-1.72	0.41	17.46	<0.0001
Explant	1	0.71	0.23	9.05	0.0026
BA	1	0.61	0.10	37.18	<0.0001
IBA	1	-0.84	0.13	40.07	<0.0001
Explant*BA	1	-0.25	0.06	19.28	<0.0001
Explant*IBA	1	0.13	0.06	4.02	0.0450
BA*IBA	1	-0.05	0.02	4.68	0.0306

Table 2.2. Poisson regression summary table for the number of shoots per petri dish from 3-week old TDZ pretreated 'Marion' leaf and petiole explants on WPM supplemented with BA and IBA in a $2 \times 5 \times 6$ factorial experiment. Whole experiment was conducted three times.

Model equation (non-significant parameters not included)

LOG (No. shoots/petri dish) =

$0.77 + BA* 0.69 - 0.48*IBA + 0.47*Explant – 0.07*BA^2 -0.58*IBA*Explant$

Summary of Fit

Mean of Response: 3.1361 Deviance:1621.0295 Pearson ChiSq: 1955.748

SCALE (Pearson): 1.6679 Deviance/DF: 2.3059 Pearson ChiSq/DF: 2.7820

Analysis of Deviance

Source	DF	Deviance	Deviance/DF
Model	16	3895.4214	243.4638
Error	703	1621.0295	2.3059
Corrected Total	719	5516.4509	-

Type III (Wald) Tests

Source	DF	ChiSq	Pr >ChiSq
BA	1	308.3009	<0.0001

IBA	1	28.4415	<0.0001
Run	2	0.8085	0.6675
Explant	1	13.4922	0.0002
BA^2	1	314.1789	<0.0001
BA*IBA	1	3.6827	0.0550
BA*Run	2	0.4908	0.7824
BA*Explant	1	3.7991	0.0513
IBA^2	1	1.0194	0.3127
IBA*Run	2	0.0884	0.9567
IBA*Explant	1	19.9973	<0.0001
Run*Explant	2	2.6307	0.2684

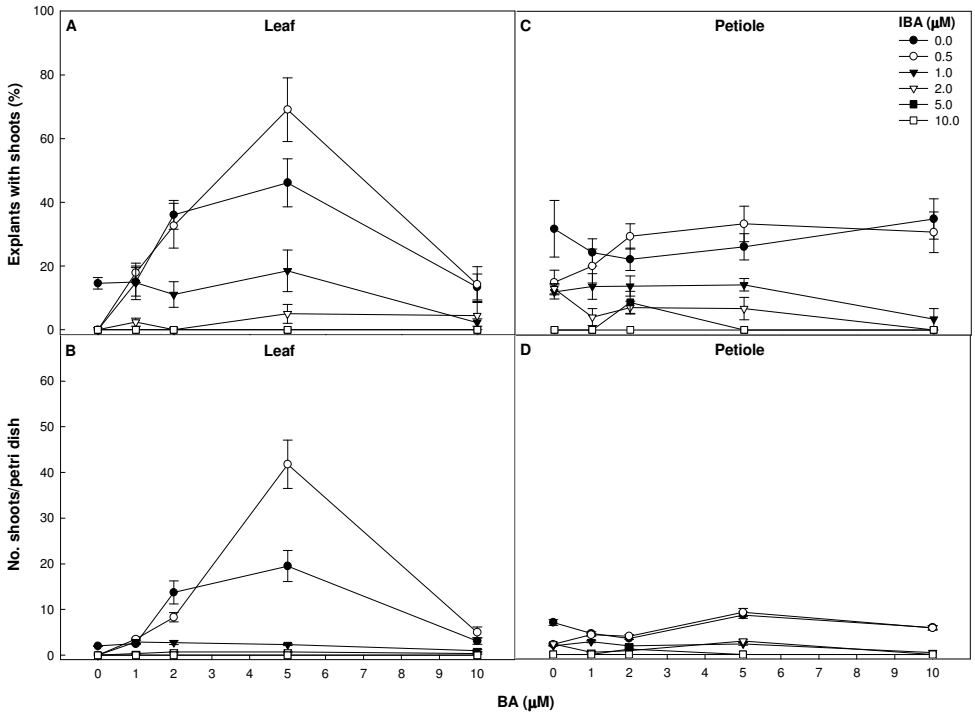

Figure 2.1. Effect of BA and IBA concentration on shoot regeneration rate (%) and number of shoots per petri dish for leaf (A and B, respectively) and petiole (C and D, respectively). Vertical bars, which represent standard error, that are overlap are not significantly different.

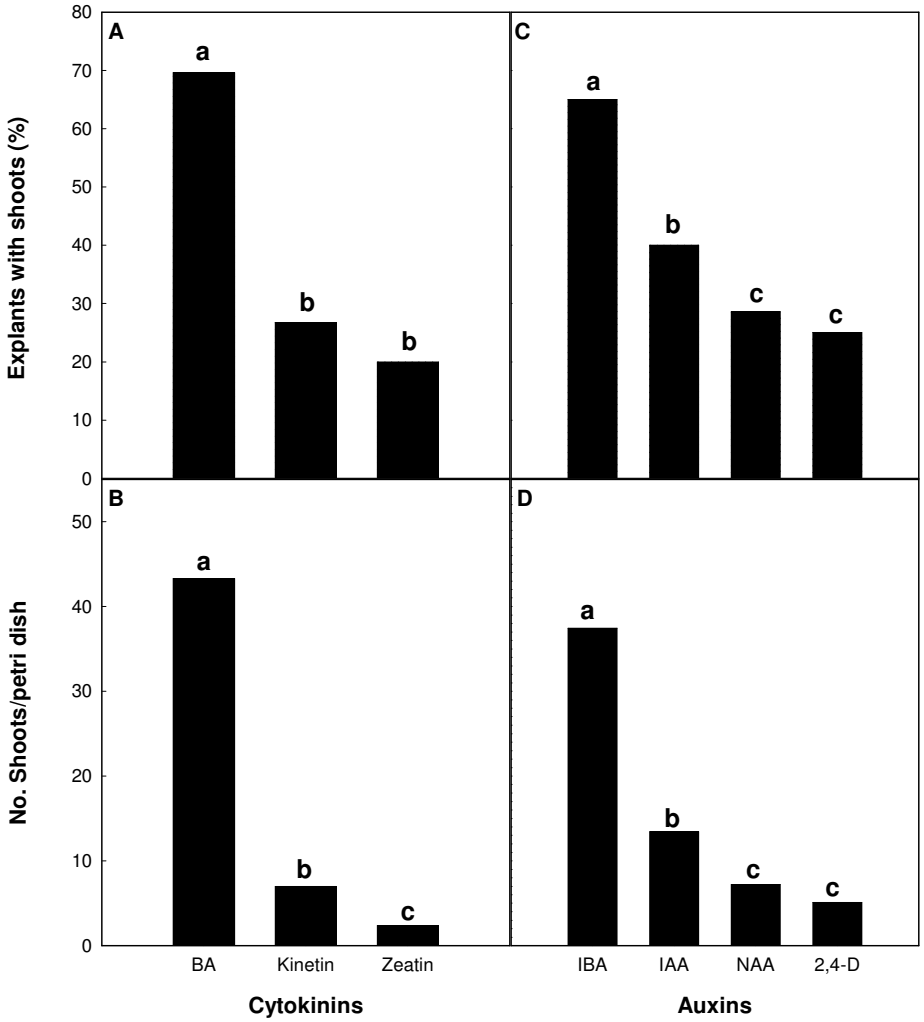

Figure 2.2. Effect of BA, kinetin, and zeatin on shoot regeneration. (A) Explants with shoots (%) and (B) Number of shoots per petri dish when IBA (0.5 µM) was combined with each cytokinin separately. Effect of 0.5 µM IBA, NAA and 2,4-D on shoot. (C) Explants with shoots (%) and (D) Number of shoots per petri dish when BA (5 µM) was combined with each auxin separately. Bars with same letters are not significantly different according to LSD, $P \leq 0.05$.

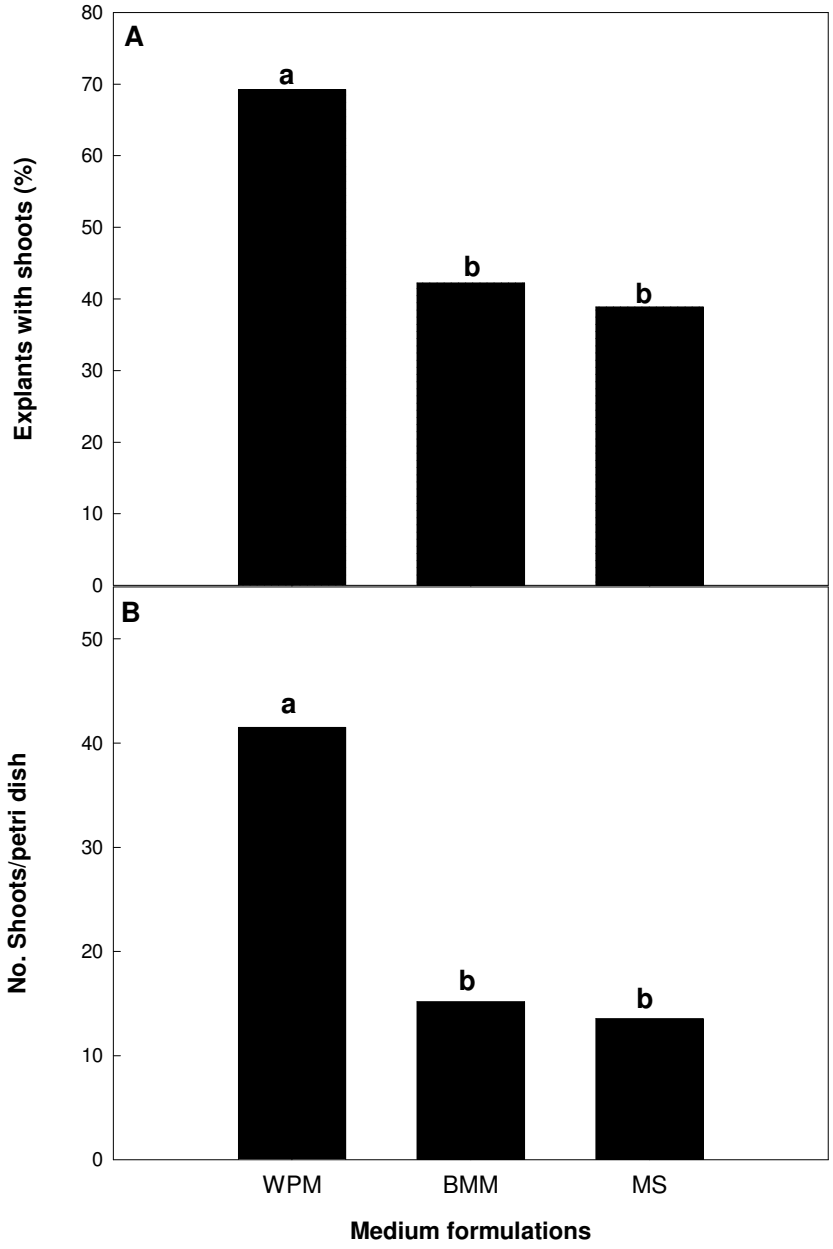

Figure 2.3. Effect of medium salts formulation [Woody Plant Medium (Lloyd and McCown, 1980), Blackberry Multiplication Medium (Reed, 1990), and MS salts and vitamins (Murashige & Skoog, 1962)] on the (A) Explants with shoots (%) and (B) Number of shoots per petri dish from regenerating 'Marion' leaf explant. Bars with same letters are not significantly different according to LSD, $P \leq 0.05$.

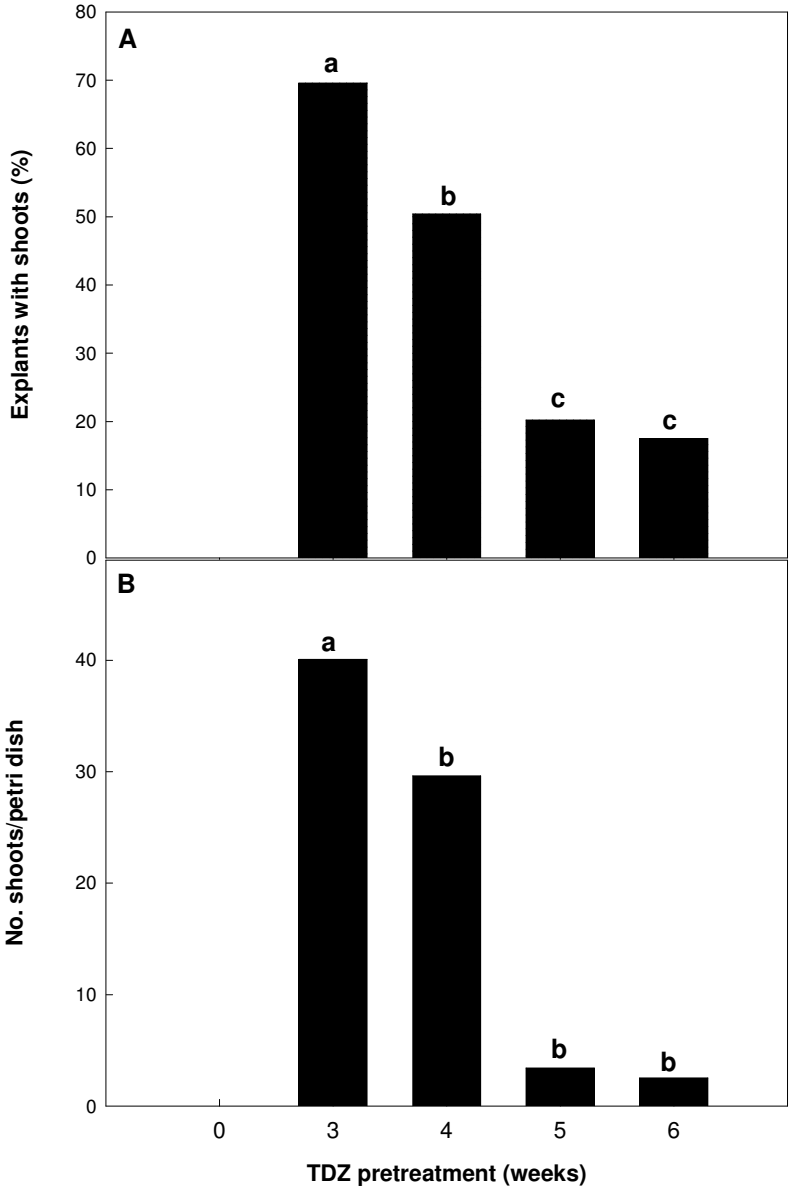

Figure 2.4. Effect of 0, 3, 4, 5 and 6 weeks of TDZ pretreatments on (A) Explants with shoots (%) and (B) Number of shoots per petri dish from regenerating 'Marion' leaf explants. Bars with same letters are not significantly different according to LSD, $P \leq 0.05$.

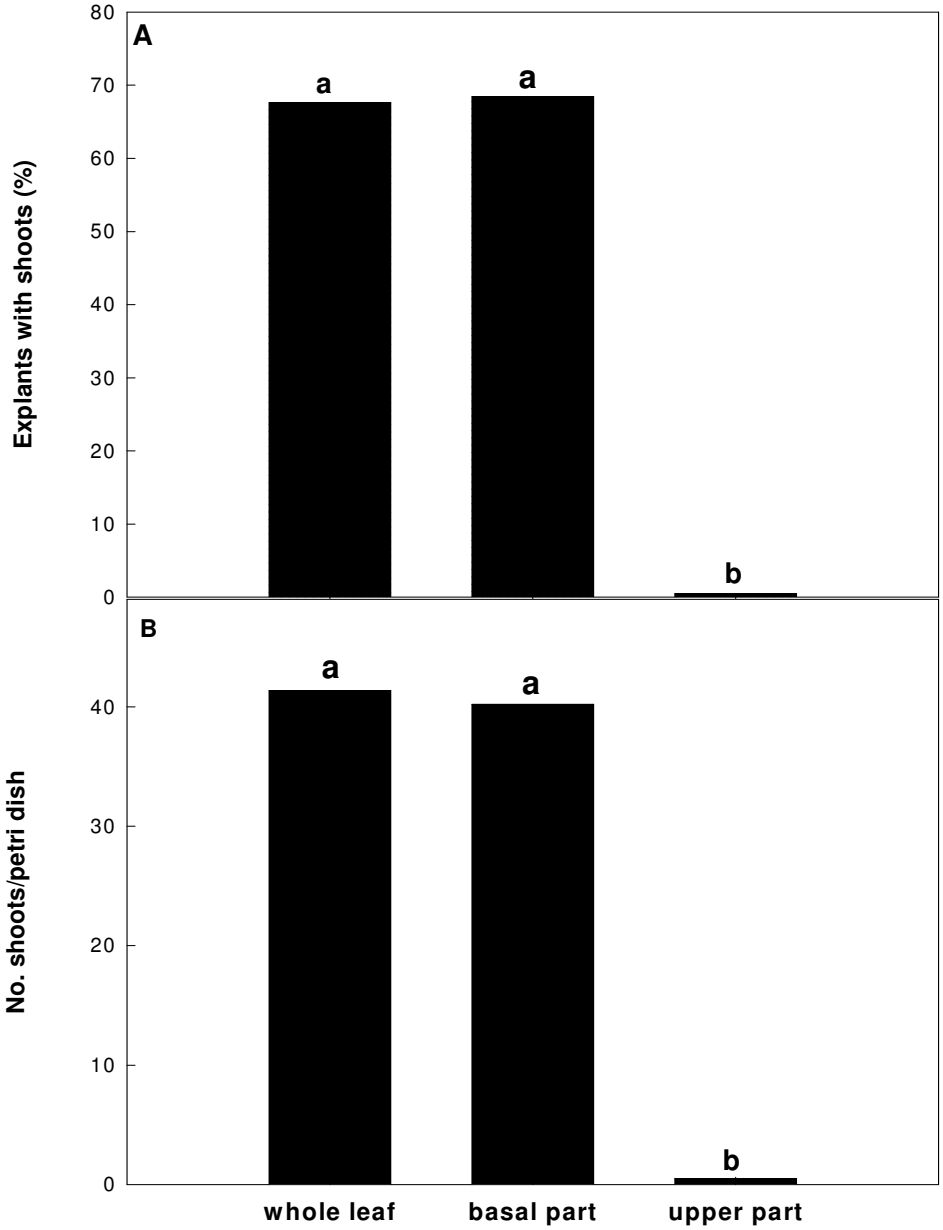

Figure 2.5. Effect of leaf integrity on (A) Explants with shoots (%) and (B) Number of shoots per petri dish. Bars with similar letters are not significantly different according to LSD, $P \leq 0.05$.

Chapter 3. *Agrobacterium tumefaciens*-mediated transformation of 'Marion' blackberry leaf explants: an assessment of factors influencing the efficiency of *uidA* gene transfer

3.1. Abstract

The development of an *Agrobacterium*-mediated transformation protocol for a species requires the identification and optimization of the factors affecting T-DNA delivery and subsequent plant regeneration. Having developed an efficient *in vitro* regeneration system for 'Marion' blackberry, we described here the optimization of major factors affecting T-DNA delivery. The expression of an intron-containing *uidA* gene coding for β-glucuronidase was used as an indicator of successful T-DNA delivery and GUS gene expression. A number of parameters that are known to influence genetic transformation were studied and optimized during the early steps of an *Agrobacterium tumefaciens*-mediated transformation procedure, as determined by the percentage of leaf explants showing *uidA* expression and the absence of leaf necrosis at the end of cocultivation. Factors produced significant differences in T-DNA delivery included: *A. tumefaciens* strain and plasmid vector, sonication treatment of the mix of inoculum and leaf explants, presence of iron chelator in the cocultivation medium, density of *Agrobacterium* cells during infection, duration of inoculation and cocultivation, presence and concentration of virulence gene inducer acetosyringone in the

inoculation medium and cocultivation medium, and presence, concentration and type of surfactant during inoculation. Using the optimized protocol, about 20% of the leaf explants showed blue staining 7 d after cocultivation. Putative transgenic shoots obtained during the optimization experiments, however, were all GUS negative, indicating the necessity of further evaluation of factors that lead to the recovery of real transgenic plants. While no transgenic 'Marion' plants were recovered, the investigation of factors that influence T-DNA delivery is an important first step in *Agrobacterium*-mediated transformation of 'Marion' leaf tissue.

3.2. Key words

Agrobacterium tumefaciens, transformation, acetosyringone, cocultivation, sonication, vacuum infiltration, *Rubus*, blackberry, GUS, kanamycin, hygromycin

3.3. Abbreviations

AS: Acetosyringone · GUS: β-Glucuronidase · MES: 2-(N-Morpholino) ethanesulfonic acid · *nptII*: Neomycin phosphotransferase II gene · *uidA*: β-glucuronidase gene from *Escherichia coli* · *hpt*: hygromycin phosphotransferase gene · CAT: chloramphenicol acetyl transferase · SAM, S-adenosylmethonine · SAMase: S-adenosylmethonine hydrolase · BA: benzyladenine · CaMV: cauliflower mosaic virus · GA_3: gibberellic acid · TDZ:

thidiazuron · MS: Murashige and Skoog medium · PCR: polymerase chain reaction · Tnos: nopaline synthase terminator · WPM: woody plant medium

3.4. Introduction

'Marion' is one of the most important blackberry cultivars in the world (Finn et al., 1997) but it has poor winter hardiness. Periodic, severe freeze damage, which happens at temperatures below -5°C, remains a characteristic of the industry and is the most serious limiting factor that prevents 'Marion' from having constant production year to year (Strik, 1992). To overcome this problem, improved cultivation techniques have been developed (Bell et al., 1995; Cortell and Strik, 1997) but a genetic solution would be much more satisfactory. Using hardy selections as parents to introduce hardiness into 'Marion' has been another major method to improve its winter tolerance, however, it is been a slow process and it is impossible to introduce only the trait of improved cold tolerance, which is a complex trait controlled by a multigene family, without simultaneously disrupting other existing desirable traits. Recombinant DNA technology is a powerful tool for the introduction of foreign genes into long-lived perennials and for fundamental studies of gene expression (Humara et al., 1999a). By using this technology, difficulties associated with traditional breeding technologies are minimized, and the time necessary to produce genetic changes in woody species is shortened (Cheliak and Rogers, 1990). Therefore, the application of genetic engineering could become a valuable complementation to classical plant breeding in

creating new cultivars with desirable new characters and widening prospects for genetic improvement of existing elite cultivars in many crops.

Many genes isolated from bacteria and plants have been reported to increase cold tolerance of plants via genetic transformation (Jaglo-Ottosen et al., 1998; Steponkus et al., 1998; Haake et al., 2002; Novillo et al., 2004; Parvanova et al., 2004). This progress has shed new light on developing improved ‘Marion’ with increased cold tolerance and studying the mechanisms of low temperature stress adaptation.

Development of an efficient regeneration method followed by an efficient system for transformation with useful genes is essential for utilizing the genetic transformation method to improve 'Marion's cold hardiness. An efficient organogenesis regeneration system for ‘Marion’ using leaf and petiole explants has been established (Meng et al., 2004). After establishing the regeneration protocol for ‘Marion’, we continued our studies to establish a method for transforming ‘Marion’. *Agrobacterium tumefaciens*-mediated transformation was chosen as the method because of its several advantages over direct gene delivery methods, such as particle bombardment, including integration of a well-defined DNA sequence, potentially low copy number, high co-expression of the introduced genes, and preferential integration into active regions of the chromosome, etc. (Birch, 1997; Gheysen et al., 1998). *Agrobacterium*-mediated transformation of *Rubus* species, including red raspberry, blackberry and their hybrids has been reported by some groups (Graham et al., 1990a and b; 1995; Hassan et al.,

1993; Mathews et al., 1995; Kokko and Kärenlampi, 1998). The β-glucuronidase (GUS) gene has been used most widely, including in *Rubus*, as a marker because of the simple staining process and easy detection of the expression in the transformed tissues (Martin, 2002). The objective of this study was to use a GUS reporter gene to optimize the conditions for T-DNA delivery into 'Marion' leaf cells from which hopefully whole plants could be regenerated, for future establishment of an efficient transformation system.

There has been only one report of successful blackberry transformation, in which McNicol and Graham (1989) described transforming ‘Loch Ness’ using *Agrobacterium* strain LBA4404 carrying the binary vector PBI121.X containing the *nptII* and GUS genes (no intron contained). In that paper, no details such as the *Agrobacterium* concentration and selection pressure were reported in the protocol, and the regeneration system was different than what we are using for ‘Marion’. The putative transformants were not obtained through antibiotic selection, and rooting under selection was not conducted either. Finally, while straight forward, we were not able to produce any transient expression of GUS gene in our preliminary experiments using their protocol. Transformation procedures cited above, for other *Rubus* genotypes, with different strains, vectors, types of selection and genotypes as well as regeneration systems were basically incomparable. Therefore, the transformation parameters that would possibly influence the efficiency of T-DNA delivery and

recovering of transformants into shoots, usually species- and tissue-specific, needed to be identified and optimized for ‘Marion’ leaf explants.

We report here a comprehensive and systematic examination of a series of factors possibly affecting transformation of ‘Marion’ based on the transient expression of GUS gene, including strain of *A. tumefaciens* and plasmid vector, *Agrobacterium* cell density, length of inoculation and cocultivation, inoculation and cocultivation conditions, selection agents, virulence gene inducer, and wounding procedures.

At the same time, we tried to recover transgenic shoots throughout the optimization procedure. While all the putative transgenic shoots in our experiments turned out to be negative, our optimized parameters will be valuable information for the further efforts to obtain transgenic ‘Marion’ blackberry.

3.5. Materials and methods

3.5.1 Plant materials and culture media

‘Marion’ blackberry stock cultures were provided by Sakuma Bros. (Mt. Vernon, Wash.) and Cedar Valley Nursery (Centralia, Wash.) and maintained on blackberry multiplication medium (Reed, 1990) with modification described by Meng et al. (2004) (hereafter referred to as BMM), which is a modified MS medium (Murashige and

Skoog, 1962) with vitamins, supplemented with 0.2 g chelated iron, 3% sucrose, 1.0 mg•L^{-1} BAP, 0.1 mg•L^{-1} IBA, and solidified with 0.2% phytagel, pH 5.7. BAP and IBA were sterilized by autoclave along with the medium at 121°C for 20 min at 105 kPa. All antibiotics were filter-sterilized through 0.22 mm Millipore filters (http://www.millipore.com/), and added to medium cooled to 50-60°C after autoclaving as above. Sixteen stem segments with 1-2 leaf buds on each and leaf blades excised were placed on BMM in GA7 magenta boxes (Magenta Corp., Chicago) and subcultured every 8 weeks. The stock cultures were maintained at 25°C under a 16 h photoperiod (photosynethethic photon flux (*PPF*) ≈ 50 µmol•m^{-2}•s^{-1}].

Leaf explants were excised from 3-week-old stock cultures unless otherwise stated. After removing the petiole, the remaining leaf blades were used in the subsequent experiments.

3.5.2. Effect of cefotaxime on the shoot organogenesis of 'Marion' leaf explants

With the aim of evaluating whether cefotaxime had a side effect on the shoot organogenesis of 'Marion' leaf explants, preliminary sensitivity tests were conducted. The stock solution of cefotaxime (50 g·L^{-1}) was made by dissolving cefotaxime powder (Claforan®, Roussell S.A.) in sterile distilled water and filter sterilized (ProX 0.22 µm membrane). The non-transformed leaf explants were placed on the regeneration medium (2.3 g WPM, 5 µM BA, 0.5 µM IBA, 30 g sucrose, pH 5.7)

containing different concentrations of cefotaxime at 0, 250, 500, 750, or 1000 mg·L^{-1}. After 3 weeks of culture, the regeneration efficiency was evaluated. The concentration that did not affect the regeneration efficiency would be used in the selection medium to recover transgenic shoots.

3.5.3. Effect of selective agents on the shoot organogenesis of 'Marion' leaf explants

Sensitivity tests were conducted to assay the resistance threshold of non-transformed 'Marion' leaf explants to kanamycin and hygromycin. The stock solution of kanamycin (50 g·L^{-1}) was made by dissolving kanamycin powder (Sigma, St. Louis, Mo.) in sterile distilled water and filter sterilized. Hygromycin stock solution (50 g·L^{-1}) was from Sigma Co. (St. Louis, Mo.). These stock solutions were added to the cooled media (about 50-60°C) after autoclaving. The non-transformed leaf explants were placed on regeneration medium supplemented with 250 mg•L^{-1} cefotaxime and either kanamycin (Sigma, St Louis, Mo.) at 0, 10, 20, 30, 40, and 50 mg•L^{-1} or hygromycin (Sigma, St. Louis, Mo.) at 0, 5.0, 7.5, 10, and 15 mg•L^{-1} . After 3 weeks of culture, the resistance threshold of non-transformed leaf explants to the selection agents was evaluated based on tissue browning and lethality. The lowest concentration of either antibiotic that completely inhibited the regeneration would be used in the selection medium to recover transgenic shoots.

3.5.4. Optimization of rooting condition under selection

When shoots regenerated from normal regeneration medium were taller than 0.5 cm they were transferred to rooting medium (4.43 g MS, 30 g sucrose, 0.2 g Sequestrene iron 138, 250 mg cefotaxime, per liter, pH 5.7) supplemented with either kanamycin at 0, 15, 25, 35, 45, 50 mg•L^{-1} or hygromycin at 0, 5, 7.5, 10, 15 mg•L^{-1}. Medium containing no selective agent was the control. The threshold concentration that completely inhibited rooting was used for examining whether or not the putative transgenic shoots were real.

3.5.5. *Agrobacterium tumefaciens* bacterial strains and Ti plasmid vectors

Agrobacterium tumefaciens was used as host for all the plasmids. To evaluate the infectivity of *A. tumefaciens* strains and plasmid vectors in the transformation of 'Marion', five *A. tumefaciens* strains of different opine groups including AGL-1 (Lazo et al., 1991), EHA101 (Hood et al., 1986), agropine strain EHA105 (Hood et al., 1993), octopine strain LBA4404 (Hoekema et al., 1983), and nopaline strain C58 (Zambryski et al., 1983) in conjunction with seven plasmids pTOK233 (Hiei et al., 1994), pIG121-Hm (Hiei et al., 1994), pGiPTV-HPT, and pTOK47 (Jin et al., 1987)/pGPTV-HPT, pCAMBIA1305.1 (Centre for the Application of Molecular Biology to International Agriculture - Canberra, Australia), and p35SGUSINT (Vancanneyt et al., 1990) were used. Eight gene constructs from the combination of the above strains and plasmids,

each containing an intron-*uidA* gene, including AGL-1 (pIG121-Hm), AGL-1 (pTOK233), EHA101 (pIG121-Hm), EHA105 (pCAMBIA1305.1), EHA105 (pGiPTV HPT), EHA105 (pTOK47/pGiPTV HPT), LBA4404 (pTOk233), and C58 (p35SGUS INT), were screened. For the subsequent optimization experiments, the best strain and plasmid combination obtained in this experiment would be used to further evaluate other factors influencing 'Marion' leaf explant transformation.

3.5.6. *Agrobacterium* culture

Glycerol stocks of each *A. tumefaciens* strain were streaked onto solid YEP medium (10 g Bacto-yeast extract, 10 g Bacto-peptone, 5 g NaCl, per liter of medium), pH 7.0, containing 50 mg•L^{-1} hygromycin and/or 50 mg•L^{-1} kanamycin for 2-3 d at 27°C in the dark. A single colony for each *Agrobacterium* strain was suspended in 5 ml liquid YEP medium containing 50 mg•L^{-1} kanamycin and/or hygromycin (50 mg•L^{-1}) and incubated at 250 rpm on a rotary shaker at 27 ºC for 18-20 hours. 100-ml cultures were started with 2 ml of the preceding 5 ml at the same condition. Cultures were then centrifuged at 3,000 rpm for 20 minutes to yield a bacterial pellet. For the initial experiment to evaluate the best *Agrobacterium* strain and plasmid combination, the pellet was resuspended in *Agrobacterium* induction medium [2.3 g WPM, 10 g glucose, 5 µM BA, 0.5 µM IBA, 100 µM acetosyringone (Sigma, St. Louis, Mo.), per liter of medium, pH 5.2] to an OD_{600}=1.0 [acetosyringone was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions and then added to the medium after

autoclaving; the pH of the media was adjusted to 5.2 before autoclaving]. Bacterial suspensions were prepared immediately prior to use.

3.5.7. *Agrobacterium* inoculation

Agrobacterium suspensions supplemented with the optimized concentration of acetosyringone were used for inoculation at the optimized density. Leaf explants were transferred into 125 ml flasks containing inoculum, usually allowing 10 ml of inoculum per 50 explants, ensuring that all explants were completely submerged. After adding the explants, the flasks were gently shaken at 25 rpm at room temperature for 1 minute as the initial length of inoculation (for the experiments optimizing the length of inoculation, 0.5, 1, 3, 5, or 10 minutes was applied, and the optimized length will be used for the subsequent experiments). Inoculum was then pipetted out, and the infected explants were removed from the tubes, placed on a sterile Whatman no. 1filter paper to remove excess bacteria and then placed onto cocultivation medium [2.3 g WPM, 10 g glucose, 20 g sucrose, 0.25 g MES, 5 µM BA, 0.5 µM IBA, 0.2 g Sequestrene iron 138, 100 µM acetosyringone (initial concentration), per liter of medium, pH 5.2].

3.5.8. Sonication

To determine the effect of sonication on T-DNA transfer and regeneration efficiency, explants were transferred into 50 ml falcon tubes containing inoculum at the optimized density or resuspension medium without *Agrobacterium* at the ratio of 10 ml to 50 explants, respectively. The experiments were carried out in a stainless steel ultrasound bath (Elma Inc., Model D-78224 Singen/Htw.; frequency 50/60 kHz, power 300 W), an ultrasound sonicator that has adjustable power output. The 50 ml falcon tubes were capped and immersed into the bath sonicator with the lower part of the tube touching the bottom of the bath, and it was subjected to sonication to investigate the combination effect of duration (0", 15", 30", 1', 2', and 5') and percentage of power output (20, 40, 60, 80, 100, 120, or 140%) on T-DNA delivery and regeneration efficiency. After being sonicated, leaf explants were transferred to flasks and shaken for 1'. The optimized sonication treatment was determined by comparing its effect on T-DNA delivery and leaf health, which was used for the subsequent experiments.

3.5.9. Effect of thiol compounds in the inoculation medium on T-DNA delivery

To investigate the effect of thiol compounds in the *Agrobacterium*-mediated T-DNA delivery into 'Marion' leaf explants, L-cysteine (Sigma, St. Louis, Mo.) and Sequestrene Fe 138 (Novartis, Greensboro, N.C.) were first added to the inoculation medium, at different concentration levels. L-cysteine was dissolved in dimethyl sulfoxide and Sequestrene Fe 138 in sterile distilled water to make stock solutions and then added to the medium after autoclaving at 0, 100, 200, or 400 $mg \bullet L^{-1}$, respectively.

The same concentrations of both compounds were then added to the cocultivation medium, respectively, to investigate the effect of their presence during cocultivation on T-DNA transfer and leaf health. The optimized concentration of the better of the two thiol containing compounds, if any, was used in the subsequent experiments.

3.5.10. *Agrobacterium* cell density and length of inoculation

To evaluate the optimum *Agrobacterium* cell density and length for inoculating 'Marion' leaf explants, *Agrobacterium* pellet was resuspended in *Agrobacterium* induction medium (2.3 g WPM, 20 g sucrose, 10 g glucose, 5 µM BA, 0.5 µM IBA, 100 µM acetosyringone, per liter of medium, pH 5.2) to an OD_{600}=0.1, 0.5, 1.0, 2.0, or 2.5 for 0.5, 1, 3, 5, and 10 minutes, respectively. The optimized density and inoculation time, determined by the T-DNA delivery and by the absence of tissue necrosis, will be used for future experiments.

3.5.11. Acetosyringone concentration

In the initial protocol, 100 µM acetosyringone was added to the inoculation medium and cocultivation medium. To evaluate if acetosyringone promotes T-DNA delivery and optimize its concentration during inoculation and cocultivation, acetosyringone was first added in the *Agrobacterium* suspension supplemented at 0, 50, 100, 200, 400, 600, or 800 µM. The optimized concentration was applied in the inoculation medium

to optimize its concentration in the cocultivation medium by adding acetosyringone at 0, 50, 100, 200, 400, 600, or 800 µM. Having its concentration optimized in both media, acetosyringone would be used in the rest of the experiments accordingly.

3.5.12. Surfactants in the inoculation medium

Surfactant Silwet (Sigma, St. Louis, Mo.) and Tween 20 (Sigma, St. Louis, Mo.) were added in the inoculation medium at 0.01, 0.1, 0.5, or 1.0% [v/v], respectively, to evaluate if surfactant would increase T-DNA delivery and, if it would, identify the optimum type and concentration, which would be used in the subsequent experiments.

3.5.13. Vacuum infiltration

The effect of vacuum infiltration during inoculation on T-DNA delivery and leaf condition were evaluated. Before shaking, the inoculation medium containing leaf explants was vacuumed using 125 ml vacuum flasks containing the *Agrobacterium* suspension by an in-house vacuum (24-27 inch Hg). Batches of 30-40 leaf explants were transferred to a vacuum infiltration flask containing 10 ml of *A. tumefaciens*. Four vacuum infiltration treatments were employed by applying vacuum to the inoculum for 0.5, 1, 2, 5, or 10 minutes and quickly released. Explants kept in *Agrobacterium* suspension without a vacuum were used as control. The inoculum was then transferred to regular 125 ml flasks for sonication treatment and shaking.

3.5.14. Cocultivation

The cocultivation was performed as in the initial protocol for 3 d in the dark at 25±2°C except for the in the experiment optimizing cocultivation length, where 1, 2, 3, 4, 5, and 6 d of cocultivation treatment were compared. The optimized length will be used in the rest of experiments. After cocultivation, the explants were washed four times with sterile distilled water and one time with sterile wash solution (2.3 g WPM, 30 g sucrose, 5 µM BA, 0.5 µM IBA, pH 5.7, per liter of medium) containing filter-sterilized cefotaxime (500 $mg \cdot L^{-1}$) (Himedia, India), blotted dry on sterile filter paper, and transferred to (1) selection medium (2.3 g WPM, 30 g sucrose, 5 µM BA, 0.5 µM IBA, pH 5.7, per liter of medium) containing optimized concentration of filter-sterilized cefotaxime to prevent *Agrobacterium* growth and appropriate selective agents (hygromycin or kanamycin at optimized concentration) to optimize T-DNA delivery or obtain transgenic shoots, or (2) regeneration medium containing cefotaxime without selective agents as control. A portion of the explants in the selection medium was collected for GUS assay 7 d later, and the remaining portion was subcultured every 2 weeks to obtain transgenic shoots.

3.5.15. Selection and recovery of plantlets

After cocultivation for 3 d (initial time, and the length of cocultivation were optimized and used in the subsequent experiments), the explants were transferred to selection medium (2.3 g WPM, 5 µM BA, 0.5 µM IBA, 30 g sucrose, 250 mg•L^{-1} cefotaxime, 0.2 g Sequestrene iron 138, pH 5.7) kept continuously in the dark for 4 d. From the 5th day, the culture was placed under 16 h photoperiod. Except for the experiment to identify the best combination of *Agrobacterium* strain and plasmid vector, in which no explants were used for obtaining transgenic shoots, a portion of the culture was used for histochemical β-Glucuronidase (GUS) assay 1 week after the end of cocultivation, and the remaining portion of the explants were used for obtaining transgenic shoots and were subcultured every 2 weeks. When the putative transgenic shoots were taller than 0.5 cm, they were transferred to the rooting medium (4.43 g MS, 30 g sucrose, 0.2 g Sequestrene iron 138, pH 5.7) containing selection agents. The cultures were maintained under a 16 h photoperiod (15 mmol·m^{-2}·s^{-1}) at 25±2°C.

3.5.16. Histochemical β-Glucuronidase (GUS) assays

The expression of the *uidA*-intron gene in transformed leaf explants was assayed as described by Jefferson et al. (1987) with some modifications. 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronide (X-Gluc, Sigma, St. Louis, Mo.) was used as a substrate. Leaf explants were incubated in 50 ml falcon tubes overnight at 37°C in a GUS assay mix (1 mM X-Gluc in 50 mM phosphate buffer pH 7.5, supplemented with 10 mM EDTA and 0.1% Triton X-100). The GUS reaction was stopped by removing the GUS

assay mix and washing the explants with 70% ethanol until destaining was complete and the ethanol remained colourless. For the experiments optimizing T-DNA delivery, histochemical GUS assays were performed on leaf explants 7 d after the end of cocultivation. The washed explants were examined under a dissecting microscope for the number of explants producing blue spots. Non-transformed explants were used as controls. Explants showing at least one blue spot/section were scored as GUS expression. Leaf explants cut from the putative transgenic shoots were also examined for GUS expression to confirm real transgenic shoots.

3.5.17. Experimental design and statistical analysis

The tested parameters were evaluated stepwise during the optimization process, and conditions considered as optimal at one stage were used in the following step, which eventually resulted in the optimized conditions for foreign gene delivery into 'Marion' leaf explants. Means of percentage of the leaf explant surface covered with blue spots or sectors were calculated. Explants were allocated randomly to treatments and the Petri dishes or containers were organized in a completely randomized design with three replicates. Each experiment was conducted three times. For each treatment, the tabulated data were an average of about 60-100 explants from three independent sets of experiments with roughly same number of explants each. Data were analyzed using the SAS program Version 8.0 (SAS Institute, Cary, N.C.). A probability level of 5% (=0.05) was chosen for all statistical inferences. The analysis of variance and the Least

Significant Difference (LSD) method ($P \leq 0.05$) was used to detect differences among treatments.

3.6. Results and discussions

3.6.1. Effect of cefotaxime on the shoot organogenesis of 'Marion' leaf explants

Antibiotics such as cefotaxime and carbenicillin commonly are employed in *Agrobacterium*-mediated plant transformation to eliminate *Agrobacteria* after the gene transfer occurs because proliferation of *Agrobacteria* can reduce regeneration (Graham et al., 1990a; Hassan et al., 1993). For transformation of *Rubus*, the inhibition effect of cefotaxime and carbenicillin varied for different *Agrobacterium* strains (Graham et al., 1990a; Hassan et al., 1993). Cefotaxime was used in our experiments and sufficiently controlled *Agrobacterium* growth at 250 mg•L^{-1} in the selection medium. Fiola et al. (1990) and Sarma et al. (1995) reported that cefotaxime inhibited *Rubus* embryo growth and Sitka spruce somatic embryogenesis and development, respectively. Graham et al. (1990a) reported that cefotaxime greatly inhibited regeneration of *Rubus* even at the concentration as low as 50 mg•L^{-1}. However, according to Yepes and Aldwinckle (1994) it enhanced callus induction and growth in apple and *Antirrhinum majus* (Holford and Newbury, 1992) tissue culture.

To determine if cefotaxime had a negative effect on 'Marion' leaf regeneration and if the negative effect would require use some other kind of antibiotic, the regeneration

efficiency of ‘Marion’ leaf explants in a series of media supplemented with different levels of cefotaxime was examined. Cefotaxime did not affect shoot organogenesis at concentrations lower than 500 mg•L^{-1} either positively or negatively (Table 3.2). Regeneration efficiency was reduced dramatically when the concentrations of cefotaxime were higher than 750 mg•L^{-1}. Therefore, cefotaxime at 250 mg•L^{-1} to inhibit the overgrowth of the residual *Agrobacterium* colonies after cocultivation without compromising the regeneration efficiency of ‘Marion’ was used.

3.6.2. Optimization of the concentration of selection agents

In plant transformation systems, only a small fraction of the plant cells are transformed. A selection strategy to inhibit the growth of untransformed cells while allowing the growth, division, and regeneration of transformed cells, is essential to obtain transgenic shoots. Kanamycin and hygromycin have been used commonly in plant genetic engineering acting as selection agents. Kanamycin inhibits the growth of plant cells by binding to the 30S ribosomal subunit, thereby inhibiting initiation of plastid translation (Wilmink and Dons, 1993). Neomycin photosphotransferase II (*npt II*) gene from *E. coli* encodes neomycin phosphotransferase II, which is capable of phosphorylating aminoglycoside antibiotics including kanamycin, neomycin and geneticin (Fraley et al., 1986) and confers resistance on its host cells to these antibiotics. Hygromycin is an amino glycoside antibiotic produced by *Streptomyces hygroscopicus*, which inhibits protein synthesis by interfering with translocation and

causing mistranslation at the 80S ribosome (Bashir et al., 2004). The hygromycin phosphotransferase (*hpt*) gene from *E. coli* codes for a kinase that inactivates hygromycin through phosphorylation.

Fusing the antibiotic resistant genes with eukaryotic promoters has resulted in the development of vectors that permit selection for resistance to the according selection agents and hence suitable markers for plant transformation systems. The *npt* II and *hpt* selection systems are used widely in plant transformation because selection for resistance can be applied to isolated cells, callus, somatic embryos, tissue explants, and whole plants (Bashir et al., 2004; Fraley et al., 1986).

Because each of the constructs used have *nptII* (conferring resistance to kanamycin) and *hpt* (conferring resistance to hygromycin) genes as the selection markers in the T-DNA cassettes, kanamycin and hygromycin were the selection agents of choice. In theory, 'Marion' cells transformed with the *npt* II and *hpt* gene can detoxify the kanamycin and hygromycin in the selection medium, and remain alive, but non-transformed cells will die because they are highly sensitive to antibiotics and lack the phosphotransferase to detoxify these antibiotics. If too low a concentration of antibiotic is used, the non-transformed cells may overgrow and inhibit the growth and regeneration of transformed cells (Kapaun and Cheng, 1999). Using too high concentration of the antibiotic, however, not only kills the nontransformed cells, but also inhibits regeneration from transformed cells (Wilmink and Dons, 1993).

Therefore, it is necessary to optimize the concentration of these two antibiotics to be used for selecting transformed cells, to reduce or avoid the emergence of non-transformed shoots without compromising regeneration efficiency.

The effects of kanamycin and hygromycin on the regeneration of 'Marion' explants and on the rooting capacity of the regenerated shoots was examined and concentrations greater than 40 mg•L^{-1} kanamycin or 10 mg•L^{-1} hygromycin led to complete inhibition of shoot organogenesis from non-transformed 'Marion' leaf explants (Table 3.3). The shoots did not root under the surface of the rooting medium containing 35 mg•L^{-1} kanamycin or 7.5 mg•L^{-1} hygromycin (Table 3.4).

In *Rubus* transformation, kanamycin (Graham et al., 1990a; Graham et al., 1995; Mathews et al., 1995) and hygromycin (Mathews et al., 1995) have been used as selection agents, but Swartz and Stover (1996) indicated that transformed *Rubus*, compared to other genera, were not recovered easily when kanamycin was used as the selection agent. Graham et al. (1990a) reported that *nptII* was not a suitable marker through kanamycin selection for several *Rubus* genotypes including the red raspberry 'Autumn Bliss' and an SCRI selection (8242E6), the blackberry and raspberry hybrids 'Tayberry', 'Tummelberry', and 'Sunberry', and 'Loch Ness' blackberry, and therefore the transformants had to be screened solely by GUS assay. Graham et al. (1995) recovered only two transgenic plants from 800 internode inoculations of MD-ETCE-1 ('Black Satin' × 'Tayberry') using kanamycin at 10 mg•L^{-1}, and found that at

this level kanamycin did not reduce the number of transformed, kanamycin resistant, shoots formed. Mathews et al. (1995) found that *hpt* gene to be more efficient than *nptII* for recovering transformants in *Rubus*, and hygromycin gave clear cut-off points in arresting the growth of control raspberry tissues, compared to kanamycin. According to Martin's experience with *Rubus* transformation (2002), hygromycin selection system produced fewer escapes than kanamycin did.

We have tried to select transformants using kanamycin and hygromycin. We were not able to recover any putative transgenic shoots from *nptII* kanamycin system. According to Swartz and Stover (1996), in *Rubus*, transgenic shoots were recovered at concentration of 10-30 mg•L^{-1} and higher rates of kanamycin (> 30 mg•L^{-1}) reduced recovery of transformed shoots, primarily due to necrosis of surrounding non-transformed tissues. Therefore, using kanamycin to select transformants may not be a suitable method for 'Marion' leaf explants, because we had to use 40 mg•L^{-1} or greater concentration to inhibit regeneration of non-transformed cells and at this level of selection pressure, cell necrosis would lead to the failure of the regeneration of the transformed cells. By using *hpt* hygromycin selection system (10-20 mg•L^{-1}), we had no problem obtaining putative transgenic shoots. While the later negative results from GUS assay and rooting and final browning of tissue in the sustained selection medium showed that they were not real transgenic shoots, considering the high selection pressure, we excluded the possibility that they were escapes but proposed that this was the result of chimerism, which will be discussed in later sections. However, our results

indicated that, in agreement with those from the previous experiments in transformation of other *Rubus* genotypes, *hpt* hygromycin system was a better selection scheme than *nptII* kanamycin system for 'Marion' leaf explants.

3.6.3. *Agrobacterium tumefaciens* strains and Ti plasmid vectors

Of the various factors influencing frequency of *Agrobacterium*-mediated transformation, *Agrobacterium* strains and plasmids play one of the most important roles in the transformation process, as they are responsible not only for infectivity but also for the efficiency of gene transfer (Cheng et al., 1997; Bhatnagar and Khurana, 2003). *Agrobacterium* strains can be classified as octopine, nopaline and L, L-succinamopine types, depending on which opine synthesis is encoded for the T-DNA.

Agrobacterium strain has been shown to greatly influence the transformation success of *Rubus* (Martin, 2002). While among 35 strains of *A. tumefaciens* strains, A208, A281, ACH5, C58, and TR105 were found to be the most effective at forming tumors on leaf disks of ten red raspberry genotypes *in vitro* (Owens y de Novoa and Connor, 1992), de Faria et al. (1997) found that LBA4404, was the most efficient of seven *A. tumefaciens* strains. Transgenic *Rubus* plants have been obtained after transformation conducted by the *Agrobacterium* strains and plasmids like LBA4404/PBI121X (Graham et al., 1990b; 1995), C58/(pGV3850+pJIT106) and C58/(pGV3850+pJIT54) (Hassan et al., 1993), or C58C1/pGV2260 and EHA101/pEHA101 (Kokko and

Kärenlampi, 1998) with different transformation efficiency depending on the genotypes. The results of the previous experiments mentioned above underscores the importance of testing the various *A. tumefaciens* strain and plasmid combinations in developing a transformation system for a specific *Rubus* genotype under study.

The suitability of different strains harboring various plasmids for transformation of 'Marion' leaf explants was evaluated after the explants were incubated for 1 min at a bacterial density of OD600=1.0, followed by co-cultivation for 3 d and selection for 7 d. The explants were subjected to the GUS histochemical assay after washing. Among the eight strain and plasmid combinations, *A. tumefaciens* strain LBA4404 harboring superbinary vector pTOK233 was found to be the most efficient with respect to T-DNA delivery, with about 5% explants showing GUS expression, followed by EHA105 harboring pCAMBIA1305.1, pTOK47/pGiPTV HPT, and pGiPTV-HPT, respectively (Table 3.5). EHA101, C58 and AGL-1, regardless of what plasmids they harbor in this experiment, did not show any capacity to transfer T-DNA into leaf cells (Table 3.5).

LBA4404 is an octopine strain with Ach5 chromosomal background (Hoekema et al., 1983). EHA 101 derived from supervirulent wild-type strain A281 (Hood et al. 1986, 1993). EHA105, an agropine-type bacterium, is a hypervirulent bacterium derived from EHA101 whose oncogenic progenitor is the supervirulent wild type strain A281.

C58 is nopaline-type wild type strain. AGL1 is a derivative of EHA101 (Lazo et al., 1991).

Plasmid pTOK233 is a superbinary vector, a derivative of the superbinary vector pTOK162 (Komari, 1990), carrying additional copies of *vir* genes (*virB*, *virC*, and *virG*) from the supervirulent plasmid pTiBo542 and having the *nptII* (conferring resistance to aminoglycosidic antibiotics like kanamycin), *hpt* (conferring resistance to hygromycin) and intron-*uidA* genes in the T-DNA region, all of which were regulated by the CaMV35S promoter and nopaline synthase gene (nos) terminator. Because pTOK233 has an intron-interrupted *uidA* gene, the expression of *uidA* only occurs in transformed plant cells and tissues rather than in *A. tumefaciens* itself. Vector pGPTV contained ß-glucuronidase reporter gene (*uidA*) and two plant selectable marker genes *nptII*, *hpt* fused to the nopaline synthase (*nos)* promoter. The plasmid pCAMBIA1301 is a normal binary vector originating from the Center for Application of Molecular Biology to International Agriculture, Canberra, Australia (CAMBIA). It contains an intron-containing GUS gene to ensure detection of plant-specific glucuronidase expression, *hpt* gene for selection in plants, and the *nptII* gene, all of which are driven by the CaMV35S promoter. Plasmid pIG121Hm contains the *hpt* and *nptII* genes, both for plant selection, and an intron-containing GUS gene. Plasmid p35SGUSINT has the intron containing GUS reporter gene and *nptII* (nos-nptII-nos) as the selectable marker for kanamycin resistance. The GUS gene is controlled by the CaMV 35S promoter and polyadenylation signal, while the *nptII* gene is under control of the nos promoter and

termination signals. pGiPTV-HPT is constructed based on the binary pGPTV-HPT vector (Becker et al., 1992) with the addition of an intron into the *uidA* gene, under the control of the CaMV 35S promoter, to restrict the GUS expression in plant cells. With the addition of the plasmid pTOK47, a supervirulent plasmid containing the *virB*, *virC* and *virG* regions, pGiPTV-HPT is supposed to have increased virulence.

Consequently, *A. tumefaciens* strain LBA4404 harboring the superbinary pTOK233, with the highest T-DNA delivery efficiency among all the constructs tested, was then used as a model system to optimize different transformation parameters for high efficiency gene transfer to leaf explants of 'Marion'.

3.6.4. Effect of sonication treatment on T-DNA delivery and leaf explant health

Sonication has been reported to enhance the delivery of naked DNA into tobacco protoplasts (Joersbo and Brunstedt, 1990) and seedlings (Zhang et al., 1991). For *Agrobacterium*-mediated transformation, sonication treatment has also successfully enhanced the transformation of many target tissues in different plant species (soybean cotyledonary nodes by Meurer et al. [1998]; soybean embryogenic suspension culture by Trick and Finer [1997]; soybean immature cotyledons by Santarém et al.[1998]; stone pine cotyledons by Humara et al. [1999a; 1999b]; mature zygotic embryos of loblolly pine by Tang [2003]; shoot apices of sunflower by Weber et al. [2003]; black locust cotyledons by Zaragoza et al. [2004]). In the sonication-assisted

Agrobacterium-mediated transformation (SAAT) (Trick and Finer, 1997), the target tissues were subjected to ultrasound while immersed in an *Agrobacterium* suspension. The enhanced transformation rates were believed to be due to the energy released by cavitation (Frizzel, 1988), which causes large numbers of small wounds on the surface of and deep within the target tissues (Santarém et al., 1998) and which facilitates the infection by *Agrobacterium* (Trick and Finer, 1997). Furthermore, the wounding may induce the production of signal phenolic compounds (Stachel et al., 1985) and enhance the accessibility of putative cell-wall binding factors (Lippencott and Lippencott, 1969) to the bacterium. Santarém et al. (1998) found that the enhancement of transient GUS expression due to SAAT treatment was neither genotype specific among three soybean cultivars tested nor vector specific between two different binary plasmids.

The duration and intensity of sonication had dramatic effect on the transient expression of GUS in plant tissues (Santarém et al., 1998). Because sonic waves cause rupture of the cell walls, which allows more thorough *Agrobacterium* penetration into explant tissue, longer and higher power treatment of sonication may have greater effect, but an overly strong treatment results in immediate cell lysis (Joersbo and Brunstedt, 1992) and leaf explant damages, leading to lower regeneration efficiency. A certain degree of ultrasound treatment is needed, giving a moderate rupture of cell walls, which could be applied to improve transformation.

To determine if sonication can assist in *Agrobacterium*-mediated transformation of 'Marion' leaf explants and how long and how much intensity should be used, the effect of duration (0', 15″, 30″, 1′, 2′, 5′) and intensity (20%, 40%, 60%, 80%, 100%, 120%, 140% power) of sonication on GUS gene expression, in the presence (explants were sonicated in the inoculation medium) or absence (explants were inoculated after sonication in the liquid regeneration medium) of *Agrobacterium* was investigated. To evaluate the effect of sonication on the regeneration potential, part of the explants treated with sonication in the absence of *Agrobacterium* were transferred to regeneration medium and the shoot organogenesis results were compared with those from standard regeneration experiments (Meng et al., 2004).

We found that sonication treatment did not improve T-DNA delivery when the explants were not immersed in the inoculation medium, and at longer/higher energy treatment levels the T-DNA delivery efficiency was significantly reduced (Table 3.6). Regeneration was not affected by sonication when the treatment was 1) as short as 15″ regardless of the intensity and 2) when the intensity was less than 60% power output regardless of the treatment time up to 5′. At other treatment levels, generally speaking, the longer the treatment and the higher intensity applied, the more likely regeneration efficiency decreased, resulting in many calli rather than shoots formed. This was consistent with the previous observation that wounding in 'Marion' leaf explants caused callus formation and reduced direct shoot organogenesis (Meng et al., 2004).

When the leaf explants were treated with *Agrobacterium* without sonication, an average of about 5% of the leaf explants showed blue spots. A tremendous enhancement of GUS expression was observed when certain levels of sonication were applied along with *Agrobacterium* (Table 3.6). While many combinations of length and intensity (≥ 60%) of ultrasound significantly enhanced the T-DNA delivery, treatments ranging from 0.5' to 1′ at 60% power output gave the highest efficiency. Power output higher than 60% for certain length of treatment significantly increased the number of explants producing blue spots, but the extent of improvement was diminished. Some strong treatments (e.g. 80% power for ≥ 1', 100% - 140% power for ≥ 0.5', etc.) produced worse results than zero sonication treatment, probably due to the severe tissue disruption and death of explants.

While successful in many plant species and target tissues, using sonication to facilitate *Agrobacterium*-mediated transformation has neither been reported on leaf explants nor in *Rubus*. In the present study, 'Marion' leaf explants were very responsive to sonication, which is useful for *Agrobacterium*-mediated infection. The best results were obtained at the moderate levels of treatment (lower energy ultrasonic frequency and relatively short time). At 60% power output, 0.5-1 min ultrasound treatment doubled the T-DNA delivery efficiency to about 11% leaf explants showing blue spots. The average GUS expression obtained with 0.5- and 1-minute sonication treatments did not differ statistically, and in the absence of *Agrobacterium* neither affected regeneration potential. Whether or not the shoot organogenesis is affected when the

Agrobacterium is present is unknown. To minimize such possibility, we chose the 0.5' treatment for further experiments.

3.6.5. Effect of iron chelator and L-cysteine in the inoculation and cocultivation medium on T-DNA delivery and leaf explant health

We have found that sonication treatment significantly increased T-DNA delivery, but it seems that the level of improvement could have been limited by the tissue damage caused by longer and higher energy treatment.

Ultrasound treatment, vacuum infiltration, and even inoculation of *Agrobacterium* itself are all disruptive processes and could trigger hypersensitive responses in the plant tissue. As a result, phenolic compounds or enzymes are released causing oxidation-browning and, if not controlled, eventually tissue necrosis, both of which will dramatically reduce the T-DNA delivery and regeneration efficiency.

It has been reported that thiol compounds in iron chelator and L-cysteine inhibit wound- and pathogen-induced responses, through inhibiting the polyphenol oxidases and peroxidases, thereby increasing the capacity for *Agrobacterium*-mediated transformation (Olhoft and Somers, 2001; Olhoft *et al*., 2001). Therefore, the goal of this experiment was to investigate the role of thiol compounds such as Sequestrene (Fe-EDDHA) and L-cysteine in increasing the frequency of transformed ‘Marion’

leaves and protection of leaf tissue from the wound- and pathogen-induced responses.

L-cysteine and Sequestrene iron 138 were added to the inoculation medium and cocultivation medium, respectively, at 0, 100, 200, and 400 mg•L^{-1}, to investigate their effects step by step. In this experiment, in which ultrasound was applied for 0.5' at 60% power output, we wanted to see if the addition of thiol compounds would improve T-DNA delivery. It should be noted that 200 mg•L^{-1} Sequestrene iron 138 is present in the cocultivation medium all the time as a standard protocol because the cocultivation medium was made based on regeneration medium that contained 200 mg•L^{-1} Sequestrene iron 138 as nutrient source of iron. Therefore, except for studying the effect of Sequestrene iron 138 in the cocultivation medium, all the other three experiments were performed with Sequestrene iron 138 present in the cocultivation medium at 200 mg•L^{-1} .

The presence of L-cysteine either in the inoculation medium or cocultivation medium caused dramatic decrease in the frequency of transformed explants and severe tissue necrosis (Table 3.6). This indicated that ‘Marion’ leaf explants were sensitive to L-cysteine for some reason and it is not beneficial to use this compound in a transformation system. The presence of Sequestrene iron 138 in the inoculation medium did not improve T-DNA delivery either, when compared with the standard conditions, but its presence was not detrimental to leaf explants until the concentration was raised to 400 mg•L^{-1}. When Sequestrene iron 138 was not present in the

cocultivation medium, the frequency of transformed explants was dramatically reduced and tissue necrosis increased, which indicated that this compound was indispensable in the cocultivation medium. The optimum concentration of Sequestrene iron 138 was 200 mg•L^{-1} (Table 3.6), but it is not clear whether the effect was due to its nutritional value as iron source or the anti-browning effect of the thiol group. We are inclined to believe that the latter was true because the cocultivation was only 3 d and malnutrition symptoms should not be expressed that quickly. While it is hard to explain why the Sequestrene iron 138 did not show positive effect when it is in the inoculation medium, such phenomenon was consistent with the previously reported results, in which L-cysteine and other thiol groups containing compounds improved transformation efficiency only when they were present in the cocultivation medium (Olhoft and Somers, 2001; Olhoft *et al*., 2001).

3.6.6. Effect of *Agrobacterium* cell density and inoculation time on T-DNA delivery and leaf explant health

It has been observed that higher *Agrobacterium* densities yielded more T-DNA delivery events and therefore increased the transformation frequency in many plant species (Confalonieri et al., 1994; Drake et al., 1997; Cheng et al., 2004). At the same time, however, the detrimental effects of the *Agrobacterium* infection on explants at high density were also reported, which was believed to be related to the hypersensitive response of plant cells as part of plant defense against pathogens (Humara et al., 1999a;

Orlikowska et al., 1995). Such an effect caused a reduction in the number of explants forming buds during the bud induction period and ultimately resulted in lower recovery of the transformed cells into transgenic shoots (Orlikowska et al., 1995).

The detrimental effect on explants related to *A. tumefaciens* infections was diminished by decreasing the bacterial density, leading to an increase in the number of explants showing regenerative processes (Humara et al., 1999a). For some recalcitrant species, however, a higher density of *Agrobacterium* is necessary to improve transformation frequency (Cheng et al., 2004). In such cases, a short inoculation time was usually applied to reduce the detrimental effect of the *Agrobacterium* on explants (Zhao et al., 2000 and 2001).

A factorial experiment was set up to optimize *Agrobacterium* cell density and the length of inoculation for transforming 'Marion' leaf explants. The combinations of six different bacterial densities (OD_{600}=0.1, 0.5, 1.0, 1.5, 2.0, or 2.5) and five inoculation durations (0.5, 1.0, 3.0, 5.0, 10.0 minutes) were tested with 'Marion' leaf explants. The treatment producing the highest T-DNA delivery frequency without tissue necrosis and *Agrobacterium* overgrowth during cocultivation would be considered as the best condition for inoculation. Uninfected control explants did not show GUS expression (Table 3.8). *Agrobacterium* at the density of OD_{600}=0.1 or 0.5 did not produce any T-DNA delivery, regardless of the length of inoculation up to 10 minutes. Increasing the OD_{600} to between 1.0 and 2.0 caused substantial increases in the

number of explants producing blue spots. *Agrobacterium* density higher than 2.0, however, resulted in no increase in the percentage of explants showing GUS expression when inoculation time was up to 5 minutes, and significant decrease when the time was raised to 10 minutes, probably due to the detrimental effect of bacterium overgrowth and tissue necrosis. Increasing the inoculation time to 3 minute increased the GUS gene transfer when the optical density of *Agrobacterium* was at 2.0, but a longer inoculation time (5 minutes) did not give further improvement in T-DNA delivery and bacterium overgrowth and tissue necrosis were observed. Furthermore, when the inoculation time was increased to 5 and 10 minutes, all explants showed necrosis when the optical density was 0.5 or higher. While increasing the inoculation time to 3 minutes resulted in significant improvement of GUS expression, even longer treatment for 5 minutes had no more positive effect and for 10 minutes the T-DNA delivery frequency was significantly reduced, which was concurrent with the occurrence of *Agrobacterium* overgrowth and tissue necrosis.

In the research of *Agrobacterium*-mediated transformation of *Rubus*, leaf and petiole tissues inoculated in an *Agrobacterium* suspension with an optical density of 0.5-0.6 at 600 nm for 30-60 minutes were used to successfully transform red raspberries (Mathews et al., 1995). The *Agrobacterium* density in that report seemed too low for the best T-DNA delivery and inoculation time too long to keep explants healthy for transformation of ‘Marion’ leaf explants.

Consequently, without sacrificing leaf health the best percentage of uidA-expressing explants was found when the leaf explants were inoculated for 3 minutes in the *Agrobacterium* suspension with a density (OD_{600} nm) of 2.0.

3.6.7. Effect of acetosyringone concentration in the inoculation and cocultivation medium on T-DNA delivery and leaf explant health

Most dicot tissues, when wounded, exude phenolic compounds that have been found to be essential for induction of the virulence genes present in Ti plasmids (Stachel et al., 1985; Shimoda et al., 1990) whose protein products are directly involved in T-DNA processing from the Ti plasmid and the subsequent transfer of T-DNA from the bacterium to plant (Veluthambi et al., 1988). The presence of these virulence gene inducers such as 4-acetyl-2,6-dimethoxyphenol (acetosyringone) in the inoculation and/or cocultivation media was crucial for efficient T-DNA delivery mediated by *A. tumefaciens* for various types of explants in many plant species (Atkinson and Gardner, 1991; Lulsdorf et al., 1991; James et al. 1993; Cheng et al., 1997; Amoah et al., 2001; Henzi et al., 2000; De Clercq, 2002). There were also reports, however, suggesting that acetosyringone was not necessary for genetic transformation for certain explants (De Kathen and Jacobsen, 1990; Enriquez-Obregon et al., 1998) or *Agrobacterium* strains (De Kathen and Jacobsen, 1990; Nadolska-Orczyk and Orczyk, 2000). In *Rubus*, a positive effect of acetosyringone on *Agrobacterium*-mediated transformation was suggested in two reports (Hassan et al., 1993; Mathews et al., 1995).

In our standard transformation protocol, the *Agrobacterium* inoculation and cocultivation medium were both supplemented with 100 µM acetosyringone. Experiments were conducted to investigate whether this virulence gene inducer is needed in either medium to obtain *Agrobacterium*-mediated gene transfer to 'Marion' leaf explants and whether the efficiency can be improved. Acetosyringone was first added in the inoculation medium at different concentrations (0, 20, 50, 100, 200, 400, 600, and 800 µM), with a constant 100 µM in the cocultivation medium. Then the same range of concentrations in the cocultivation medium were examined, keeping the optimum concentration determined from the previous experiment in the inoculation medium.

The presence of the inducer acetosyringone in either inoculation or cocultivation medium was indispensable for transformation (Table 3.9). In the inoculation medium, no gene transfer events were observed at 0 and 50 µM acetosyringone. The percentage of GUS expressing explants significantly increased when 100 µM was supplemented in the inoculation medium, but it did not increase when acetosyringone concentration was increased up to 800 µM. Such results indicated that the *Agrobacterium* needs to be preinducted for its virulence to function and the preinduction level could be improved to the maximum level with increased acetosyringone concentration. A phytotoxic effect of acetosyringone on plant tissue, however, was also reported (Godwin et al., 1991). In our experiments, short exposure of high concentration of acetosyringone up to 800 µM did not cause visible damage to 'Marion' leaf explants

because no necrosis of leaf tissue was found either right after inoculation or over time in cocultivation medium.

During the cocultivation stage, the presence of acetosyringone with 100 µM or higher concentration was essential for transformation as well (Table 3.9). Acetosyringone at 600 µM concentration resulted in production of the maximum number of explants with GUS expression. A phytotoxic affect was observed with the 800 µM treatment and may contribute to the dramatic decrease in the percentage of leaf explants showing blue spots observed at these higher concentrations.

Most transformation research reported the use of acetosyringone at no more than 200 µM. Hassan et al. (1993) reported that 20 µM acetosyringone significantly improved number of crown galls formed in transformation of 'Cherokee' x 'VSPB-1' blackberry. Mathews et al. (1995) used 50 µM acetosyringone to successfully transform several raspberry cultivars, in which experiment the *Agrobacterium* cell density was OD_{600} = 0.5-0.6. In our report, the preinduction of the *Agrobacterium* with acetosyringone in inoculation required the acetosyringone concentration to be 100 µM and 600 µM in the cocultivation medium, neither dispensable for an optimum induction of virulence genes. Besides the possibility that 'Marion' is very recalcitrant to *Agrobacterium*, the high density of *Agrobacterium* (OD_{600} = 2.0) used in our experiments could be another reason for the necessity of a high level acetosyringone. Such optimum conditions were then adopted in the further optimization experiments.

3.6.8. Effect of surfactant in the inoculation medium on T-DNA delivery efficiency and leaf explant health

The surfactant Silwet L-77 has been shown to improve the *Agrobacterium*-mediated transformation efficiency of floral-dip methods in *Arabidopsis* (Clough and Bent, 1999), *Muscari armeniacum* embryogenic cultures (Suzuki and Nakano, 2002), and 'Bobwhite' spring wheat (Cheng et al., 1997). A similar result was reported in *M. armeniacuma* embryogenic cultures when Tween 20 was added to the inoculation medium, in which T-DNA delivery efficiency was doubled (Wu et al., 2003). The enhancement of T-DNA delivery by surfactants was believed to result from the reduction in surface tension and/or eliminating certain substances that inhibit *Agrobacterium* attachment, therefore allowing better *Agrobacterium* penetration into the plant tissues (Cheng et al., 2004).

The effect of two types of surfactant, Silwet L-77 and Tween 20, in the inoculation medium, was evaluated based on T-DNA delivery efficiency as well as the explant mortality. Leaf explants were inoculated in the presence of 0, 0.1, 0.5, and 1.0% Silwet L-77 and Tween 20, respectively.

Tween 20 was found to have a significant positive effect on the GUS expression. T-DNA delivery was improved at 0.1% and 0.5% of Tween 20 (Table 3.10), but tissue

browning was observed in the latter treatment. Low concentrations of Silwet L-77 (0.01%) did not produce any positive effect. At higher concentrations, Silwet L-77 appeared to be too toxic to the 'Marion' leaf tissues even when only a small amount (0.1%) was added to the inoculation medium, and T-DNA delivery was dramatically reduced accordingly (Table 3.10). At concentrations greater than 0.5%, there was severe tissue browning and at 1% no explants survived.

Based on this result, Tween 20 at a concentration of 0.1% was used routinely in the subsequent experiments.

3.6.9. The effect of vacuum infiltration on T-DNA delivery frequency

The use of vacuum infiltration to assist *Agrobacterium*-mediated transformation was first reported by Bechtold et al. (1993), and followed and/or modified by Chang et al. (1994), Katavic et al. (1994), Richardson et al. (1998), and Clough and Bent (1999), for transforming *Arabidopsis thaliana in planta*. Other plants such as wheat (Amoah et al., 2001), petunia (Tjokrokusumo et al., 2000), rice (Datta et al., 2000; Dong et al., 2001), sunflower (Hewezi et al., 2002), and apricot (Petri et al., 2004) have also been transformed by this method. Vacuum infiltration was also used to enhance transformation frequency of detached tissues in *Agrobacterium*-mediated systems (Rossi et al., 1993; Kapila et al., 1997; Schob et al., 1997; Llave et al., 2000; Qing et al., 2000; Trieu et al., 2000).

Applying a vacuum to plant organs in the presence of *Agrobacterium* removes intercellular fluids and air, which are replaced by *Agrobacterium* when the vacuum is released. Our experiment was thus designed to investigate the effects of the duration of vacuum treatments on *Agrobacterium*-mediated T-DNA delivery into 'Marion' leaf explants and tissue health by using the in-house vacuum equipment. Six vacuum infiltration treatments were employed. The differential parameter was the elapsed time (0, 0.5, 1, 2, 5, and 10'). Explants kept in *Agrobacterium* suspension without a vacuum were used as controls.

Vacuum infiltration treatment did not significantly improve the percentage of transformed explants as compared with a control (Table 3.11). Leaves were greatly affected by vacuum treatment longer than 5 minutes and, as a result, many explants developed severe necrosis and died. Therefore, this treatment was not adopted in our transformation system.

3.6.10. Length of cocultivation on T-DNA delivery and leaf explant health

The cocultivation time is an important factor for transformation efficiency. While 2 d cocultivation has been routinely used in some transformation protocols (Holford et al., 1992; Muthukumar et al., 1996), prolonged cocultivation periods of 3 to 4 d seemed to have been more commonly used (Cao et al., 1998; Jia et al., 1989; Dong et al., 1991; Ducrocq et al., 1994; Mourgues et al., 1996). A longer duration of cocultivation

usually yielded more efficient T-DNA delivery on various tissues or cells, but it frequently resulted in *Agrobacterium* overgrowth and more cell damage was observed, therefore subsequently reducing the regeneration potential of transformed shoots (Cervera et al., 1998; Cheng et al., 1997; Clercq et al., 2002; De Bondt et al., 1994 and 1996; Jin et al., 1999).

For *Rubus*, the length of cocultivation has a significant effect on gall formation. Hassan et al. (1993) found that 3 or 4 d of cocultivation, with all strains except for A281, resulted in significantly more gall formation than one or two day treatments. In the successful transformation of *Rubus*, cocultivation was reported as either one day (Graham et al., 1990b), 2 d (Mathews et al., 1995), 2 to 4 d (Kokko and Kärenlampi, 1998), or 3 to 4 d (Hassan et al., 1993).

We compared the percentage of GUS positive explants from six cocultivation times (0, 1, 2, 3, 4, 5 and 6 d) to find the best length for transformation without compromising the health of the leaf explants (Table 3.12). Explants were cultured for 1-6 d on a co-cultivation medium, respectively, and then *Agrobacteria* were killed or inhibited with antibiotic. Having no cocultivation and 1 d of cocultivation resulted in no GUS expression (Table 3.12). Dramatically less GUS expression was observed following 2 d (about 9% leaf explants showing blue spots) than 3 d of cocultivation (about 21%). The highest GUS expression was observed from the 3 or 4 d of cocultivation, but *Agrobacterium* overgrowth was found in the 4 d of cocultivation. GUS expression was

not further improved after 5 or 6 d, and excessive *Agrobacterium* growth resulted in browning of tissues and death of some explants.

Therefore, a 3 d cocultivation was considered the optimum and was used in the subsequent experiments.

3.6.11. Recovery of transgenic shoots

Throughout the whole process in our optimization experiments, we tried to recover transgenic shoots.

At the beginning, we used selection medium containing 35 mg•L^{-1} kanamycin or 7.5 mg•L^{-1} hygromycin. All we got from the selection medium containing kanamycin was callus rather than shoots. Therefore, in the subsequent selection effort, we used hygromycin as the selection agent. When regenerated shoots were about 0.5 cm long, they were removed from the explants and transferred to rooting medium containing hygromycin. When the putative transgenic shoots were taller than 1 cm, one leaf blade would be cut from the plant for GUS analysis. Unfortunately, no GUS positive leaf was found and no plantlet in the antibiotic containing rooting medium formed roots, which indicate the false positive nature of all regenerants. We raised the concentration of hygromycin to 10 mg•L^{-1} to reduce the chance of the occurrence of escapes, but the putative transgenic shoots were still GUS negative. Such results indicated that

regeneration of escapes was not directly due to inefficient antibiotic selection pressure because our preliminary experiments showed that hygromycin at concentration greater than 7.5 mg•L^{-1} completely inhibit regeneration. Instead, the protection of non-transformed cells from the selective agent by the surrounding transformed cells or the remaining *Agrobacterium* in the explants were probably the cause of the regenerated escapes.

We have tried to use three different strategies to recover transgenic shoots. In the first strategy, we encouraged all the regenerable cells to produce shoots by starting the selection at low concentration of antibiotics and then gradually increasing the pressure to kill the escapes, no matter whether the escapes came from the explant area not touching medium or chimera shoots (the regenerants are composed of transformed and no-transformed cells). In the reversed strategy, we started with a very high concentration of antibiotics and gradually reduced the pressure, trying to make sure that the competition from non-transformed cells is completely inhibited, therefore allowing the transformed cells to fully use their regeneration potential. In the third strategy, we kept using the same selection pressure to recover transgenic shoots. All three strategies turned out to be unsuccessful.

We obtained more than a thousand putative transgenic shoots out of tens of thousand leaf explants. It is difficult to explain how all the regenerants we obtained were from non-transformed cells rather than transformed cells. In grape, Colby et al. (1991)

reported that transformed cells were not those capable of regenerating and those regenerable cells were not able to be transformed by *Agrobacterium*.

In another attempt to solve the problem, we tried changing the pretreatment method. We inoculated 1-2 week old plantlets (TDZ pretreated) then brought them back to TDZ medium to grow another 1-2 weeks, hoping the transformed cells would divide and grow into a large area of transformed leaf tissue containing those regenerable cells. When these leaves are cut for regeneration, they are expected to have higher chance to recover to real transformants than those obtained by single step TDZ pretreatment. With this method, however, we did not find large blue areas on leaf explants from GUS assays. While we did obtain putative transgenic shoots, they were all GUS negative and did not root under selection.

3.7. Summary and conclusion

The most critical task in the development of an *A. tumefaciens*-mediated transformation system is the establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated. In another words, the standard of "optimum" has to be the conditions that are a compromise between the T-DNA transfer efficiency and regeneration potential. Multiple factors are involved in achieving this goal, which influence the success or failure of the transfer of the gene of interest into plants and their subsequent stable integration and expression. These

factors can be generally grouped as follows: type and age of the tissue to be transformed, *Agrobacterium* strain and plasmid vector, extent of time and conditions for inoculation and cocultivation of the tissue with *Agrobacterium* (including wounding process and compounds that enhance T-DNA delivery, etc.), growth of *Agrobacterium* with respect to the transformed plant cells, plant tissue necrosis caused by *Agrobacterium* and treatments, etc. The different factors can affect transformation differently, depending in part on the plant species and even genotypes within a species. Since nobody has convincingly been able to transform blackberry, and transformation protocol has been proved to be genotype specific, we had to take a comprehensive examination to identify and optimize parameters influencing these factors.

We developed an efficient shoot organogenesis system using 3-week old, TDZ-pretreated, leaf explants (Meng et al., 2004). As a general rule, young plants are easier to transform than old ones, but we did not find a difference in T-DNA delivery efficiency between 2- and 3-week old 'Marion' leaf explants (data not shown). Therefore, the type and age of the tissue to be transformed in all the experiments were 3-week old TDZ- pretreated leaf explants. LBA4404 harboring superbinary vector pTOK233 was the most efficient among all the combinations of *Agrobacterium* strains and plasmid vectors tested. Having the best explant (type and age) and *Agrobacterium* strain/vector identified, the further experiments were to optimize conditions for transformation, which means to increase T-DNA delivery efficiency but avoid the overgrowth of *Agrobacterium* and tissue necrosis. Overgrowth of *Agrobacterium* has a

negative effect on the survival rate of the transformed cells and increases the number of copies of T-DNA inserts in the transformed plant, which can eventually cause silencing of the transgene. Therefore, overgrowth of *Agrobacterium* and direct tissue necrosis resulted in dramatically reduced chances of regenerating complete plants from the transformed tissue and the silencing of the transgene. Keeping this in mind, we identified and optimized factors that enhanced T-DNA delivery without sacrificing good leaf explant condition, including duration (30") and intensity (60% power output) of sonication treatment, type (Sequestrene iron 138), concentration (200 mg•L^{-1}) and usage (applied in the cocultivation medium) of thiol group containing compound, *Agrobacterium* cell density (optical density 2.0 at 600 nm), duration of inoculation (3'), concentration of virulence gene inducer acetosyringone in the inoculation (100 µM) and cocultivation (600 µM) medium, type (Tween 20) and concentration (0.1%) of surfactant in the inoculation medium, duration (3 d) of cocultivation. We also found that while the use of a vacuum enhanced transformation of many plant species it did not work with 'Marion' leaf explants. By combining the best conditions for each parameter we examined, the frequency of transformation, expressed as percentage of explants showing blue spots, was about 20%, four fold higher than what we had had at the beginning.

While we were not able to obtain transgenic shoots, our investigation is an important first step in the utilization of *Agrobacterium* in the transformation of 'Marion' leaf tissue.

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Table 3.1 Ag*robacterium tumefaciens*-mediated transformation of *Rubus* spp.

Genotype	Type[a]	Explant[b]	Strain/plasmid	SA[c]	Reporter[d] gene	Target[e] gene	TF[f] (%)	Confirmation	Reference
8242E6	R	I	LBA4404/PBI121.X	kan	GUS	-	66.7	GUS assay/Dot blot	McNicol and Graham, 1989; Graham et al., 1990a, b
Tayberry	H	I	LBA4404/PBI121.X	kan	GUS	-	80.0	GUS assay/Dot blot	
Sunberry	H	L	LBA4404/PBI121.X	kan	GUS	-	33.3	GUS assay/Dot blot	
Loch Ness	B	I	LBA4404/PBI121.X	kan	GUS	-	42.8	GUS assay/Dot blot	
Black Satin × Tayberry	H	L	C58/pJIT106	kan	GUS	-	0.33	Southern blot	Hassan et al., 1993
		I	C58/pJIT54	kan	CAT	-	0.25	Southern blot	
Dirksen × Shawnee	B	C	C58/pJIT54	kan	CAT	-	1.50	Rooting	
Meeker	R	L	EHA105/pAG1452	hyg	-	SAMase	15.9	Southern blot	Mathews et al., 1995
		P	EHA105/pAG1452	hyg	-	SAMase	49.6	Southern blot	
Chilliwack	R	L	EHA105/pAG1452	hyg	-	SAMase	0.9	Southern blot	
		P	EHA105/pAG1452	hyg	-	SAMase	0.7	Southern blot	
Canby	R	L	EHA105/pAG1452	hyg	-	SAMase	2.4	Southern blot	
	R	P	EHA105/pAG1452	hyg	-	SAMase	8.1	Southern blot	
	R	L	EHA105/pAG1552	gen	-	SAMase	1.3	Southern blot	
	R	P	EHA105/pAG1552	gen	-	SAMase	3.6	Southern blot	
R. arcticus	R	I	EHA101/pEHA101	kan	GUS	-	3.3	Southern blot	Kokko and Kärenlampi, 1998
Ruby	R		GV2260/pBin19	kan	-	*DefH9iaam*	-	Southern blot	Mezzetti et al., 2002

[a] R, raspberry; B, blackberry; H, raspberry × blackberry hybrid.
[b] I, internodes; L, leaves; C, cotyledons; P, petiole.
[c] SA, selective agent; kan, kanamycin; hyg, hygromycin; gen, geneticin.
[d] GUS, β-Glucuronidase; CAT, chloramphenicol acetyl transferase.
[e] SAMase, S-adenosylmethonine hydrolase; *DefH9iaam*, placenta/ovule-specific auxin-synthesizing gene
[f] TF, transformation frequency.

Table 3.2 Effect of *Agrobacterium* antibiotic cefotaxime on shoot organogenesis.

Cefotaxime concentration (mg•L^{-1})	Regeneration efficiency (%) [z]
0	64.7 a
250	66.3 a
500	63.8 a
750	41.1 b
1000	25.0 c

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.3 Effect of kanamycin and hygromycin on shoot organogenesis of 'Marion' leaf explants.

Selection agent (mg•L^{-1})	Regeneration efficiency (%) [z]
Kanamycin	
0	65.3 a
10	53.5 b
20	33.3 c
30	9.8 d
40	0.0 e
50	0.0 e
Hygromycin	
0	65.3 a
5	39.3 b
7.5	7.6 c
10	0.0 d
15	0.0 d

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.4 Effect of kanamycin and hygromycin on rooting of 'Marion' regenerants.

Selection agent (mg•L^{-1})	Rooting efficiency (%) [z]
Kanamycin	
0	100 a
15	90.0 b
25	66.7 c
35	0.0 d
45	0.0 d
50	0.0 d
Hygromycin	
0	100.0 a
5	75.0 b
7.5	0.0 c
10	0.0 d
15	0.0 d

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.5 Efficiency of different combinations of *Agrobacterium tumefaciens* strain and plasmid, based on the GUS histochemical staining of leaf explants of 'Marion' blackberry 7 days after cocultivation.

Strain	Plasmid	Percentage of GUS+ explants (%) [z]
LBA4404	pTOK233	5.3 a
EHA105	pCAMBIA1305.1	1.6 b
EHA105	pTOK47/pGiPTV HPT	1.0 b
EHA105	pGiPTV-HPT	1.0 b
AGL-1	pIG121-Hm	0.0 c
AGL-1	pTOK233	0.0 c
EHA101	pIG121-Hm	0.0 c
C58	p35SGUS INT	0.0 c

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.6 The effect of length and intensity of sonication on the T-DNA delivery and leaf health in 'Marion' blackberry.

Sonication		In the absence *Agrobacterium*		In the presence of *Agrobacterium*	
Length (min)	Intensity	Percentage of GUS+ explants (%) [z]	Adverse effect on regeneration	Percentage of GUS+ explants (%)[z]	Tissue necrosis (during cocultivation)
0	0	4.8 b	no	4.3 b	no
0.25	20	4.6 b	no	3.7 b	no
0.5	20	4.3 b	no	4.2 b	no
1.0	20	3.9 b	no	3.5 b	no
2.0	20	4.2 b	no	4.1 b	no
5.0	20	4.5 b	no	4.5 b	no
0.25	40	3.8 b	no	4.3 b	no
0.5	40	3.5 b	no	5.5 b	no
1.0	40	3.7 b	no	4.4 b	no
2.0	40	4.0 b	no	3.8 b	no
5.0	40	3.7 b	no	4.0 b	no
0.25	60	4.3 b	no	7.2 c	no
0.5	60	5.0 b	no	11.1 d	no
1.0	60	4.6 b	no	10.5 d	no
2.0	60	5.1 b	no	9.2 cd	no
5.0	60	5.1 b	no	8.7 cd	yes
0.25	80	3.8 b	no	7.4 c	no
0.5	80	3.9 b	no	7.5 c	no
1.0	80	2.4 ab	no	6.6 bc	no

2.0	80	2.4 ab	no	4.1 b	yes
5.0	80	1.2 a	yes	3.9 b	yes
0.25	100	5.2 b	no	7.1 bc	no
0.5	100	3.0 ab	no	8.3 c	no
1.0	100	1.4 a	yes	4.3 b	yes
2.0	100	1.1a	yes	4.0 b	yes
5.0	100	1.0 a	yes	2.8 ab	yes
0.25	120	4.5 b	no	8.0 c	no
0.5	120	1.5 a	yes	8.8 c	no
1.0	120	1.0 a	yes	3.7 b	yes
2.0	120	1.7 a	yes	4.4 b	yes
5.0	120	1.0 a	yes	2.5 ab	yes
0.25	140	4.9 b	yes	6.8 bc	no
0.5	140	0.9 a	yes	2.0 a	yes
1.0	140	1.3 a	yes	1.5 a	yes
2.0	140	1.5 a	yes	1.1 a	yes
5.0	140	0.6 a	yes	0.0 a	yes

[z] Values within a column with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.7 The effect of the L-cysteine and chelated iron in the inoculation and cocultivation medium on T-DNA delivery efficiency and leaf explant health in 'Marion' blackberry.

Treatment	Percentage of GUS+ explants (%) [z]	Tissue necrosis (during cocultivation)
Chelated iron in the inoculation medium (mg•L^{-1})		
0	10.9 a	no
100	9.3 a	no
200	10.0 a	no
400	3.5 b	yes
Chelated iron in the cocultivation medium (mg•L^{-1})		
0	6.9 b	no
100	8.8 ab	no
200	10.9 a	no
400	5.7 b	yes
L-cysteine in the inoculation medium (mg•L^{-1})		
0	10.1 a	no
100	4.4 b	yes
200	1.7 c	yes
400	0.0 d	yes
L-cysteine in the cocultivation medium (mg•L^{-1})		
0	10.6 a	no
100	2.5 b	yes
200	0.0 c	yes
400	0.0 c	yes

[z] Values with different letters in each treatment are significantly different at $P \leq 0.05$ by LSD.

Table 3.8 The effect of *Agrobacterium* cell density and inoculation time on the T-DNA delivery efficiency and leaf explant health in 'Marion' blackberry.

Cell density (OD_{600})	Inoculation time (min)	Percentage of GUS positive explants (%)[z]	Bacterium overgrowth (during cocultivation)	Tissue necrosis (during cocultivation)
0.0	0.0	0.0	no	no
0.1	0.5	0.0 a	no	no
0.5	0.5	0.0 a	no	no
1.0	0.5	0.0 a	no	no
1.5	0.5	0.0 a	no	no
2.0	0.5	11.0 c	no	no
2.5	0.5	10.6 c	yes	no
0.1	1.0	0.0 a	no	no
0.5	1.0	0.0 a	no	no
1.0	1.0	10.8 c	no	no
1.5	1.0	13.0 c	no	no
2.0	1.0	12.6 c	no	no
2.5	1.0	16.3 d	yes	yes
0.1	3.0	0.0 a	no	no
0.5	3.0	0.0.a	no	no
1.0	3.0	11.7 c	no	no
1.5	3.0	11.3 c	no	no
2.0	3.0	15.1 d	no	no
2.5	3.0	16.0 d	yes	yes
0.1	5.0	0.0 a	no	no

0.5	5.0	0.0 a	no	no
1.0	5.0	10.1 c	no	no
1.5	5.0	11.9 c	no	yes
2.0	5.0	17.0 d	yes	yes
2.5	5.0	7.5 b	yes	yes
0.1	10.0	0.0 a	no	no
0.5	10.0	0.0 a	yes	yes
1.0	10.0	0.0 a	yes	yes
1.5	10.0	7.6 b	yes	yes
2.0	10.0	8.2 b	yes	yes
2.5	10.0	0.0 a	yes	yes

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.9 The effect of acetosyringone in the inoculation and cocultivation medium, respectively, on T-DNA delivery and leaf explant health in 'Marion' blackberry. The optimum concentration in the inoculation medium was determined first and then used to study the optimum concentration in cocultivation medium.

	Acetosyringone Concentration (µM)	Explants showing GUS response (%) [z]	Tissue necrosis	
			Right after inoculation	During cocultivation
Inoculation	0	0.0 a	no	no
	50	0.0 a	no	no
	100	14.8 b	no	no
	200	15.3 b	no	no
	400	14.1 b	no	no
	600	14.5 b	no	no
	800	13.9 b	no	no
Co-cultivation	0	0.0 a	-	no
	50	0.0 a	-	no
	100	14.3 c	-	no
	200	15.0 c	-	no
	400	17.1 cd	-	no
	600	18.6 d	-	no
	800	6.8 b	-	yes

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.10 Effect of surfactant present in the inoculation medium on T-DNA delivery and leaf explant health in 'Marion' blackberry.

Concentration of surfactant (%, v/v)	Percentage of GUS+ explants (%) [z]	Tissue necrosis	
		After inoculation	During cocultivation
0.00	17.8 d	no	no
Tween 20			
0.01	17.2 d	no	no
0.10	21.3 e	no	no
0.50	20.9 e	no	yes
1.00	12.7 c	no	yes
Silwet®			
0.01	18.3 d	no	no
0.10	13.1 c	no	yes
0.50	5.5 b	yes	yes
1.00	0.0 a	yes	yes

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.11 Effect of vacuum infiltration on T-DNA delivery and leaf explant health in 'Marion' blackberry.

Vacuum infiltration (min)	Percentage of GUS+ explants (%) [z]	Tissue necrosis	
		Right after inoculation	During cocultivation
0.0	22.1a	no	no
0.5	20.7 a	no	no
1.0	21.6 a	no	no
2.0	12.4 b	no	yes
5.0	3.0 c	yes	yes
10.0	2.2 c	yes	yes

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Table 3.12 Influence of length of cocultivation on T-DNA delivery and leaf explant health in 'Marion' blackberry.

Co-cultivation time (d)	Percentage explants with GUS foci (%)[z]	*Agrobacterium* overgrowth (during cocultivation)	Tissue necrosis (during cocultivation)
0	0.0 a	no	no
1	0.0 a	no	no
2	8.8 b	no	no
3	21.1 c	no	no
4	20.4 c	yes	no
5	15.7 d	yes	yes
6	16.1 d	yes	yes

[z] Values with different letters are significantly different at $P \leq 0.05$ by LSD.

Chapter 4. Overexpression of *Arabidopsis* CBFs in *Populus tremula* × *alba* enhances freezing tolerance

4.1. Abstract

Hybrid aspen *Populus tremula* × *alba* (clone 717-1B4) was transformed with the *Arabidopsis* CBF1, CBF2 and CBF3 genes controlled by either a constitutive CaMV35S (35S) promoter or a stress-inducible *Arabidopsis* rd29A gene (rd29A) promoter. The freezing tolerance, growth, sugars and proline contents of the transgenic plants with varying level of transgene expression were characterized. Two transgenic lines of 35S::AtCBF1 increased freezing tolerance 3.1°C and 2.2°C in leaves and stems respectively, when compared to wild type (WT) plants grown under non-acclimation conditions, and a 3.3°C and 3.8°C increase in the freezing tolerance of leaves and stems respectively after 4 weeks of cold acclimation at 2°C. After acclimation, there was no increase in freezing tolerance found in the rd29A::AtCBF1 line compared to the WT. The three 35S::AtCBF2 lines grown under acclimation condition had a decreased freezing tolerance, compared to the WT. All four tested transgenic 35S::AtCBF3 lines gave the most significant increases in freezing tolerance, with an average of 5.3°C increase in leaf tissue tolerance when grown under non-acclimating conditions and 6.1°C after being grown under acclimating conditions, and for stem tissues a 3.0°C and 6.3°C increase in freezing tolerance when grown under non-acclimating and acclimating conditions, respectively. Three of the six rd29A::AtCBF3 lines, after cold acclimation, were

significantly more tolerant to freezing than the WT, with an average increase of 2.5°C in leaf tissue tolerance and 3.3°C in stem tissues. The 35S::AtCBF1 and 35S::AtCBF3 lines each had a dwarf phenotype, but the dwarf 35S::AtCBF1 line grew out of this habit after 50 days in soil and the 35S::AtCBF3 line did not. Using the stress inducible promoter rd29A to drive *AtCBF3* did not improve freezing tolerance as much as did the 35S promoter, however, it did not cause retarded growth of transgenic lines, either. 35S::AtCBF1, Non-acclimated 35S::AtCBF3 lines and acclimated rd29A::AtCBF3 lines had significantly higher levels of cryoprotectant proline and soluble sugars in their leaf tissues than did the WT. Such accumulation of cryoprotectant mimics the biochemical changes in the WT during cold acclimation.

4.2. Introduction

Poor freezing tolerance is a serious problem for many crop plants. In some years it can cause severe yield loss and, in general, it can limit the geographic distribution of crop. The study of plant cold acclimation, the process whereby a plant increases its freezing tolerance in response to low, non-freezing temperatures (Hughes and Dunn, 1996; Smallwood and Bowles 2002; Thomashow, 1999), has led to significant progress in understanding how plants have evolved to survive freezing and given us the potential to apply new strategies to improve plant freezing tolerance. During cold acclimation, low, non-freezing temperatures induce the alteration of expression of hundreds of genes, some of which contribute to freezing tolerance (Guy, 1990; Smallwood and Bowles, 2002;

Thomashow, 1999). Among these genes, a family of transcription factors called CBF [CRT/DRE (C-Repeats/dehydration-responsive element) binding factor] play an important role in controlling the expression of many *cor* (cold-regulated) genes (Van Buskirk and Thomashow, 2006).

The CBF transcription factors were first discovered in *Arabidopsis* (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Stockinger et al., 1997) and then their orthologous genes were isolated from a wide range of other crop plants, including canola (*Brassica napus*) (Gao et al., 2002; Jaglo et al., 2001), soybean (*Glycine max*), broccoli (*Brassica oleracea*), sweet cherry (*Prunus avium*) (Kitashiba et al., 2002), sour cherry (*Prunus cerasus*) and strawberry (*Fragaria* × *ananassa*) (Owens et al., 2002), wheat [*Triticum aestivum* (Shen et al., 2003) and *T. monococcum* (Vagujfalvi et al., 2003)], rice (*Oryza sativa*) (Dubouzet et al., 2003), barley (*Hordeum vulgare*) (Choi et al., 2002; Francia et al., 2004; Skinner et al., 2005; Xue, 2003), and poplar (*Populus tremula* × *alba*) (Benedict et al., 2006). The wide range of species that have been found to contain CBF indicates that CBF may be highly conserved in most plant species (Jaglo et al., 2001). The CBF genes have been used to engineer freezing tolerance in several species (Table 1). Overexpression of the *Arabidopsis* CBF genes in the absence of a cold stimulus in transgenic *Arabidopsis* (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998; Kasuga et al. 1999), canola oilseed rape (Jaglo et al., 2001), strawberry (Owens et al., 2002), potato (Pino et al., 2005), and poplar (Benedict et al., 2006) results in increased freezing tolerance. Similar results were observed when CBF orthologs isolated from canola, rice,

and barley were incorporated into transgenic canola (Savitch et al., 2005), *Arabidopsis* (Amundsen, 2004; Dubouzet et al., 2003) or rice (Ito et al., 2006).

These results along with the fact that multiple *cor* genes (and their homologs) and other CRT/DRE containing target genes were also expressed when CBFs were overexpressed suggest that the CBF regulation plays the role of a master switch in the cold responsive pathway in plants (Thomashow, 2001).

While most commercial fruit and forest crops are woody plants and many suffer from cold hardiness problems, there has been little research on CBF regulon in temperate woody perennials. Trees have some unique characteristics distinguishing themselves from annual herbaceous plants, such as self-supporting, perennial structures and a long lifespan. *Populus* species have been used commonly as a model system for woody perennials. Benedict et al. (2006) showed that constitutive expression of *Arabidopsis CBF1* in *Populus* sp. increased freezing tolerance of non-acclimated leaves and stems. Transcript profiling demonstrated that there was a strong conservation of the CBF regulon between this tree species and *Arabidopsis*. Their study of induction kinetics and tissue specificity of four *CBF* paralogs showed the differential expression and different functional composition of CBF1 regulons of leaf (annual) and stem (perennial) tissues (Benedict et al., 2006).

Undesirable stunted growth has been a phenotype in transgenic plants ectopically overexpressing CBF genes under normal growing conditions (Benedict et al., 2006; Gilmour et al., 2000; Liu et al., 1998). Using stress-inducible promoters, such as rd29A, that have low background expression under normal growth condition in conjunction with the CBF genes has been one way to achieve increased freezing tolerance without retarded growth (Kasuga et al., 1999).

Sugars were found to be effective cryoprotectants *in vitro* (Carpenter and Crowe, 1988) where they protected membranes (Sanitarius, 1973; Strauss and Hausser, 1986) and cells (Livingston and Henson, 1998; Vijn and Smeekens, 1999; Taji et al., 2002) during cold stress. Similarly, proline was believed to have multiple roles in plant stress tolerance such as a mediator of osmotic adjustment (Handa et al. 1986), a stabilizer of subcellular structures (Schobert and Tschesche, 1978), a scavenger of free radicals (Saradhi et al., 1995), a buffer in cellular redox potential (Hare et al., 1998), and a major constituent of cell wall structural proteins that may provide mechanical support for cells (Nanjo et al. 1999a). Van Swaaij et al. (1985) showed that the exogenous application of proline increased potato frost tolerance. Increases in levels of soluble sugars (Gilmore et al., 2000; Guy et al., 1992; Koster and Lynch, 1992; McKown et al., 1996; Strauss and Hauser, 1986; Wanner and Junttila, 1999) and proline (Alberdi et al., 1993; Gilmore et al., 2000; Koster and Lynch, 1992; McKown et al., 1996; Rudolph and Crowe, 1985; Wanner and Junttila, 1999) are among the most important biochemical changes occurring in plants during cold acclimation. Gilmour et al. (2000) reported similar changes brought about by

CBF3 overexpression in non-acclimated *Arabidopsis*. This work reports that the overexpression of CBF genes in transgenic *Populus* resulted in the similar alteration of these two cryoprotectants.

Gilmour et al. (2004) reported that *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 had matching and redundant functional activities in cold acclimation. This was based on their similar effects on increasing the freezing tolerance of non-acclimated and acclimated plants, the biochemical composition, morphology and development of the transgenic plants, and cell ultrastructure. However, Navillo et al. (2004) determined that CBF2 negatively regulated CBF1 and CBF3 expression by analyzing *cbf2* mutant, and that the combined expression and regulation of these three genes controlled the freezing and the related stress tolerance in *Arabidopsis*.

In this study, we used a hybrid aspen (*P. tremula* × *alba*) clone to overexpress three members of CBF family (CBF1, CBF2, and CBF3), each controlled by either a constitutive promoter (35S) or a stress-inducible promoter rd29A, respectively, to determine 1) if overexpressing CBF1, CBF2 and CBF3 under either a constitutive or low-temperature inductive promoter would increase freezing tolerance as is the case in other annual species expressing CBFs, 2) which CBF construct has the most significant effect on cold tolerance improvement, 3) if using the rd29A promoter can improve freezing tolerance in *Populus* and at the same time avoid the growth retardation under normal growth conditions, which occurred when CBF1 was constitutively expressed by 35S

promoter, 4) if cryoprotectant proline and sugars increased in transgenic poplars as they did in wild type under cold acclimation, and finally, 5) if overexpressing CBF transcription factor family member(s) would be a feasible way to improve freezing tolerance of a woody perennial.

4.3. Materials and methods

4.3.1. Plant materials and growth condition

Poplar plants (*P. tremula* × *alba*) were cultured *in vitro* in a culture room at 25±1°C under cool-white fluorescent lights (40-60 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 16 h photoperiod). Plants were subcultured every 1-2 months for multiplication by cutting young stems into 2-3 node pieces and/or placing shoot tips on rooting medium (RM pH5.8). For transformation, fully expanded young leaves and stems were collected and cut into leaf discs (5-10 mm in diameter) and stem internodes (5-10 mm in length) (DeBlock, 1990).

For Northern blotting and physiological analysis, plants of WT and transgenic lines were propagated by cuttings *in vitro.* Rooted plants were transplanted into one gallon pots containing Sunshine SB40 Professional Growing Mix (Sun Gro Horticulture Inc., Bellevue, Wash.) supplemented with 7% pumice (weight/volume) and 0.14% perlite (weight/volume), and fertilized with 0.04% Osmocote (14N–4.2P–11.6K, The Scotts Company, Marysville, Ohio). Plants were grown in a greenhouse maintained at a 16 h

day (25±3 °C) and 8 h night (20±3 °C) with supplemental lighting (mercury halide lamps providing 400-500 μmol·m^{-2}·s^{-1}). After six weeks, plants of both transgenic lines and WT were divided into two groups. One group of the plants was transferred to a cold room (2 °C, 16 h photoperiod) for one week, while the other group was maintained in the greenhouse under the above greenhouse conditions. For freezing tests, 1.0 cm diameter leaf-discs were excised from each side of the two to three uppermost fully-expanded leaves, and 2 cm stem segments were harvested. Three replicates of three leaf discs or stem segments were used per assay and freezing tolerance was determined by ion leakage (Sukumaran and Weiser, 1972).

4.3.2. Construction of transformation vector

Seven expression vectors were constructed, i.e. two different plasmids containing *Arabidopsis* CBF1 cDNA (*AtCBF1*) ligated with 35S promoter, one plasmid containing *AtCBF1* with rd29A promoter, two plasmids containing *AtCBF2* with 35S and rd29A promoter, respectively, and two *AtCBF3* with 35S and rd29A promoter, respectively (Fig. 4.1E and Fig. 4.1F). All *AtCBFs* were from Thomashow Lab at Michigan State University (Jaglo-Ottosen et al., 1998). Plasmid-1 contained a 35SPromoter::*AtCBF1*::35STerminator cassette (Fig. 4.1A) ligated as a HindIII fragment into the binary vector pGAH (Onouchi *et al.*, 1991) that had been linearized with HindIII. Plasmid-2 contained a 35SPromoter::*AtCBF1*::7'Terminator cassette (Fig. 4.1B) ligated as a BamHI fragment into the BglII-cut binary vector pGA643 (An et al., 1988). Plasmid-

3 was obtained by cutting Plasmid-2 with PstI/HindIII to excise 35S promoter and replace it with rd29A promoter (Fig. 4.1C). Plasmid-4 contained a 35SPromoter::*AtCBF2*::7'Terminator cassette (Fig. 4.1D) ligated as a BclI/BamHI fragment into the BglII-cut binary vector pGA643. Plasmid-5 was obtained by cutting Plasmid-4 with BamHI/ClaI to excise 35S promoter and replace it with the rd29A promoter (Fig. 4.1E). Plasmid-6 contained a 35SPromoter::*AtCBF3*::7'Terminator cassette (Fig. 4.1F) ligated as a HindIII/BamHI fragment into the HindIII/BglII-cut binary vector pGA643. Plasmid-7 was obtained by cutting Plasmid-4 with BamHI/HindIII to excise 35S promoter and replace it with the rd29A promoter (Fig. 4.1G).

4.3.3. Poplar transformation

The *A. tumefaciens* culture, poplar transformation, recovery of transformants, and verification of the presence of transgenes in the poplar genome by PCR were conducted following the methods of Benedict et al. (2006).

4.3.4. Leaf and stem freezing tolerance assays

The freezing tolerance of leaves and stems of the WT plants and the transgenic poplar lines overexpressing *AtCBF* was measured using the electrolyte leakage test following Benedict's (2006) modification of Dexter et al. (1932) and Sukumaran and Weiser (1972).

Lethal temperature was defined as the temperature that leads to leakage of 50% of the cellular electrolytes (TEL_{50}). Leaf samples consisted of a 1.0 cm diameter leaf-disc cut from middle vein from two or three of the uppermost fully expanded leaves. Stems were cut into 2 cm segments. Three leaf discs or stem segments were placed on a piece of moistened Kimwipe toweling in a 5x150 mm test tube. Three samples tubes from each line were placed in a programmed freezer maintained at –1.0°C for 1 hour while three tubes were left in a refrigerator as a control. After initiation of ice formation at –1.5°C, the bath temperature was lowered at a cooling rate of $2°C \cdot h^{-1}$. Tubes were moved from the cooling bath at 2°C intervals and allowed to thaw in a refrigerator overnight. Each leaf disc or stem segment was placed in a vial containing 10 mL of de-ionized water. Conductivity (R0) was measured after 10 minutes of vacuum and 1 hour of shaking at 150 rpm at room temperature. To measure total conductivity (R1), samples were frozen at -80°C overnight, thawed to room temperature, and then placed under a vacuum for 10 minutes followed by shaking for 1 hour at 150 rpm in the original solution at room temperature. Ion leakage was calculated as R0/R1 x 100%.

4.3.5. DNA and RNA extraction and gel blot analysis

For PCR analysis, genomic DNA was isolated using Qiagen DNeasy Kits (Qiagen, Valencia, Calif.) from young leaves of WT and each of the transgenic lines that were *in vitro* cultured. For Northern blotting, leaf samples were collected from three individuals of 1) each 35S-transgenic line grown under warm greenhouse condition, 2) each rd29A-

transgenic line grown with one week of cold acclimation (2°C with 16 h photoperiod), and 3) WT plants grown under either the warm greenhouse conditions or after one week of cold acclimation (2°C with 16 h photoperiod). Total RNA was extracted using Q Qiagen RNeasy columns (Qiagen, Valencia, Calif.). The gel electrophoresis and blotting to Nytran nylon membranes were performed as described by Skinner and Timko (1998). RNA blots were probed using Ultrahyb (Ambion, Austin, Texas) and washed following the manufacturer guidelines. *Hv*CBF probes excluded the AP2 domain and consisted of only the C-terminal domain and 3' UTR to minimize *Hv*CBF cross hybridization. Labeled probes were generated as directed using a high Prime Labeling Kit (Roche Biochemicals, Indianapolis, Ind.).

4.3.6. Sugar extraction and analysis

Leaves of WT and transgenic plants were used for the sugar analysis. To avoid the leaf-to-leaf variation and variation due to plant age, we only selected leaves of similar age and size. The two to three uppermost fully-expanded leaves from each of three WT plants and transgenic lines, respectively, which were grown under either the warm greenhouse condition or after four weeks of cold acclimation (2°C with 16 h photoperiod), were lyophilized by Freeze Dry/Shell Freeze System (Labconco Corp., Kansas City, Mo.) and ground on liquid nitrogen. Ground tissue from each plant was transferred to a 50 ml falcon tube and stored in -80°C. Soluble sugar extraction was done as described in Dionne et al. (2001) with some modification. A sample of 0.5 to 1.0 g of the lyophilized

tissue was added with 5 mL of MCW solution (methanol-chloroform-water at 12:5:3, v/v/v) and then tubes were heated at 60°C for 30 minutes, during which the sample was mixed by vortex every five minutes. The homogenate was transferred to each of five 1.5 ml centrifuge tubes with equal volume and 250 µL of water was added to each tube for phase separation. The tubes were centrifuged at 12000 g for 4 minutes. The aqueous phase in each tube was collected and transferred to a new centrifuge tube that was evaporated to dryness on a rotary evaporator (Savant SpeedVac, TeleChem International, Sunnyvale, Calif.) and resuspended in 200 µl of water containing 50 $mg \cdot L^{-1}$ ethylene-diaminetetraacetic acid (EDTA; Na^{+}, Ca^{2+}). Samples from five tubes were pooled and filtered through a 0.45 µm filter (Gelman Sciences, Ann Arbor, Mich.) in preparation for high performance liquid chromatography (HPLC) analysis. Soluble carbohydrates were analyzed using a Waters 2695 Separation Module (Waters, Milford, Mass.) and detected with a Waters 410 Refractive Index Detector. The HPLC system was computer-controlled by the Empower software and was composed of an automatic injector (WISP 717B), Model 510 pump, and a Model 410 2414 Refractive Index Detector. Soluble sugars were separated on a Sugar-Pak I column (6.5×300 mm; Waters, Milford, Mass.) and eluted isocratically at 85°C using an EDTA (Ca^{2+}, Na^{+}, 50 $mg \cdot L^{-1}$) (Sigma, St. Louis, Mo.) solution as the mobile phase at a flow rate of 0.5 $mL \cdot min^{-1}$. Soluble sugars were identified by comparing their retention times to that of sugar standards, including anhydrous sucrose, glucose (Sigma, St. Louis, Mo.), fructose (Fisher, Fair Lawn, N.J.), raffinose, melezitose, maltose, mannose, ribitol, sorbitol, and mannitol (Sigma, St. Louis,

Mo.). Detected carbohydrates were quantified by comparison of unknown peak areas to peak area-response curves derived from standard solutions of varying concentrations.

4.3.7. Proline extraction and analysis

The lyophilized and ground leaf tissues used for sugar analysis were used for proline analysis as well using the acid ninhydrin method as described by Bates et al. (1973). Approximately 0.5 g of lyophilized tissue was homogenized in 10 ml of 3% aqueous sulfosalicylic acid (Sigma, St. Louis, Mo.) and the homogenate filtered through Whatman #2 filter paper. Acid-ninhydrin stock reagent was prepared by mixing 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation and heating at 100°C, until dissolved. This reagent was stored at 4°C and used within 24 hours. The 500 µL aqueous extract was mixed with 500 µL acid-ninhydrin and 500 µL of glacial acetic acid (Sigma, St. Louis, Mo.) and heated at 100°C for 1 hour. The reaction was terminated on ice and the mixture was warmed to room temperature and 500 µL of the cooled reaction mixture was partitioned against 2 mL of toluene (spectrophotometric grade, Sigma, St. Louis, Mo.). The chromophore containing toluene (organic phase) was pipetted from the aqueous phase and the absorbance was measured at 515 nm using UV-1601 UV-visible Spectrophotometer (Shimadzu, Pleasanton, Calif.). The proline concentration was determined by comparing with a standard curve constructed using known amounts of proline (Sigma, St. Louis, Mo.).

4.3.8. Experimental design and statistical analysis

The controlled freezing test was arranged as a completely randomized design with three replications. All experiments were conducted three times. The analysis of variance was made by the General Linear Model (GLM) Procedure of SAS program Version 8.0 (SAS Institute, Cary, N.C.). Fishers Least Significant Difference test (LSD) was used for comparison of $LT_{50,}$ soluble sugar, and proline levels between lines when the F-test for main effects was significant (P = 0.05).

4.4. Results and discussion

4.4.1. Generation of transgenic poplar plants expressing *AtCBF* transgenes

Transgenic poplar lines expressing *AtCBF* transgenes were identified by Northern blotting analysis from transgenic lines positive for the *CBF* transgene operon via PCR, under non-acclimated and acclimated conditions. Under non-acclimated condition, two, three, and four expressers were selected out of 29, 10, and 10 PCR positive lines of 35S::AtCBF1-, 35S::AtCBF2-, and 35S::AtCBF3, respectively (Fig. 4.2, Table 4.2). No expression was detected from transgenic lines of rd29A promoter grown under warm conditions (data not shown), but when acclimated, one, four, and six lines were found to accumulate detectable level of *AtCBF* transcripts from 10, 10, and 10 transgenic lines expressing *AtCBF1*, *AtCBF2,*and *AtCBF3*, controlled by rd29A promoter, respectively

(Fig. 4.2, Table 4.2). The three genes had varying efficiency for generating CBF expressers. *CBF1* was the least efficient for generating transgenic lines that accumulated detectable levels of *AtCBF1* transcripts, with an average of 8.5% from the combined result of both promoters. *CBF2* and *CBF3* were more efficient with the average of 35% and 55%, respectively (Table 2). It is not clear why there was such a difference between these three *AtCBF* genes. One postulation is that these three genes may not control the exact same regulon, therefore, some gene(s) upregulated by *AtCBF1* and/or *AtCBF2* may lead to the expression of the protein(s) that affects the recovery of real expressers *in vitro*. Having a reasonably good efficiency to generate transgenic lines that strongly express the transgene is important for the practical use of genetic engineering in cultivar improvement.

4.4.2. Effects of *AtCBF* overexpression on leaf and stem freezing tolerance

Non-acclimated (25°C in greenhouse) and 4-week cold acclimated (2°C in cold room) plants from transgenic lines showing detectable levels of *AtCBF* transcripts and WT plants were selected for freezing tests to evaluate the effect of *AtCBF* overexpression on freezing tolerance of leaf and stem tissues. The two independent transgenic lines of *35S::AtCBF1* had significantly higher freezing tolerance (an average of 3.1°C and 2.2°C increase for leave and stem tissue, respectively) than WT plants at non-acclimated condition (Fig. 4.3A). Similar results were found after acclimation (Fig. 4.3A). The leaf and stem tissues of the one transgenic poplar of *AtCBF1* controlled by the rd29A promoter, however, was not more freezing tolerant than the WT. This was true whether

or not the plants had received cold acclimation (Fig. 4.3B). Constitutive expression of *AtCBF1* enhanced freezing tolerance in *Arabidopsis* (Jaglo et al. 2001; Jaglo-Ottosen et al., 1998), strawberry (Owens et al., 2002), potato (Pino et al., 2005), and poplar (Benedict et al., 2006) in non-acclimated or acclimated plants as well as in our studies with leaf and stem tissues of 35S::AtCBF1. Overexpression of *CBF1* controlled by cold-inducible promoter rd29A has not been reported. Unexpectedly, our results found that the rd29A::AtCBF1 line was not different in freezing tolerance from the WT after acclimation. One explanation for this could be that, while detectable, the transgene expression was not high enough to increase freezing tolerance. We suspect that the inability of the rd29A to induce strong expression of the transgene could be the reason and this will be discussed more fully in the discussions of the rd29A::AtCBF2 and rd29A::AtCBF3 lines. Another possibility could be that the gene was inserted in a position causing the interruption of some household gene whose mutation (due to interruption) negatively affected the tree fitness, therefore, the freezing tolerance was impaired. More transgenic lines having detectable transgene expression need to be analyzed to confidently draw a conclusion.

Leaf and stem tissues from three independent 35S::AtCBF2 lines grown in the greenhouse had no difference in freezing tolerance relative to the WT (Fig. 4.3C). Following four weeks of cold acclimation at 2°C, the WT and transgenic lines had increased freezing tolerance whereas those in the non-acclimated condition and the rd29A::AtCBF2 lines with cold acclimation were significantly less cold tolerant (Fig.

4.3C). Four independent rd29A::AtCBF2 lines had similar freezing tolerance to the WT when grown under non-acclimating or acclimating condition (Fig. 4.3D). CBF2 was reported to have matching functional activities with CBF1 and CBF3 based on the observation of their effect on freezing tolerance, morphology, growth, and composition change of compatible solutes such as soluble sugars and proline (Gilmour et al., 2004). However, when a *cbf2* mutant without the *CBF2* gene function was analyzed it was found that CBF2 was a negative regulator of CBF1 and CBF3. Therefore, a proper induction of the downstream cold-regulated genes could be achieved through the combination effect of the three genes (Navillo et al., 2004). Our freezing test results with 35S::AtCBF2 lines or rd29A::AtCBF2 lines were generally consistent with Navillo's conclusions. We postulate the following situations occurring:

- For 35S::AtCBF2 lines, under non-acclimating condition. While *AtCBF2* was constitutively expressed, the endogenous CBF1 and CBF3 had no or little expression and therefore *AtCBF2*'s effect was not seen. In contrast, under acclimating conditions, endogenous CBF1 and CBF3 expression were induced in the WT and transgenic lines, but down regulated by AtCBF2 to some extent in the latter, leading to the difference in increased freezing tolerance between the two.
- For rd29A::AtCBF2 lines, under non-acclimating conditions there was no effect of AtCBF2 effect because of the presence of a cold-inducible promoter. With acclimating conditions, there was still no difference in freezing tolerance between the WT and transgenic lines and suggesting that the rd29A promoter doesn't have the capacity to strongly induce transgene expression.

The most significant increases in freezing tolerance were found in the four tested transgenic lines overexpressing *35S::AtCBF3*. There was an average increase of 5.3°C cold tolerance under non-acclimating conditions and 6.1°C after acclimation in leaf tissues, and 3.0°C and 6.3°C in stem tissues, respectively (Fig. 4.3E). For the six rd29A::AtCBF3 lines, as expected none showed an increase in freezing tolerance when non-acclimated. However, three of the six were significantly more tolerant to freezing (+2.5°C and +3.3°C for leaves and stem, respectively) than the WT after acclimation (Fig. 4.3F). The extent of increase in freezing tolerance by overexpression under non-acclimation conditions was greater for *35S::AtCBF3* than for *35S::AtCBF1*, with the former having an average increase of 5.3°C and 3.0°C for leaf and stem tissue, respectively, vs. the latter 3.1°C and 2.2°C. Transcriptional activators CBF1 and CBF3 were reported to have matching functional activities based on their similar effects on increasing the freezing tolerance of non-acclimated and cold-acclimated plants, the biochemical composition, morphology and development of the transgenic plants, and cell ultrastructure, but they may not control the identical regulon (Gilmour et al., 2000). Therefore, the different extent of the freezing tolerance enhancement in poplars could have reflected the differences between the regulons activated by CBF1 and CBF3 overexpression. It also may be a factor of a limited number of transgenic lines recovered had different levels of *AtCBF1* and *AtCBF3* expression. For whichever reason, in our study of the two 35S::AtCBF1 lines and four 35S::AtCBF3 lines, overexpression of *AtCBF3* constitutively in poplar gave greater freezing tolerance improvement than did

AtCBF1. Overexpressing *rd29A::AtCBF3* had less effect than *35S::AtCBF3*. First of all, only three of the six transgenic lines showing detectable transgene expression had greater freezing tolerance than WT after acclimation. Second, the extent of the freezing tolerance improvement by overexpression of rd29A::AtCBF3 (2.5 °C and 3.3 °C for leaf and stem tissue, respectively) was less than that from 35S::AtCBF3 (5.3 °C and 3.0 °C for leaf and stem tissue, respectively). Considering that four weeks of cold acclimation is long enough for low temperature induction, we reason that the capacity of *rd29A* to induce transgene expression is not as strong as that of constitutive promoter 35S. As we discussed previously, this could also explain why rd29A::AtCBF1 did not have any increase in freezing tolerance as CBF1 protein activity was not as strong as CBF3.

4.4.3. Effects of overexpressing *AtCBF1* and *AtCBF3* on poplar plant growth

The WT and those AtCBF transgenic poplars that had significantly increased freezing tolerance with non-acclimating condition, i.e. two 35S::AtCBF1 lines, four 35S::AtCBF3 lines or after cold acclimation, i.e. three rd29A::AtCBF1 lines, were evaluated for their growth rate. When grown *in vitro*, while the two 35S::AtCBF1 lines and four 35S::AtCBF3 lines grew much slower than the WT plants with shorter internodes and fewer leaves, the three rd29A::AtCBF3 lines were not stunted (Fig. 4.4A). The plant height of three plants from each line was measured after the rooted plants were transplanted into the soil and grown in the greenhouse for 100 days. Initially, the 35S::AtCBF1 lines had retarded growth but they gradually caught up with the WT after

about 50 days in greenhouse (Fig. 4.4B). The 35S::AtCBF3 lines also grew much slower than the WT, and the difference between the two became greater the longer they were allowed to grow in the greenhouse (Fig. 4.4C). The transgenic lines overexpressing *AtCBF3* controlled by rd29A promoter did not have a different rate of growth than the WT (Fig. 4.4D).

Prostrate growth habit is a phenotype found to be associated with cold acclimation and increased freezing tolerance in flax (Omran et al., 1968) and wheat (Roberts, 1990). Dwarf phenotypes that have increased freezing tolerance have been found in transgenic *Arabidopsis* (Gilmour et al., 2000; Liu et al., 1998) overexpressing 35S::*AtCBF3* and transgenic tomato (Hsieh et al. 2002a, Hsieh et al. 2002b) and poplar (Benedict et al., 2006) overexpressing 35S::*AtCBF1* under normal growing conditions. The coincident occurrence of increased freezing tolerance and stunted growth in naturally acclimated plants and unacclimated *AtCBF1/AtCBF3* transgenic plants, further supports the critical role played by CBF1 and CBF3 as key regulators in change of genes expression during cold acclimation. Our observations on growth characteristics agree with the previously mentioned results. It is interesting that, under normal temperatures in the greenhouse, 35S::AtCBF1 lines changed from slow, stunted growth to normal growth after 50 d, while 35S::AtCBF3 lines remained dwarf. In *Arabidopsis*, while *CBF1* overexpression induced *COR* gene expression and increased plant freezing tolerance without a low temperature stimulus there was not any negative effect of *CBF1* overexpression on plant

growth (Jaglo-Ottosen et al., 1998). *CBF1* overexpression, however, did produce dwarf transgenic *Arabidopsis* plants (Gilmour et al., 2000; Liu et al., 1998).

Based on the positive correlation between the level of CBF expression and the level of expression of the target COR gene (Liu et al., 1998; Gilmour et al., 2000), and the degree to which the plants were stunted (Liu et al., 1998), Gilmour et al. (2000) proposed that the difference effect on growth and development between *CBF1*- and *CBF3*-expressing plants was due to the different levels of CBF-induced target gene expression. Our results showed different degrees of increased freezing tolerance between 35S::AtCBF1 and 35S::AtCBF3 that might arise from the different level of *CBF* expression and the level of expression of *COR* gene. This may have led to the different growth characteristics, such as the ability to resume normal growth after beginning with stunted growth as described above. Using the stress-inducible rd29A promoter for the overexpression of CBF1 in transgenic *Arabidopsis* (Kasuga et al., 1999) and tobacco (Kasuga et al., 2004) minimized the negative effects on plant growth while at the same time improving freezing tolerance. In our study, rd29A::AtCBF3 lines had normal plant growth under normal temperature, and, while not as tolerant to freezing as 35S::AtCBF1, they did give a greater freezing tolerance than the WT after acclimation.

4.4.4. Effects of overexpression of *AtCBF* genes on changes of soluble sugar and proline levels in leaf tissue

Soluble sugar and proline levels in leaves of acclimated and non-acclimated plants from the WT and transgenic lines used in the growth characteristic study were measured using an HPLC and a spectrophotometer, respectively.

Under non-acclimating conditions, the levels of glucose, fructose, and sucrose in 35S::AtCBF1 was approximately 3-4, 6-8, and 2-3 fold-higher, respectively, than those in the WT (Fig. 4.5A, Fig. 4.5B, and Fig. 4.5C). For 35S::AtCBF3 lines, the figures obtained under the same condition were 4-6, 11-16, and 5-7 fold-higher, respectively (Line 1-4 in Fig. 4.5D, Fig. 4.5E, and Fig. 4.5F). Under non-acclimated condition, there were no significant changes in the sugar levels found in rd29A::AtCBF3 lines (Line 5-7 in Fig. 4.5D, Fig. 4.5E, and Fig. 4.5F). Four weeks of low, nonfreezing temperature promoted a significant accumulation of glucose, fructose and sucrose in leaves of the WT and transgenic lines overexpressing *35S::AtCBF1* and *35S::AtCBF3*, with each sugar level at least twice the values as those of the unacclimated leaves. With cold acclimation, the sugar levels of the transgenic lines overexpressing *rd29AS::AtCBF3* were not as high as those expressing *35S::AtCBF3* but were significantly higher than the WT.

Proline levels in WT and transgenic lines under non-acclimated condition and during cold acclimation are shown in Fig. 4.6. Changes in proline levels followed a trend similar to those for the soluble sugars under acclimated and non-acclimated conditions.

The accumulation of compatible solutes such as soluble sugars (Guy et al., 1992; Koster and Lynch, 1992; McKown et al., 1996; Ristic and Ashworth, 1993; Wanner and Junttila, 1999) and proline (Dorffling et al., 1997; Koster and Lynch, 1992; Levitt, 1980; Wanner and Junttila, 1999; Xin and Browse, 1998) in plant organs during cold acclimation has been widely documented. Such accumulation contributes to freezing tolerance enhancement by reducing the rate and extent of cellular dehydration, sequestering toxic ions, and/or protecting macromolecules against dehydration-induced denaturation (Steponkus, 1984). Overexpression of *CBF3* in *Arabidopsis* correlated with the accumulation of soluble sugar and proline and increases in freezing tolerance (Gilmour et al., 2000). Glucose, fructose, sucrose, and raffinose are soluble sugars commonly found to accumulate during cold acclimation (Gilmour et al., 2000; Koster and Lynch, 1992). Our study in the WT and the CBF1 and CBF3 transgenic poplars, while not detecting raffinose, agreed with other studies regarding the change in the soluble sugar levels brought on by cold acclimation and the overexpression of *AtCBF1* and *AtCBF3* in poplar. Gilmour et al. (2000) found that CBF3 had no effect on the transcription of the genes controlling sucrose levels in plant cells, such as Suc-phosphatesynthase (SPS) and Suc-synthase (SuSy) (Winter and Huber, 2000), and there have been no reports of such a relationship between CBFs and soluble sugars. For proline, one of the two known genes controlling its expression, *P5CS* (Strizhov et al., 1997; Yoshiba et al., 1997), was upregulated by *CBF3* overexpression in *Arabidopsis* (Gilmour et al., 2000). However, whether the proline accumulation is the cause or consequence of the enhanced freezing tolerance remains controversial (Gilmour et al., 2000; Wanner and Junttila, 1999). These

uncertainties need to be further deciphered to give a better picture of the pathways controlling cold acclimation, CBF regulon, and sugar and proline changes, etc. Our first finding of the key role that CBF1 and CBF3 played in the coordinated regulation of biochemical alteration of proline and sugars and freezing tolerance enhancement in a woody perennial should help with this deciphering.

4.5. Conclusions

This study found that the integrated control of cold acclimation with CBF1 and CBF3, previously reported in *Arabidopsis* is also present in a woody perennial, *P. tremula* × *alba*, as demonstrated by the key role they play in inducing multiple biochemical, morphological, and physiological changes that act to increase plant freezing tolerance. Gene construct *AtCBF1* showed the lowest efficiency in generating CBF protein expressers. Overexpressing *AtCBF1*, *AtCBF3*, rather than *AtCBF2*, constitutively increased freezing tolerance of transgenic poplar under acclimated and non-acclimated conditions, as did the overexpression of *AtCBF3* induced by exposure to low-temperature. Based on the analysis from a limited number of transgenic lines, we found that *35S::AtCBF3* construct gave the greatest degree of increased freezing tolerance, unfortunately this construct also had the most serious stunted growth. Using the rd29A promoter gave us less gain in freezing tolerance but avoid stunting under normal growth conditions. *AtCBF1* and *AtCBF3* overexpression was correlated with an increase of cryoprotectant levels (proline and soluble sugars), along with an improvement in freezing

tolerance. Taking these results together, we propose that the overexpression of CBF transcription factor family members (*CBF1* and *CBF3*) would be a reasonable and feasible way to improve freezing tolerance of a woody perennial by genetically engineering.

4.6. Reference

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Table 4.1 Successful enhancement of plant freezing tolerance by overexpression of *CBF* genes reported in the literature.

Gene	Origin	Transgenic species	Citation
CBF1	*Arabidopsis*	*Arabidopsis*	Jaglo-Ottosen et al. (1998)
CBF1	*Arabidopsis*	*Arabidopsis*	Kasuga et al. (1999)
CBF3	*Arabidopsis*	*Arabidopsis*	Liu et al. (1998)
CBF3	*Arabidopsis*	*Arabidopsis*	Gilmour et al. (2000)
CBF1, 2, 3	*Arabidopsis*	canola	Jaglo et al. (2001)
CBF1	*Arabidopsis*	strawberry	Owens et al. (2002)
CBF4	*Arabidopsis*	*Arabidopsis*	Haake et al. (2002)
OsCBF3	rice	*Arabidopsis*	Dubouzet et al. (2003)
CIG-B (*PaCBF*)	sweet cherry	*Arabidopsis*	Kitashiba et al., (2004)
HvCBF3	barley	*Arabidopsis*	Amundsen (2004)
BNCBF5, 17	canola	canola	Savitch et al. (2005)
CBF1	*Arabidopsis*	potato	Pino et al. (2005)
CBF1	*Arabidopsis*	aspen	Benedict et al. (2006)
OsDREB1 (*OsCBF*)	rice	rice	Ito et al. (2006)

Table 4.2 Efficiency of generating transgenic poplar (*Populus tremula* × *alba*) plants overexpressing *AtCBF1, AtCBF2,* and *AtCBF3,* driven by 35S and rd29A promoter, respectively.

Gene	Promoter	No. Northern positive/ PCR positive	Efficiency (%)	Average (%)
AtCBF1	35S	2/29	7	
AtCBF1	rd29A	1/10	10	8.5
AtCBF2	35S	3/10	30	
AtCBF2	rd29A	4/10	40	35.0
AtCBF3	35S	4/10	40	
AtCBF3	rd29A	6/10	60	55.0

Table 4.3 . Example of transgenic plants with improved freezing tolerance

Gene function	**Gene**	**Gene product**	**Transformed species**	**Reference**
Encoding enzymes that synthesize osmotic and other protectants	*codA*	choline oxidase (glycine betaine synthesis)	tobacco	Parvanova et al., 2004
	COR15a	cold induced gene	*Arabidopsis*	Steponkus et al., 1998
	P5CS	pyrroline carboxylate synthetase (proline synthesis)	tobacco	Parvanova et al., 2004
	SPE	spermidine synthase	*Arabidopsis*	Kasukabe et al., 2004

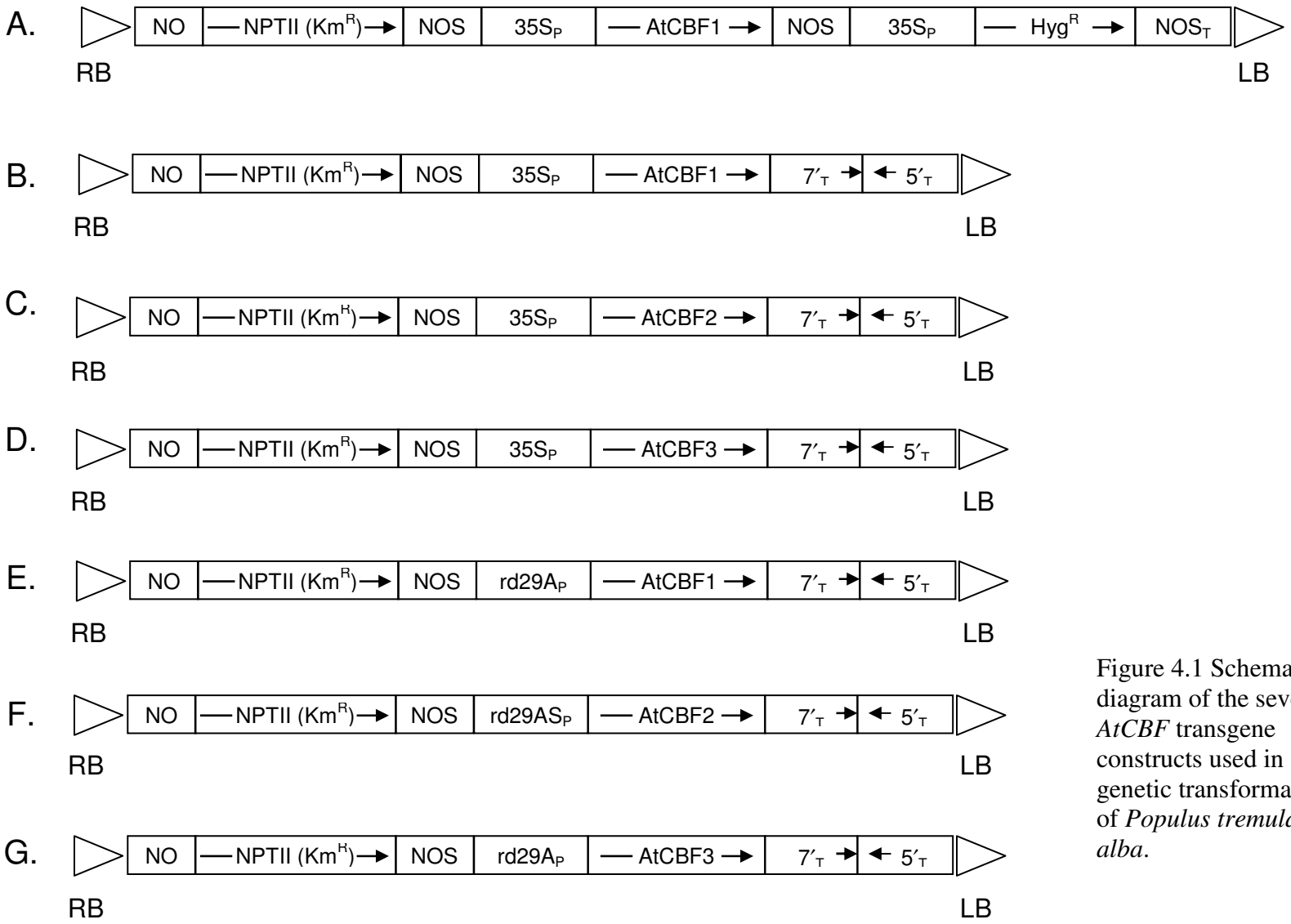


Figure 4.1 Schematic diagram of the seven *AtCBF* transgene constructs used in genetic transformation of *Populus tremula* × *alba*.

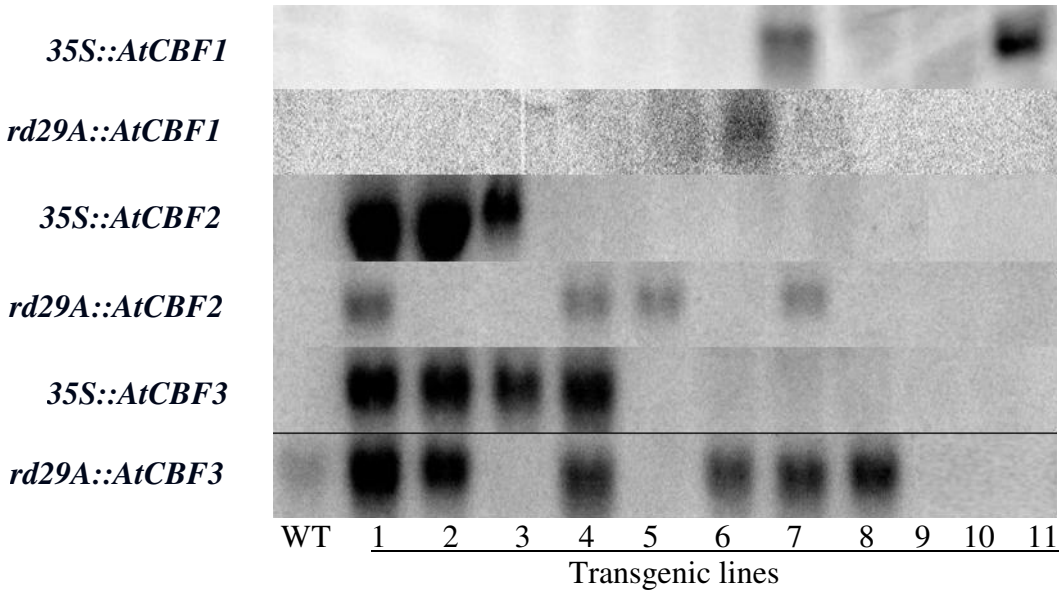


Figure 4.2 Expression analyses of the *AtCBF* genes in transgenic *Populus tremula* × *alba* plants by Northern blotting. Before isolating the total RNA, the transgenic lines overexpressing *AtCBFs* driven by 35S promoter were grown in the greenhouse (25°C) and those driven by rd29A promoter were grown in a cold room (2°C) for four weeks.

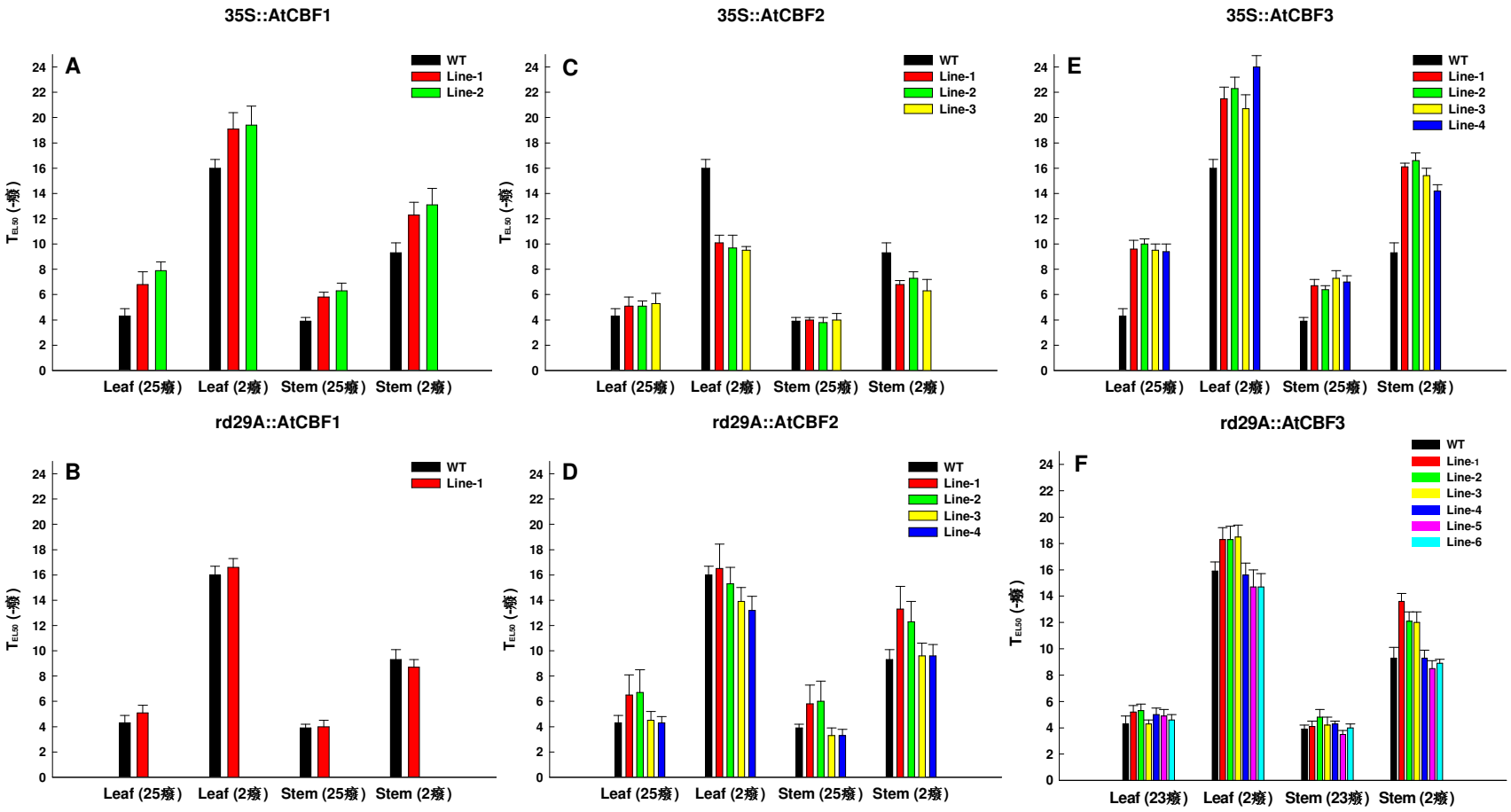
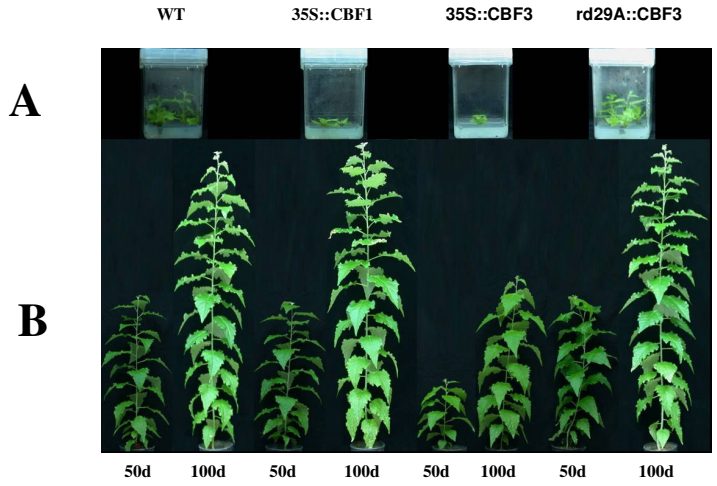
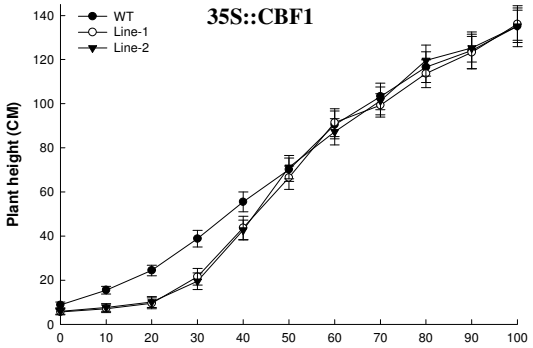
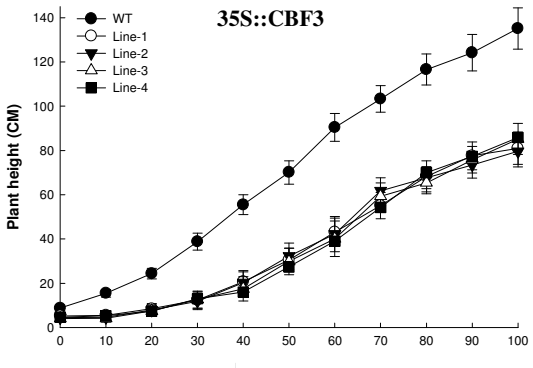


Figure 4.3 Freezing tolerance, based on TEL_{50} measurements, of leaf and stem tissues of WT and transgenic poplars (*Populus tremula* × *alba*) having detectable *AtCBF* transgene expression grown without acclimation (25°C) or with four weeks cold acclimation (2°C). A, WT and 35S::CBF1 lines; B, WT and rd29A::CBF1 line; C, WT and 35S::CBF2 lines; D, WT and rd29A::CBF2 lines; E, WT and 35S::CBF3 lines; F, WT and rd29A::CBF3 lines.







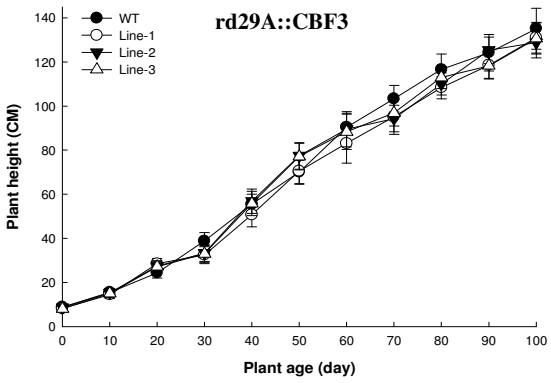
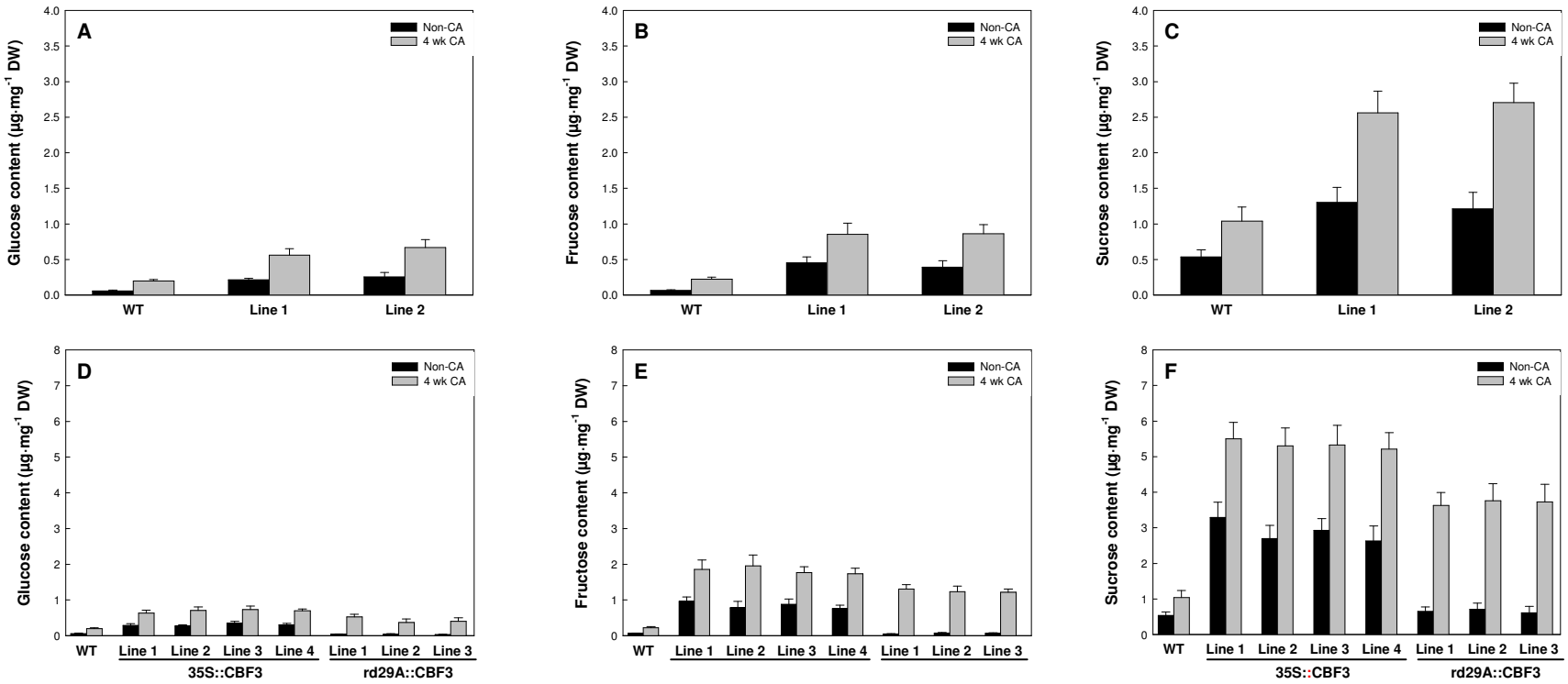


Figure 4.4 Growth characteristics of the WT and AtCBF-expressing transgenic *Populus tremula* × *alba* lines. (A) Morphology of 4-week old in vitro grown poplars. (B) Morphology of poplars grown in soil. (C), (D), and (E) Average plant height during 100-days growth on soil in wild-type and 35S::CBF1, 35S::CBF3, and rd29A::CBF3, respectively.


A
Non-CA
4 wk CA
Glucose content (µg·mg-1 DW)
WT
Line 1
Line 2
B
Frucose content (µg·mg-1 DW)
C
Sucrose content (µg·mg-1 DW)
D
Glucose content (µg·mg-1 DW)
Line 3
Line 4
35S::CBF3
rd29A::CBF3
E
Fructose content (µg·mg-1 DW)
F
Sucrose content (µg·mg-1 DW)

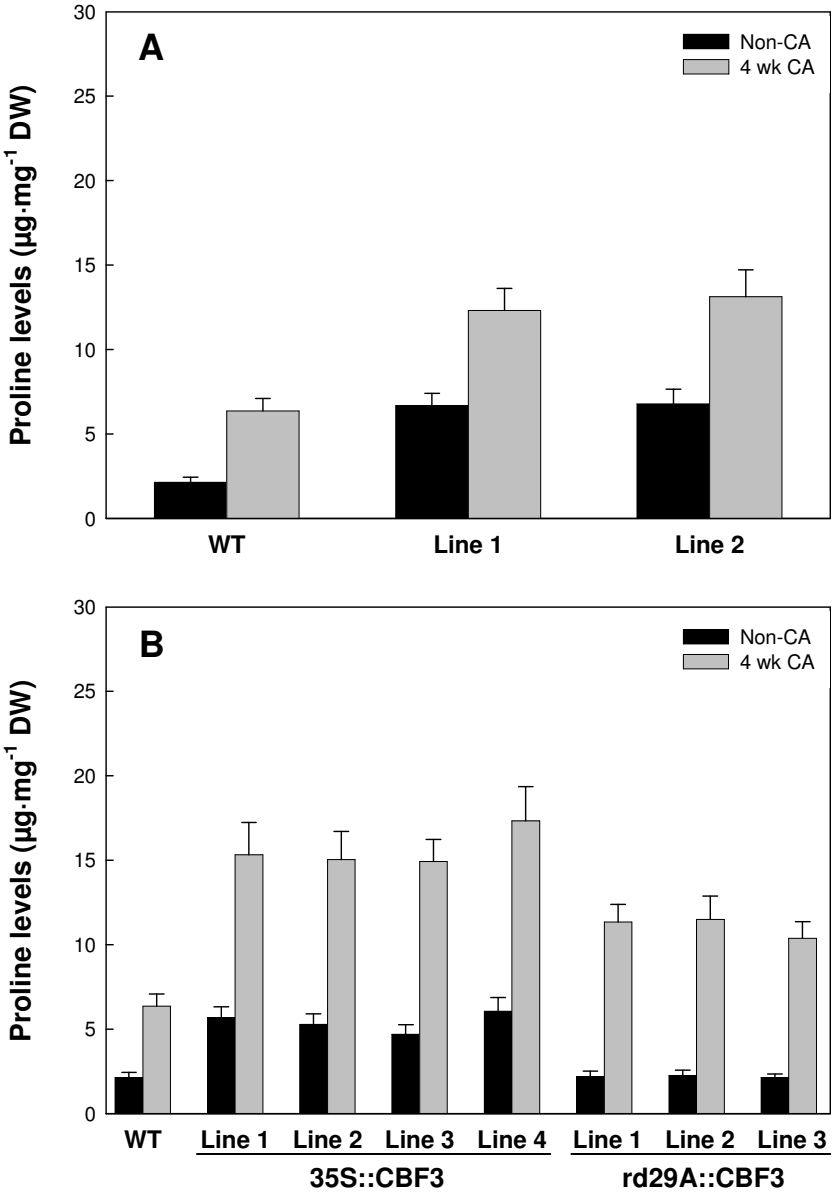


Figure 4.6 Effect of overexpression of *AtCBF1* and *AtCBF3* on proline levels in leaf tissues of the WT and transgenic *Populus tremula* × *alba* lines that had a significant enhancement in freezing tolerance. Leaf tissues were sampled from nonacclimated plants grown in the greenhouse (25°C) (Non-CA) and cold acclimated plants grown in a cold room at 2°C for 4 weeks (4 wk CA). A, Proline levels of the WT and 35S::CBF1 lines; B, Proline levels of the WT, 35S::CBF3, and rd29A::CBF3 lines.

Chapter 5. Conclusion

The goal of this research was to investigate the feasibility of improving the cold hardiness of 'Marion' blackberry through genetic engineering, i.e. *Agrobacterium tumefaciens*-mediated genetic transformation. An efficient transformation system for 'Marion' and a powerful transgene(s) that functions in the woody perennials are the prerequisite for eventual success. The work described in Chapter 2 and Chapter 3 of this dissertation was done in hopes of accomplishing the former task, and that in Chapter 4 was done for the latter.

The successful production of transgenic plants is based on the efficient regeneration of whole plants from the cultured cells containing chromosomes that have been integrated with the transgenes. Therefore, in the first step, an efficient *in vitro* shoot regeneration system was established for 'Marion' using leaf and petiole explants, by optimizing plant growth regulators' concentrations and combination, mineral salt formulations, TDZ pretreatment conditions, etc., Optimum shoot formation was obtained when stock plants were incubated in the TDZ pretreatment medium for three weeks before culturing leaf explants on the regeneration medium (WPM with 5 µM BA and 0.5 µM IBA) in darkness for 1 week and then they were transferred to light photoperiod (16-hour photoperiod at photon flux of ≈50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 23 °C $\pm$ 2 °C for 4 weeks**.** Under these conditions, about 70% of leaf explants formed approximately 40 shoots per petri dish that could be harvested and rooted to form plantlets.

Following the establishment of the *in vitro* shoot regeneration system, a series of factors that could affect the production of transgenic 'Marion' were examined comprehensively and systematically based on the transient expression of GUS gene and tissue necrosis that would reduce the regeneration potential of transformed cells. LBA4404, harboring superbinary vector pTOK233, was the most efficient among all the combinations of *Agrobacterium* strains and plasmid vectors tested. Factors that would enhance T-DNA delivery without sacrificing the regeneration potential of leaf explants were identified and optimized, including duration (30") and intensity (60% power output) of sonication treatment, type (Sequestrene iron 138), concentration (200 $mg \bullet L^{-1}$) and usage (applied in the cocultivation medium) of thiol group containing compounds, *Agrobacterium* cell density (optical density 2.0 at 600 nm), duration of inoculation (3'), concentration of virulence gene inducer acetosyringone in the inoculation (100 µM) and cocultivation (600 µM) medium, type (Tween 20) and concentration (0.1%) of surfactant in the inoculation medium, duration (3 days) of cocultivation. Vacuum treatment, which has enhanced the transformation of many plant species, did not work with 'Marion' leaf explants. By combining the best conditions for each parameter we examined, the frequency of transformation, expressed as percentage of explants showing blue spots, was improved four times higher than what we had had at the beginning. However, our effort to recover transgenic shoots was not successful. Our protocol will be valuable information for the further efforts to obtain transgenic 'Marion' blackberry.

Overexpression of *Arabidopsis* transcription factor CBF genes has been shown to increase freezing tolerance of a number of annual plants, but little research on the CBF regulon had been done in temperate woody perennials. Considering that blackberry is a woody perennial and it was rather difficult to obtain transgenic 'Marion', it was important to use a woody plant model to find out if CBFs can increase freezing tolerance of woody plants as they did in other annual species, which would lead us to determine whether or not the further effort to establish a transformation system for 'Marion' would be worthwhile. A hybrid aspen clone (*P. tremula* × *alba*) was used to overexpress three members of CBF family (*Arabidopsis* CBF1, CBF2, and CBF3), each driven by either a constitutive promoter (35S) or a stress-inducible promoter (rd29A). Results showed that *AtCBF1* and *AtCBF3* constitutively increased freezing tolerance of transgenic poplar under acclimated and non-acclimated conditions, as did the overexpression of *AtCBF3* induced by exposure to low-temperature. *35S::AtCBF3* lines had the greatest degree of increased freezing tolerance, but at the same time showed the most serious stunted growth. Using the rd29A promoter gave us less gain in freezing tolerance but there was no stunting given normal environmental conditions for plant growth. *AtCBF1* and *AtCBF3* overexpression was correlated with an increase of cryoprotectant levels (proline and soluble sugars), along with an improvement in freezing tolerance.

Combining all of these results, we conclude that the establishment of an *in vitro* regeneration system and the optimization of factors influencing transformation for

‘Marion’ should contribute to the final success in producing transgenic ‘Marion’. Once such success is achieved, overexpression of *CBF3* driven by rd29A promoter in ‘Marion’ would be a reasonable and feasible way to improve its cold hardiness.