DIFFERENTIAL EXPRESSION OF COLD RESISTANT RELATED GENES IN *POPULUS NIGRA* L. CLONES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

EVRİM ZEYBEK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

BIOLOGY

JULY 2014

Approval of the thesis:

DIFFERENTIAL EXPRESSION OF COLD RESISTANT RELATED GENES IN *POPULUS NIGRA* L. CLONES

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ABSTRACT

DIFFERENTIAL EXPRESSION OF COLD RESISTANT RELATED GENES IN *POPULUS NIGRA* L. CLONES

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July 2014, 185 pages

Poplar has been adopted as a model perennial woody species for forest tree genetics to understand molecular processes of growth, development and responses to environmental stresses. In this study, seasonal changes in antioxidant enzymes activities including glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and catalase (CAT) were examined in black poplar (*Populus nigra* L.) clones. It was found that GR, APX and DHAR activities increased with low air temperatures in winter, whereas they decreased under high temperatures. CAT activity indicated exactly the opposite seasonal variations. The identification and characterization of differentially expressed genes responsive to low temperatures were also carried out by using microarray techniques. Although 4421 genes differentially expressed in cold resistant genotype, 4187 genes were significantly expressed in cold sensitive genotype (fold change ≥ 5, p value ≤ 0.05). It was observed that especially the genes related with carbohydrate metabolic processes and sugar transport activities, i.e., beta-galactosidase, beta-1,3-glucanases and monosaccharide transporters were significantly up-regulated in cold resistant genotype. Glutathione peroxidases and gibberellin 20-oxidase were also over-expressed as defense related genes. In cold sensitive genotype, especially transcription factors including AP2 domain containing protein, WRKY-type DNA

binding protein and myb-like protein were significantly up-regulated. Genes involved in scavenging of reactive oxygen species, i.e., cytochrome P450, 5-oxoprolinase-like protein and metallothioneins were also highly expressed. These genes identified by comparative transcriptome analyses may play key roles in protecting black poplars against low temperature stress.

Informations generated from this study can be used for further understanding of the molecular mechanisms of cold tolerance in black poplars. Low temperature related antioxidant enzyme activities and gene expression data could be effectively used in breeding programmes for the purpose of high productivity in low temperature areas.

Keywords: *Populus nigra*, low temperature, antioxidant enzymes, gene expression

ÖZ

POPULUS NIGRA L. KLONLARINDA SOĞUĞA DİRENÇLİ GENLERİN FARKLI İFADELERİ

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Temmuz 2014, 185 sayfa

Kavak ağaçları, büyüme, gelişme ve çevresel uyarılara karşı verilen cevaplarda meydana gelen moleküler olayları anlayabilmek için kullanılan model organizmalar olarak kabul edilmektedir. Bu çalışmada, karakavağın düşük sıcaklık koşullarıyla başa çıkabilme mekanizmasını anlamak için ilk olarak glutatyon redüktaz (GR), askorbat peroksidaz (APX), dehidroaskorbat redüktaz (DHAR) ve katalaz (CAT) antioksidan enzimlerinin mevsimsel aktivite değişimleri incelenmiştir. GR, APX ve DHAR aktiviteleri düşük sıcaklıklar altında artarken, hava sıcaklığının yükselmesiyle birlikte enzim aktiviteleri azalmaktadır. CAT enzim aktivitesi ise tam tersine bir mevsimsel değişim göstermektedir. Soğuğa dayanıklı ve hassas klonların farklı sıcaklıklar altındaki gen ifadeleri mikroarray yöntemi aracılığıyla incelenmiş olup, karakavak klonlarında soğuk stresine karşı etkin genler belirlenmiştir. Soğuğa dayanıklı genotipte 4421 gen istatistiksel olarak anlamlı şekilde ifade edilirken, soğuğa hassas genotipte 4187 gen belirlenmiştir (kat değişimi ≥ 5 ve p değeri ≤ 0.05). Soğuğa dirençli genotipte, özellikle beta-galaktosidaz, beta-1,3-glukanaz ve monosakkarit taşıyıcısı gibi karbonhidrat metabolizması ve şeker iletimi ile ilgili genler istatistiksel olarak önemli düzeyde ifade edilmiştir. Bununla birlikte, savunma sistemi ile ilgili olan glutatyon peroksidaz ve gibberellin 20-oksidaz genleri de

yüksek seviyede ifade edilmiştir. Soğuğa hassas genotipte ise, özellikle AP2 domain içeren protein, WRKY-tip DNA bağlanma proteini and myb-benzer protein gibi transkripsiyon faktörlerinin gen ekpresyonunda artış meydana gelmiştir. Reaktif oksijen türlerinin detoksifikasyonunda görevli olan sitokrom P450, 5-oksoprolinaz ve metallotionein proteinleri de istatistiksel olarak anlamlı düzeyde ifade edilmiştir. Karşılaştırmalı transkriptom analizleri ile belirlenen bu genler, karakavağın düşük sıcaklık stresine karşı tolerans göstermesinde önemli roller oynamaktadır.

Bu çalışmadan elde edilen bilgiler, karakavağın soğuk koşullar altında yaşayabilmesi için gerekli olan moleküler mekanizmaların daha iyi anlaşılmasına katkı sağlayacaktır. Düşük sıcaklık ile ilgili antioksidan enzim aktiviteleri ve gen ifade verileri, soğuğa karşı dayanıklı bireylerin belirlenmesi amacıyla kullanılabilecek ve böylece karakavak ıslah programı açısından yeni bir döneme girilecektir.

Anahtar Kelimeler: *Populus nigra*, düşük sıcaklık, antioksidan enzimler, gen ifadesi

To my son, my life, EFE…

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Zeki Kaya for his support, guidance and advice throughout the research. I would also like to express my regards to Assoc. Prof. Dr. Sertaç Önde and Prof. Dr. Ş. Şebnem Ellialtıoğlu for their great encouragements, suggestions and criticism all through my thesis. I express my sincere appreciation to all members of thesis examining committee for their criticism and comments.

I would like to thank to Dr. Metin Karadağ, Central Anatolian Forestry Research Institute and Poplar and Fast-Growing Forest Trees Research Institute for their collaboration. Dr. Akkın Semerci, Ercan Özyürek and Alptekin Karahan who help with their broad knowledge about field observations are also gratefully acknowledged.

I would also like to express my special thanks to Kubilay Yıldırım, Asiye Uluğ, Pelin Acar and Bircan Taşkıran for their great help and friendship during collection of samples and experiments.

I would like to give my special thanks to Assist. Prof. Dr. Ayten Tekpınar and Çiğdem Kansu for their intimacy, support and patience. Moreover, all my colleagues at laboratory are acknowledged for their friendship.

Last but not least, I would like to express my deepest appreciation to my family, especially my mom, for their endless love, patience, insight, support and encouragements all through my life. I would also like to thank to Onur Yıldırım, Gizem Yıldırım and Deniz Yıldırım for their great help during collection of samples and experiments.

Finally, special thanks go to my sweetheart, Serdar, for his love, support, patience and help in software programming.

This study was supported by the State Planning Organization (DPT) Grant No: BAP-08-11-DPT-2002K120510. It was also financially supported by The Scientific and Technological Research Council of Turkey (TUBITAK) TOVAG-110O570 and General Directorate of Forestry, Ankara, Turkey.

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ROS	Reactive Active Oxygen Species
SOD	Superoxide dismutase
APX	Ascorbate peroxidase
GR	Glutathione reductase
DHAR	Dehydroascorbate reductase
MDHAR	Monodehydroascorbate reductase
CAT	Catalase
GPX	Guaiacol peroxidase
AsA-GSH	Ascorbate-glutathione cycle
GSH	Reduced glutathione
GSSG	Oxidized glutathione
DHA	Dehydroascorbic acid
AA	Ascorbic acid
ABA	Abscisic acid
BF	Bud flush
LA	Leaf abscission
EDTA	Ethylenediaminetetraacetic acid
RNase	Ribonuclease
DEPC	Diethyl pyrocarbonate
COR	Cold-regulated genes
DRE	Dehydration responsive elements
DREB	Dehydration responsive element binding
CRT	C-repeats
AP2	APETALA2
MYB	Myeloblastosis
LRR-RLK	Leucine-rich repeat receptor like kinases
V-PPase	H^+-translocating inorganic pyrophosphatase
MT	Metallothionein
ACC oxidase	1-Aminocy- clopropane-1-carboxylate oxidase
HCA	Hierarchical clustering algorithm

CHAPTER 1

INTRODUCTION

Forests cover 31% of total land area which is just over 4 billion hectares (FAO, 2010). They contain more than 90% of terrestrial biodiversity and provide suitable habitat for those species. There are lots of economic and ecological values ensured by forests, such as carbon sequestration, protection of watershed, increased air quality, recreational habitats and basic energy needs, i.e., fuelwood. In addition to these significances, trees ensure a chance to study biological processes which is not found in other plant species. Forest trees include adaptive gene complexes related with long-lived perennial growth. Population dynamics, adaptation on geographic and long-term temporal scales and the production of extensive secondary growth are other important issues which may be searched by using forest trees.

The genus *Populus*, includes poplars, cottonwoods and aspens, is the most abundant woody genus found in temperate forests throughout the world (Rae *et al*., 2007). It has been adopted as a model perennial woody species for forest tree genetics due to *Populus trichocarpa* genome sequencing (Tuskan *et al*., 2006), its small genome size, availability of genetic maps, high growth rate, ease of clonal propagation, suitability for genetic transformation, *in vitro* propagation capabilities (Wullschleger *et al*., 2002) and the possibility of obtaining interspecific hybrids (Taylor, 2002). Because of these features, *Populus* is convenient to study tree biochemistry, physiology, morphology, genetics and molecular biology.

There are four native poplar species, i.e., *Populus nigra* L. (black poplar), *Populus tremula* L. (aspen poplar), *Populus alba* L. (white poplar) and *Populus eupratica*

Oliv. (euphrate poplar) in Turkey. Black poplar has a wide distribution along rivers and streams. Aspen stands naturally occur in mountaineous areas throughout Anatolia. White poplars are seen along watercourses and in areas with difficult conditions (drainage, saltiness and heavy soil etc.). Euphrate poplar is found along the Euphrate, Goksu and Botan rivers in south and southeast Turkey (Toplu, 2008).

Black poplar has considerable contributions in both rural and national economies in Turkey. Therefore, *Populus nigra* is the most widely studied and commercialized one among these species. *Populus nigra* var. i*talica*, *Populus usbekistanica* var. *afghanica* and *Populus nigra* var. *caudina* are the most commonly cultivated varieties of black poplar (Toplu, 2008).

1.1 *Populus nigra* L. (Black Poplar)

Black poplar (*Populus nigra* L., x = 19, 2n = 2x = 38) is a pioneer tree species of the riparian forest ecosystem. It has also been cultivated on private lands and has a wide distribution in Turkey. Black poplar is an important species in terms of social, economic and ecological interest. It provides significant contributions to the national and the rural economy.

1.1.1 Taxonomy of Black Poplar

Black poplar is an angiosperm which belongs to genus *Populus* of the *Salicaceae* family. Although there are some disagreements about the number of species in the genus *Populus*, 29 species were accepted in recent times according to Eckenwalder (1996) classification scheme. These species are subdivided into 6 sections based on relative morphological similarity and crossability. These sections, i.e., *Abaso, Turanga, Leucoides, Aigeiros, Tacamahaca* and *Populus*, are morphologically and ecologically quite distinct groups. The major barriers for hybridization in the genus arise between the sections (Eckenwalder, 1996).

Black poplar belongs to the *Aiegiros* section of the genus *Populus*. However, there are some questions related with the status of black poplar and the relationships between the sections Aigeiros (cottonwoods) and Tacamahaca (balsam poplars). These two species are distinctive vegetatively and ecologically, but there are no sharp differences in the morphology of flowers and inflorescences. Because of these reasons, they could be grouped in a single section. Nevertheless, the recent evidences keep these species in different sections. This issue affects the placement of black poplar into the *Aiegiros* section. Actually, black poplar is very similar to some of the balsam poplars. It has also weird crossability relationships with both Northern American cottonwoods and balsam poplars (Eckenwalder, 1996). Black poplar has a wide range and there is clear human intervention in these areas. This fact makes the taxanomy of the species particularly complex (Cagelli and Lefèvre, 1997). New researches may be useful to solve the classification dilemmas at the section and species levels.

1.1.2 Biology and Ecology of Black Poplar

Black poplar is a typical tree species in riverine areas. This species is heliophilous, therefore it colonizes open areas by means of seeds or cuttings. Although black poplar usually forms local populations, isolated trees or large blocks of pure or mixed stands are also occured. It may reach 20-30 m (rarely 40 m) height with a diameter up to 1.5 m. Juvenile bark is usually clear and smooth, however mature bark is very rough, longitudinally fissured and has dark grey patterns. There are important variations of leaf shape even within a single tree. Although the blade of juvenile leaves is usually flat, sinuous formations occur through the margins (Figure 1.1). The blade of adult leaves is more or less similar to a rhombic form terminated by a narrow point. The upper surface of the blade is shiny dark green, glabrous, leathery and flat (Mottl and Úradníček, 2003).

Figure 1.1 Phenotypical profile of black poplar A. General appearance of black poplars in Behiçbey Nursery. B. Early formation of leaves C. Adult leaves* and D. Bark* (*The Bulgarian Flora Online, http://www.bgflora.net/families/salicaceae/ populus/populus_nigra/ populus_nigra_en.html, last visited on June 2014).

They are dioecious trees, thus male and female flowers are found on separate individuals. They grow fastly and arrive the reproductive stage around 10-15 years. Flowers are clustered in pendulous catkins. Male catkins are short in length (<10 cm) and thin (5 mm when open). There are 6 to 30 stamens (generally 10 to 20) with small purple anthers. Female catkins are green and also short in length (8 to 10 cm when receptive). There are approximately 50 flowers per catkin. Black poplar is wind pollinated and the subsequent ripening and seed maturation process lasts 4-6 week. The female catkins are lengthening during this period and swelling green fruit

capsules appear. About 20-50 fruit capsules and up to 225 seeds are developed on each catkin (Figure 1.2). Seeds surrounded at the base by long silky hair are dispersed by wind and water. There is a short viability and seeds need specific conditions for germination. Black poplar is affected by hydrological controls at every stages of its life cycle. Black poplar also readily propagates vegetatively from broken branches and cuttings.

Figure 1.2 Catkin, flower and fruit structures of black poplar. A. Male catkin B. Male flowers C. Female catkin D. Female flowers E. Fruits (The Bulgarian Flora Online, http://www.bgflora.net/families/salicaceae/populus/populus_nigra/populus_ nigra _en. html, last visited on June 2014).

1.1.3 Distribution of Black Poplar

Black poplar has a large distribution through Europe. Northern Africa and central and west Asia are also covered by this species. The natural range of black poplar

includes the British Isles, China, the Caucasus and large parts of the Middle East (Vanden Broeck, 2003). The natural distribution area of black poplar is given in Figure 1.3.

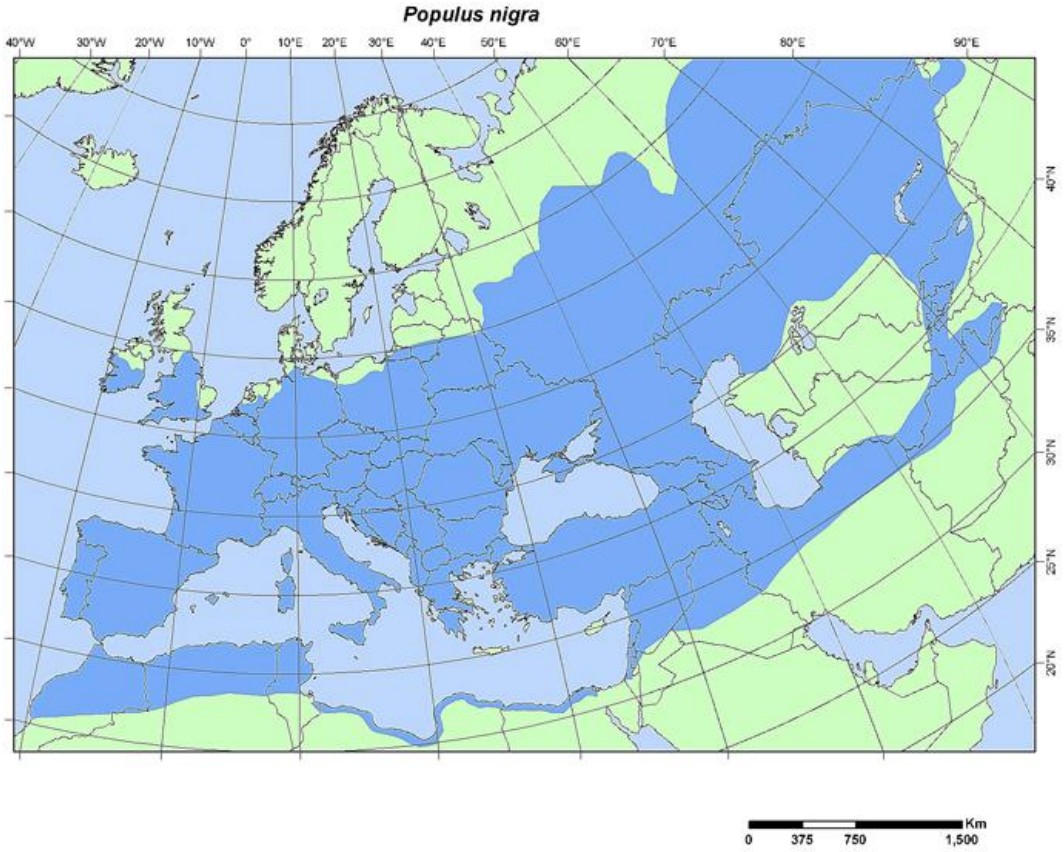


Figure 1.3 Distribution map of *Populus nigra* L. The blue color represents the natural distribution area of black poplar (EUFORGEN 2009, http://www.euforgen.org, last visited on June 2014).

1.1.4 Importance and Uses of Black Poplar

Black poplar is an important species in terms of social, economic and ecological interest. It plays a major role in breeding programmes throughout the world. Black poplar has lots of desirable features which provide the usage of this species as a parent pool in breeding programmes. Black poplar is hybridized with *Populus deltoides*, *Populus maximowiczii* and *Populus trichocarpa*. Resultant hybrids indicate high adaptability to various environmental conditions, rooting ability and resistance to diseases (Cagelli and Lefèvre, 1995). *Populus* × *euramericana* is the most common hybrid of black poplar. It was the result of hybridization between the American *Populus deltoides* and the European black poplar *(Populus nigra).* These hybrids combine some favourable characteristics of the American species (fast growth, good wood quality, resistance to relevant leaf diseases) with favourable traits of the black poplar (Cagelli and Lefèvre, 1995). The progenies of these hybrids can be easily propagated by cuttings. The breeding programmes with *Populus deltoides*, native *Populus nigra* and *Populus* × *euramericana* are also undertaken in Turkey to improve the wood quantity and quality. Fast growth, wood quality and frost resistance are desired characteristics and they may be combined in a hybrid. *Populus nigra* × *Populus nigra* and *Populus deltoides* × *Populus nigra* hybrids have also been selected by means of nursery and field clone trials in Turkey (Toplu, 2005).

Black poplar is widely distributed in East European for the purpose of soil protection and afforestation in polluted areas (Vanden Broeck, 2003). In some of these countries, up to 50% of the production of poplar wood comes from black poplar. The wood of black poplar is utilized as round wood for rural constructions, plywood, furniture, packaging, particle boards and matches (Cagelli and Lefèvre, 1995).

In addition to their valueble wood products, *Populus* genus is also important for carbon sequestration, bioremediation, nutrient cycling, biofiltration and diverse habitats. There has been an increasing interest about renewable energy sources and possible alternatives to fossil fuels in recent years. Poplar species have also received

attention for the production of biomass as a renewable energy source (Benetka *et al*., 2002).

In softwood flood plain forests, *P. nigra* is a keystone species because it is highly adapted to water dynamics and sediment movement. Black poplar is used as an indicator species for ecological purposes. Poplars have been accepted as centers for biodiversity in recent times. Most of threatened species in floodplain forests are dependent on poplars (Rotach, 2003). Therefore, conservation of the genetic resources of black poplar in riparian ecosystems is very important. The importance of restoration of these ecosystems has also been increased because poplar species can control the natural flood. Therefore, conservation of black poplar genetic resources is very important (Vanden Broeck, 2003).

1.1.5 Threats to Genetic Resources

Although black poplar is important in terms of social, economic and ecological interests, it is one of the most threatened species in Turkey and Europe. The major threats to black poplars' genetic resources may be grouped into three categories (Lefèvre *et al*., 2001). The first threat is human activities which change the riparian ecosystems across the distribution area. Urbanization of floodplain areas and displacement of the native poplar stands by agriculture are some of these activities changing the ecosystems. Regulation of floods is also a type of human activities which have affected the regeneration capacities of these species. Although the black poplars still show high regeneration capacities locally, there are significant threats for native populations in some regions of Europe. The second threat is overexploitation of the native individuals for wood sources and planting the faster growing hybrid poplars instead of them. Gene introgression is the final threat for genetic resources of poplar species. The pollen and seed pools in native stands are promoted by a few clones. The introduced hybrids and pure black poplars are under risks. There are also ideas that introduction of foreign species' genes into the native

black poplar could lower the effective population size and reduce the overall fitness of seedlings of native black poplar (Cagelli and Lefèvre, 1995).

1.1.6 Black Poplar in Turkey

Turkey has wide forest ecosystem diversity due to the variable climatic and topographic conditions. Black poplars are widely distributed in central, eastern and southeastern parts of Turkey. *Populus nigra* var. i*talica*, *Populus usbekistanica* var. *afghanica* and *Populus nigra* var. *caudina* are the most widely cultivated varieties of black poplar (Toplu, 2008).

Wood demand is increasing in Turkey. In fact, Turkey has started importing wood in recent years (Ministry of Forest and Water Affairs, 2012). Fast growing species may fill the gap, therefore poplar plantations seem to be the most efficient way to meet this demand. Wide natural distribution, considerable genetic variation, vegetative propagation and ease of hybridization properties make poplars favourable for plantation programs (Brown *et al*. 1996). Ease of planting and short rotation ages are other important features of poplars (Isık and Toplu, 2004).

Black poplar plays a significant role for both the rural and the national economy. Turkey has approximately 125000 hectares of poplar plantations. Although various black poplar clones constitute 60000 ha of these plantations, the remaining parts, i.e., 65000 ha are covered by hybrid poplars (*P.x.euramericana, P.deltoides*). Annual poplar wood production is about 3.5 million cubic meters in Turkey according to recent inventories. Approximately 40% of this production, i.e., 1.4 million m^3/year comes from black poplar clones (Ministry of Forest and Water Affairs, 2012).

Even though its economical and ecological values, black poplar is under risk of disappearance in native stands in Turkey due to human pressure. Both urban expansion and mismanagement of natural resources in rural areas endangers the existence of black poplars. A diverse genetic variation lies in the natural stands of

black poplar in Turkey. To conserve the genetic resources of black poplar, *in situ* and *ex situ* conservation programmes have been in progress in Turkey.

In situ conservation studies have been in progress under the scope of the European Forest Genetic Resources Programme (EUFORGEN). The major objective of this conservation is to sustain genetic diversity for retaining the species' adaptation to environmental changes. During these studies, five natural populations of black poplar have been found in the Eastern Anatolia, i.e., in the basins of the Melet, Kelkit, Munzur, Karasu and Pülümür rivers (Toplu and Kucukosmanoglu, 2003). As mentioned previously, there are many threats for native populations of black poplars that reduce the suitable areas for *in situ* conservation. Therefore, dynamic conservation studies should often be supplemented with *ex situ* conservation.

Ex situ conservation studies have been performed since 1962 by the Poplar and Fast Growing Forest Trees Research Institute in Izmit. However, wider conservation programme was started in 1990 throughout the Turkey. 310 black poplar individuals were selected in different regions of Turkey between 1990 and 1993. Two hundred and ninety-seven individuals from this collection were selected for the experimental nurseries in Izmit and Ankara. Two hundred and twenty-one clones were selected once again and this collection was duplicated in Erzurum. In 1995, 760 black poplar individuals were once more selected from their natural populations and used for establishment of experimental nurseries in Izmit, Erzurum and Ankara. Native clones and hybrids resulted from cross-pollination of *Populus nigra* × *Populus nigra* were included in those nurseries. Six clone banks and many clonal field trials have been established by using these germplasms in different climatic zones of the country to determine the growth performances and adaptability to different ecological conditions. According to these studies, 5 commercial black poplar individuals called Gazi, Anadolu, Kocabey, Geyve and Behiçbey have been determined and they were registered by the International Poplar Commission. During the conservation studies of black poplar, major priority has been given to selection of frost-resistant individuals (Toplu, 2005).

1.2 Low Temperature and Its Effects on Plants

Plants are sessile organisms and they encounter different environmental changes throughout their lifespan. However, plants have developed natural mechanisms during their evolution to overcome different types of abiotic and biotic stresses. Abiotic stress induces different types of responses such as morphological, biochemical and molecular changes which concern the survival of plants. Some of these abiotic factors are temperature, water availability, air quality, light intensity, mineral nutrient, salinity and heavy metals.

Plants quickly recognize these adverse alterations in their environment and rapid transient and/or seasonal responses are indicated. Genetic adaptation and acclimation are two phenomena contributing to the plant's tolerance of stress factors in their abiotic environment. Adaptation to the environment is described by genetic changes in the whole population. These changes have been fixed by natural selection over many generations. On the other hand, individual plants may also respond to environmental variations by changing their physiology or morphology. In this manner, they can better survive in that environment. Plants have developed strong perception and signal transduction pathways that are essential mechanisms to exist.

Among the limiting environmental conditions, low temperature or cold stress is one of the major abiotic stresses affecting the plants' life. It may affect plants in terms of germination, morphology, growth, reproduction, cell membrane, photosynthesis, water transport and yield. Low temperature includes chilling (0-15°C) and freezing (<0°C) temperatures that inhibit plants in many ways. Plant species differ from each other in terms of their ability to tolerate low temperature. In most cases, plants in temperate climatic regions are considered chilling resistant, while plants from tropical and subtropical origins are thought to be chilling sensitive.

Temperature values that are not optimal for growth and development but also not low enough for ice formation induce chilling injury. Changing metabolic processes, reduction in enzymatic activities and inhibition of photosynthesis are some of physiological disorders experienced by plant species under these temperature conditions (Allen and Ort, 2001). This type of injury also triggers reduced growth, discoloration and lesions of leaves, vitrified (translucent) foliage and wilting. If roots are chilled, there will be some problems with water absorption mechanisms. At that time, dehydration is promoted due to disruption of water uptake from the roots and a reduction in stomatal closure. As a result, the plants may wilt. Chilling temperatures also reduce the membrane fluidity. Membrane lipids are composed of unsaturated and saturated fatty acids. Lipids including saturated fatty acids solidify at higher temperatures than those of including unsaturated ones. Therefore, membrane fluidity is affected by the relative proportion of unsaturated fatty acids (Steponkus *et al*., 1993a). Chilling sensitive plants has a higher transition temperature due to higher level of saturated fatty acids. However, chilling resistant species have high amount of unsaturated fatty acids and therefore there is a lower transition temperature. When the membrane lipids become less fluid at low temperatures, their protein components cannot function normally. Under low temperatures, the fatty acids of membranes become unsaturated and the ratio of lipids and proteins are changed that affect the membrane fluidity and structure. Cellular functions are affected in different ways due to phase change of membranes. Increased ion leakage, inhibition of the ions exchange, prevention of osmosis and diffusion are induced by increased membrane permeability. All these factors trigger the disruption of cellular homeostasis (Farooq *et al.*, 2009). Plants have another problem in terms of energy production under chilling conditions. Low temperatures may reduce the photosynthesis capacity and affect chloroplast and thylakoid membranes. It also induces plastid swelling, thylakoid vesiculation, accumulation of lipids and finally disorder of plastids (Ishikawa, 1996; Kratsch and Wise, 2000). Major systems related with photosynthesis, such as thylakoid electron transport, carbon reduction cycle and stomatal conductance are also interrupted by chilling. Photosystem II is the primary area damaged due to low temperature. It also restricts the activities of Calvin cycle

enzymes, ATP synthase, RuBisCO regeneration and photophosphorylation (Allen and Ort, 2001). On the contrary, there is no effect on the rate of photochemistry at the photosystems. The excessive energy induces the reactive oxygen species (ROS) accumulation which results in oxidative stress if they are not removed from the cell (Asada, 1996). The generation of ROS is one the most important events under chilling stress. The accumulation of ROS will have deleterious effects especially on membranes that will result in ion leakage. Plants can cope with increased level of ROS by the help of non-enzymatic and enzymatic antioxidant systems. Another effect of low temperature is the reduction of carbon export from leaves that brings the soluble carbohydrates accumulation (Strand *et al*., 2003).

Freezing temperatures also induce numerous threats for plant growth and development. When the temperature decreases under 0°C, ice formation occurs in plants. Intercellular fluid has higher freezing point due to a difference in solute concentration. The intercellular spaces are areas in which ice formation are seen first (Thomashow, 1999). The ice accumulation in these areas may induce the cell disruption due to formation of adhesions with cell walls and membranes (Olien and Smith, 1977). Dehydration is another major threat for plants caused by intercellular ice formation. Ice formation considerably reduces the water potential. Therefore, unfrozen water within the cell moves from higher to lower water potential area (intercellular space). As a result, cellular dehydration and shrinkage occur which lead to the denaturation of some proteins and precipitation of other biomolecules (Levitt, 1980; Thomashow, 1998). According to the literature, the cell membranes of plants are the major sites of freezing injury (Levitt, 1980; Steponkus, 1984; Thomashow, 1999). Cellular dehydration related to freezing induces severe membrane damage (Steponkus, 1984; Steponkus *et al*., 1993b). Expansion induced lysis, lamellar to hexagonal II phase transitions and fracture jump lesions are some types of membrane damages which are seen as a consequence of cellular dehydration under freezing (Steponkus *et al*., 1993b; Uemura and Steponkus, 1997). Cell membranes have key roles for the movements of water and nutrient. However, water and nutrient transportations are inhibited when membrane damage occur under low temperatures.

It is also shown that reactive oxygen species which is produced by freezing contribute to membrane damage (McKersie and Bowley, 1997). In addition, low temperature causes protein denaturation that may result in cellular damage (Guy *et al*., 1998).

1.2.1 Overproduction of Reactive Oxygen Species under Low Temperature

Under optimal growth conditions, reactive oxygen species (ROS) are constantly generated as a result of aerobic metabolism in plants, especially in chloroplast, mitochondria and peroxisomes due to their extremely oxidizing metabolic activities (Apel and Hirt, 2004; Sharma *et al*., 2012). ROS contain free radicals, such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and nonradical molecules, i.e., hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS production is induced when electrons are transferred to molecular oxygen (O_2) (Asada and Takahashi, 1987; Mittler, 2002). Under normal growth conditions, ROS are produced at low levels.

It is a known fact that low temperature as an environmental stress increases the production of ROS due to disruption of cellular homeostasis (Mittler, 2002; Sharma *et al*., 2012). Limitation of CO_2 fixation in chloroplast and reduction of electron transport chain in chloroplast and mitochondria are primary sources for ROS production during stress conditions (Davidson and Schiestl, 2001). High levels of ROS are very harmful to plants and eventually lead to oxidative stress (Mittler, 2002; Suzuki and Mittler, 2006). Decreasing phospholipid content, peroxidation of lipids, increasing free and saturated fatty acid content are some of threats to plant cells caused by low temperature induced oxidative stress (Mo *et al*., 2010; Sato *et al*., 2011; Chen and Arora, 2011). They also give harm to lipid, protein, carbohydrate and DNA structures. Therefore, physiological processes, such as photosynthesis, respiration, nutrient movements, transpiration, metabolic functions, enzyme activities and biochemical reactions are negatively affected (Gill and Tuteja, 2010). However, it has been demonstrated that low levels of ROS may also play major roles in stress

perception, protection and signal transduction pathways (Suzuki and Mittler, 2006). Whether ROS will be signaling molecule or destructive agents causing oxidative damage depends on their concentration. Because of multifunctional roles, the equilibrium between production and detoxification is important for plant cells to avoid any oxidative injury.

It has been known that the first generated ROS is superoxide radical ($O_2^{\bullet-}$). It is produced in photooxidation reactions, Mehler reaction in chloroplasts, electron transport chain (ETC) reactions in mitochondria, glyoxisomal photorespiration, NADPH oxidase in plasma membranes, xanthine oxidase and membrane polypeptides (Hasanuzzaman *et al*., 2013). Its production may increase the formation of more reactive ROS, such as $^{\bullet}OH$ and $^1O_2$ (Halliwell, 2006a). Decomposition of O_3 in apoplastic space, the reaction of H_2O_2 with $O_2^{\bullet-}$ (Haber- Weiss reaction) and with Fe^{2+} (Fenton reaction) induce the formation of hydroxyl radical ($^{\bullet}OH$). $^{\bullet}OH$ is thought to be the most highly reactive ROS (Gill and Tuteja, 2010). Hydroxyl radicals can react with all biomolecules and other constituent of cells, such as pigments, proteins, lipids and DNA, and are not considered as signaling molecules (Halliwell, 2006a; Moller *et al*., 2007). The primary sources of H_2O_2 production are ETC of chloroplast, mitochondria, endoplasmic reticulum, plasma membrane, β-oxidation of fatty acid and photorespiration. Photooxidation reactions, NADPH oxidase and xanthine oxidase (XOD) also induce H_2O_2 production (Sharma *et al*., 2012). It is moderately reactive but excess of H_2O_2 induces the oxidative stress in plants. Although it acts as a signal molecule at low concentrations, it causes programmed cell death at higher levels (Quan *et al*., 2008). H_2O_2 also acts as a key regulator in various processes, such as senescence (Peng *et al*., 2005), photorespiration and photosynthesis (Noctor and Foyer, 1998a), stomatal movement (Bright *et al*., 2006), cell cycle (Mittler *et al*., 2004), growth and development (Foreman *et al*., 2003). Due to its relatively long life and high permeability through membranes, it has been accepted as a second messenger (Quan *et al*., 2008). Singlet oxygen ($^1O_2$) is produced at photoinhibition and photosystem II electron transfer reactions in chloroplasts. $^1O_2$ formation is also seen when various abiotic stresses

induce closing of stomata (Gill and Tuteja, 2010). It can oxidize proteins, polyunsaturated fatty acids and DNA directly (Karuppanapandian *et al*., 2011). The formation of $^1O_2$ during photosynthesis strongly damages photosystem I and photosystem II.

1.2.2 Antioxidative Defense Mechanisms in Plants

In order to avoid the oxidative damage, scavenging of excess ROS is carried out by antioxidative defense systems composed of nonenzymatic and enzymatic components (Figure 1.4). There is a tight association between enzymatic and nonenzymatic antioxidant systems which ensure an efficient system to arrangement of ROS levels (De Gara *et al*., 2010; Foyer and Noctor, 2009; Miller *et al*., 2010; Shao *et al*., 2008). Ascorbic acid (AA), glutathione (GSH), tocopherols, carotenoids and phenolic compounds are nonenzymatic components of antioxidative defense system (Sharma *et al*., 2012). The major elements of the enzymatic antioxidant systems are superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) cycle, i.e., ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor and Foyer, 1998b). They exist in virtually all cellular compartments which indicate the significance of ROS scavenging for cell life (Mittler *et al*., 2004). High antioxidant capacity is related to increased tolerance to the environmental stresses (Zaefyzadeh *et al*., 2009; Chen *et al*., 2010).

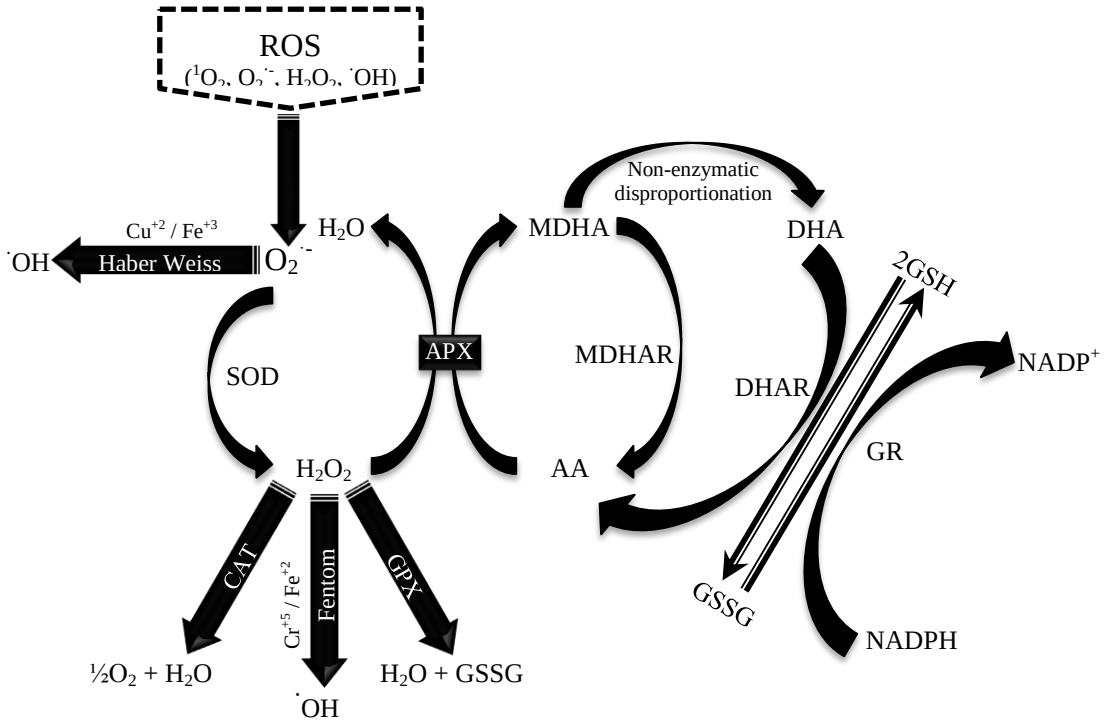


Figure 1.4 ROS and antioxidant scavenging mechanisms in plants (Gill and Tuteja, 2010). Abbreviations: AA: ascorbic acid, $O_2^{\bullet-}$: superoxide radical, APX: ascorate peroxidase, CAT: catalase, DHA: dehydroascorbate, DHAR: dehydroascorbate reductase, GPX: guaiacol peroxidase, GR: glutathione reductase, GSH: glutathione, GSSG: oxidized glutathione, H_2O: water, H_2O_2: hydrogen peroxide, MDHA: monodehydroascorbate, MDHAR: monodehydroascorbate reductase

1.2.2.1 Nonenzymatic Components of Antioxidant Defense System

Ascorbic acid (AA) is one of the most important antioxidant which has a major role against oxidative damage. It is found in most of the organelles and the apoplast (Horemans *et al*., 2000; Smirnoff, 2000). Reduced form of AA found mainly in chloroplasts under normal conditions (Smirnoff, 2000). AA is accepted the main ROS scavenging antioxidant in the aqueous phase due to its capability to donate

electrons in various enzymatic and nonenzymatic reactions. It can directly detoxify $O_2^{\bullet-}$, $^{\bullet}OH$ and $^1O_2$. AA has a major role in reduction of H_2O_2 to H_2O by AsA-GSH cycle (Noctor and Foyer, 1998b).

Glutathione (GSH) is one of the important thiol compounds which plays a major role in defense system against oxidative stress. It is found in almost all cellular components such as chloroplasts, mitochondria, endoplasmic reticulum, vacuoles, and cytosol (Noctor and Foyer, 1998b). It serves as an antioxidant in many ways. GSH is a free radical scavenger and can react with $O_2^{\bullet-}$, $^{\bullet}OH$, $^1O_2$ and H_2O_2. It has also another role in the antioxidant defense mechanism by producing AA with the AsA-GSH cycle (Noctor and Foyer, 1998b; Halliwell, 2006b).

Tocopherols are included in detoxifying of oxygen free radicals, lipid peroxyl radicals, and $^1O_2$ (Diplock *et al*., 1989). Tocopherols are produced by all plants and found in only green parts (Sharma *et al*., 2012). α-tocopherol, an abundant vitamin E compound, has the ultimate antioxidant activity (Kamal-Eldin and Appelqvist, 1996). It counteracts lipid peroxidation through scavenging of lipid peroxyl radicals and detoxifies $^1O_2$ and $^{\bullet}OH$ (Munne-Bosch and Alegre, 2004).

Carotenoids are also lipophilic antioxidants and can detoxify various types of ROS (Young, 1991). It is found in the plastids of plant tissues. They may act in different ways in terms of antioxidant activities. They can scavenge $^1O_2$ to inhibit oxidative damage and can dissipate the energy as heat. They react with triplet chlorophyll ($^3Chl^*$) or excited chlorophyll (Chl^*) molecules for inhibition the $^1O_2$ production.

Phenolic compounds which include flavonoids, tannins, hydroxycinnamate esters and lignin, are secondary metabolites abundantly found in plants (Grace and Logan, 2000). They are more efficient antioxidants *in vitro* than tocopherols and AA due to their strong capacity to donate electrons or hydrogen atoms. Polyphenols may directly scavenge ROS.

1.2.2.2 Enzymatic Components of Antioxidative Defense System

Major enzymatic antioxidative systems and their localizations in plant cells are given in Table 1.1. The most effective enzymatic antioxidant is superoxide dismutase (SOD). It is found in all subcellular compartments of aerobic organisms that are exposed to ROS mediated oxidative stress. SOD which ensures the first step of defense against the increased levels of ROS has important roles in plant stress tolerance. It catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. Plants increase their SOD activity when they are subjected to environmental stresses and higher activity is related with increased tolerance of the plants (Sharma and Dubey, 2005; Mishra *et al*., 2011).

Catalase (CAT) was the first discovered and characterized antioxidant enzyme. It catalyzes the reduction of H_2O_2 into H_2O and O_2 directly. CAT removes H_2O_2 which are produced in peroxisomes by oxidases included in β-oxidation of fatty acids, photorespiration, purine catabolism and oxidative stress (Mittler, 2002; Vellosillo *et al*., 2010). It has high specifity for H_2O_2. CAT is unique among other H_2O_2-degrading enzymes, because it does not require cellular reducing equivalent. However, CAT has lower affinity for H_2O_2 than APX (Sharma *et al.*, 2012). Environmental stresses may increase or decrease CAT activity according to intensity and stress types (Sharma and Dubey, 2005; Moussa and Abdel-Aziz, 2008; Han *et al*., 2009).

Guaiacol peroxidase (GPX) has a role in defence system by scavenging H_2O_2. GPX especially oxidizes aromatic electron donors such as guaiacol and pyragallol at H_2O_2 consuming. It can scavenge reactive O_2 forms and peroxy radicals under stress conditions (Vangronsveld and Clijsters, 1994). It is also related with various important processes, such as degredation of indole-3-acetic acid (IAA), biosynthesis of ethylene, lignin biosynthesis, wound healing, defense against biotic stresses by consuming H_2O_2 in most of the organelles (Kobayashi et al., 1996). It is different from APX in terms of sequences and physiological functions. Among the other

antioxidants, GPX may be one of the key enzymes due to its extracellular and intracellular forms. The GPX activity may change according to plant species and stress conditions (Gill and Tuteja, 2010).

The ratio of ascorbate (AsA) to dehydroascorbate (DHA) and glutathione (GSH) to glutathione disulfide (GSSG) is important for cells to percieve oxidative stress. The ascorbate-glutathione (AsA-GSH) cycle is the recycling pathway of ascorbate and glutathione. This cycle is also known as Halliwell-Asada pathway which scavenge H_2O_2. It involves consecutive oxidation and reduction of AsA, GSH, and NADPH which are catalyzed by ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). This cycle is found at least in cytosol, chloroplast, mitochondria and peroxisomes (Jiménez *et al*., 1997). AsA-GSH cycle plays key role in struggling oxidative stress caused by environmental conditions (Pallanca and Smirnoff, 2000; Sharma and Dubey, 2005).

Ascorbate peroxidase (APX) is a major component of AsA-GSH cycle. It is necessary for H_2O_2 scavenging in water-water and AsA-GSH cycles. APX needs AsA as a hydrogen donor to reduce H_2O_2 into H_2O and MDHA (Asada, 2000). APX has higher affinity for H_2O_2 than CAT which makes it more important for the management of ROS during stress (Gill and Tuteja, 2010). While APX reduces H_2O_2 levels in chloroplast and cytosol of plants, CAT performs this function in peroxisomes.

Monodehydroascorbate reductase (MDHAR) catalyzes the reduction of monodehydro asorbate (MDHA) radical produced in APX catalyzed reaction. If MDHA is not reduced rapidly, it disproportionates to AsA and DHA (Ushimaru *et al*., 1997). MDHAR catalyzes the regeneration of AsA from the MDHA radical. MDHAR represents a high specifity for MDHA as the electron acceptor and prefers NAD(P)H as the electron donor. It is found in peroxisomes and mitochondria and accompanies APX by detoxifing H_2O_2.

Dehydroascorbate reductase (DHAR) catalyzes the reduction of DHA to AsA with the help of GSH as reducing agent (Ushimaru *et al*., 1997). It has a major role in the regulation of AsA pool size and redox state (Chen and Gallie, 2006). This is important for resistance to many abiotic stresses (Gill and Tuteja, 2010). Both MDHAR and DHAR are necessary for reduced ascorbate regeneration.

Glutathione reductase (GR) is one of the important enzymes of AsA-GSH cycle. In AsA-GSH cycle, GSH is oxidized to GSSG by DHAR. GR catalyzes the NADPH dependent reaction in which GSSG is reduced to GSH. Therefore it is important for retaining the reduced GSH pool (Reddy and Raghavendra, 2006; Chalapathi Rao and Reddy, 2008). GSH is important molecule that is included in various processes in plants. The role of GR and GSH in H_2O_2 detoxification has been well proved in the AsA-GSH cycle (Noctor and Foyer, 1998b; Asada, 2000) and they have important roles for plant tolerance to various stresses. GR found in the chloroplasts, cytosol, mitochondria and peroxisomes.

1.3 Responses of Poplar to Low Temperatures

Poplar is a fast-responsive and chilling-tolerant species. Low temperatures, i.e., chilling and freezing, induce some damages to poplar species. However, poplars have a broad diversity of proteomic, metabolic, biochemical and physiological mechanisms to cope with low temperature effects. Growth cessation, accumulation of carbohydrates, pigments, polyamines, proteins and detoxifying enzymes are some of these mechanisms. Besides inducing different types of responses, exposed to low temperature triggers many pathways that causes differential gene and protein expression (Renaut *et al*., 2005).

Table 1.1 Major enzymatic antioxidant systems and their localizations in plants

Enzymatic antioxidants	Reaction catalyzed	Localization
CAT	$2H_2O_2 \rightarrow O_2 + 2H_2O$	Per, Gly, and Mit
APX	$H_2O_2 + AA \rightarrow 2H_2O + DHA$	Cyt, Per, Chl, and Mit
GPX	$H_2O_2 + GSH \rightarrow H_2O + GSSG$	Chl, Cyt, Mit, and ER
SOD	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2 + O_2$	Cyt, Chl, Per, and Mit
MDHAR	MDHA + NAD(P)H $\rightarrow$ AA + $NAD(P)^+$	Chl, Mit, and Cyt
DHAR	DHA + 2GSH $\rightarrow$ AA + GSSG	Chl, Mit, and Cyt
GR	GSSG + NAD(P)H $\rightarrow$ 2GSH + $NAD(P)^+$	Cyt, Chl, and Mit

Abbreviations: AA: ascorbic acid, APX: ascorate peroxidase, CAT: catalase, Cyt: cytosol, Chl: chloroplast, DHA: dehydroascorbate, DHAR: dehydroascorbate reductase, ER: endoplasic reticulum, Gly: glyoxisomes, GPX: guaiacol peroxidase, GR: glutathione reductase, GSH: glutathione, GSSG: oxidized glutathione, H_2O: water, H_2O_2: hydrogen peroxide, MDHA: monodehydroascorbate, MDHAR: monodehydroascorbate reductase, Mit: mitochondria, O_2: oxygen, $O_2^{\bullet-}$: superoxide radical, Per: peroxisomes, SOD: superoxide dismutase.

In the experiment conducted by Renaut *et al*. (2004), poplar species were subjected to chilling temperatures to determine the changes during cold acclimation. It was indicated that poplar is able to develop efficient tolerance mechanisms under these conditions. Chilling temperatures did not affect the survival, but the stem elongation of cold-stressed poplars was stopped. Growth retardation was also showed by other studies when *in vitro* cultured poplar plants were subjected to chilling temperatures (Hausman *et al*., 2000; Renaut *et al*., 2005). This circumstance may be one of the stress-coping mechanism which helps poplar to handle unfavourable conditions.

It is a fact that the level of carbohydrates increase as a response to low temperature stress. Carbohydrates have numerious roles such as energy supply and synthesis of new stress related compounds. They may also behave as cryoprotectants and

osmoprotectants (Ferullo and Griffith, 2001). Growth cessation may be induced due to mobilization of carbohydrates for cryoprotective processes. Glucose, fructose and sucrose are essential carbohydrates for plant growth and also being major sources of cellular energy. Low temperature treatments cause cellular perturbations, therefore the level of sucrose and its galactosides increase in poplar leaves. It was also found that raffinose and trehalose level raise with the increase in freezing tolerance. Sucrose and trehalose can stabilize membranes and proteins in the case of dehydration under freezing conditions. Stabilization is provided by the insertion of these disaccharides into the membranes (Oliver *et al.*, 1998). As a result, sucrose, glucose, fructose, trehalose and raffinose accumulated under chilling temperatures and these carbohydrates have putative roles in cold acclimation of poplar (Renaut *et al*., 2004). Although photosynthetic yield partially decreases, soluble carbohydrate levels increase under cold conditions (Renaut *et al*., 2005). Decreasing in chlorophyll fluorescence is another consequence in poplars when they are subjected to low temperatures. It was also indicated that photosystem II efficiency decreases while the pigment contents of leaves do not change under chilling temperatures (Renaut et al., 2005).

Plants can produce new proteins and enzymes, or can change their levels in response to cold stress. Proteomic analyses demonstrated that although many proteins have higher expressions in poplar subjected to chilling temperatures, much less proteins disappear or exist in lower amounts. As mentioned in previous sections, cold treatment affects the water status of plants. Poplar can synthesize new proteins, such as dehydrins and late embryogenesis abundant proteins against the dehydration (Renaut *et al*., 2004). These proteins are responsive to water stress and included in protein stabilization in membranes (Borovskii *et al*., 2002; Wang *et al*., 2002). Molecular chaperone-like proteins, including heat shock- proteins and chaperonins, stress signalling and transduction pathways proteins also increase during exposure to cold. However, the level of candidate proteins included in cell wall and energy production decrease during cold exposure which could be indirectly linked to the cessation of growth (Renaut *et al*., 2004). Increased level of dehydrins, stress protein

I and heat-shock protein 70 which are stress-related proteins are also indicated in poplar leaves (Renaut *et al*., 2005). Dehydrins are considered to be included in macromolecules or biomembranes stabilization (Close, 1997; Koag *et al*., 2003). Stress protein I shows chaperone like activity and may be necessary for enzyme stabilization (Renaut *et al*., 2005). Heat-shock protein 70 is another chaperone protein which plays roles in protein refolding and inhibition of denatured protein aggregation (Miernyk, 1997; Sung *et al*., 2001). Increased soluble sugar and protein contents in three poplar clones were also indicated by Jiang *et al*. (2011).

It is well known that low temperature induces the reactive oxygen species (ROS) production. Poplar can also develop its defence systems against ROS and other reactive compounds during low temperatures. The scavenging of these compounds is improved by increased level of detoxifying enzymes, such as ascorbate peroxidase, peroxiredoxin and thioredoxin or by pigment accumulation, i.e., chlorophylls, carotenoids and xanthophylls (Renaut *et al*., 2004; Renaut *et al*., 2005). Pigment changes in poplar stem, i.e., high carotene and xanthophyll contents were also reviewed in these studies. The coloration occurs due to increased level of carotenoids and/or anthocyanins under low temperatures (Lichtenthaler, 1987; Chalker-Scott, 1999). They are related with increased freezing tolerance. Polyamines are also considered to have functions in ROS detoxification (Kramer and Wang, 1989). Putrescine, spermidine and spermine accumulations in poplar leaves are indicated by Renaut *et al*. (2005). Polyamines may bind noncovalently to nucleic acids, acid phospholipids and lots of proteins that affect membrane permeability (Bagni and Torrigiani, 1992). Although putrescine may express the suboptimal growth conditions, spermidine and spermine may scavenge free radicals or stabilize macromolecules and membranes (Bouchereau *et al*., 1999; Larher *et al*., 2003). Increases of some detoxifying enzymes, such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase are reported in poplar clones under chilling temperatures in the study of Jiang *et al*. (2011).

Following the *Populus trichocarpa* genome sequencing in 2006 (Tuskan *et al*.), poplar DNA microarray techniques were also developed. Recent studies have analyzed the poplar transcriptome by using bioinformatic approaches to understand the various types of biological processes, including stress responses at gene level. They have tried to determine the genome transcript expression profile to indicate the gene network at different environmental conditions. To find out the roles of C-repeat binding factor (CBF) family of transcription factors in woody perennials, Benedict *et. al.* (2006) investigated the alterations in transcript profile of transgenic *Populus.* Expression of *AtCBF1* raised the freezing tolerance of leaves and stems. They studied four *CBF* paralogues (*PtCBF*s). It was demonstrated that although all *PtCBF*s were induced in leaves under cold, only *PtCBF1* and *PtCBF3* were induced in stems. They concluded that CBF family of transcriptional activators played a central role in cold acclimation of woody perennials as of the herbaceous annual such as *Arabidopsis.*

Maestrini *et al*. (2009) demonstrated the gene expression in white poplar (*Populus alba*) exposed to low temperature. 6-h and 48-h cold treatments were chosen to identify up-regulated genes because gene expression patterns absolutely changed in response to cold in these stages. 44 of 162 genes isolated within 48 h of cold treatment included DRE/LTRE cis-elements in their promoter, therefore they were assumed to belong to the CBF regulon. However, 118 of 162 genes did not contain DRE/LTRE cis-elements. Therefore, it was concluded that there might also other regulons related with cold-induced transcriptomes. They also demonstrated cDNA encoding a RD22-like protein which was early and continually up-regulated. RD22 was encoded by a dehydration-responsive gene, since it was involved in stress and defence mechanisms. A number of genes encoding enzymes related with putative carbohydrate and fatty acid metabolism were also isolated. Three E3 ubiquitin ligases encoding genes were identified in this work. Three different HSP encoding genes (two HSP70 and HSP60) were also up-regulated. Some groups of isolated genes encoded calcium-dependent proteins including calcium-binding proteins (Ca^{+2}-dependent lipid-binding protein CLB1, Ca^{+2}/calmodulin-dependent protein kinase).

Other isolated genes in this research were PIN1-like auxin transport protein, a putative IAA-amino acid hydrolase and auxin binding protein included in auxin transport.

Ping and Minxin (2010) studied expression pattern of *PtCBF5* which was a *CBF* homologue gene encoding transcription activator in *Populus tomentosa*. They showed that leaves contained the most abundant *PtCBF5*, however fewer amounts were found in stem and roots. Low temperature treatment induced the *PtCBF5* gene which was increased in 30 min after freezing and reached to a peak after 2 h in freezing. According to these results, they deduced that *PtCBF5* may play key roles in early signal transduction under cold stress.

Song *et. al*. (2013) determined the transcriptome profiling of *Populus simonii* in response to chilling stress. They demonstrated 11,626 cold-responsive genes including 5,267 up-regulated and 6,359 down-regulated. 1,085 of these genes were differentially expressed which had functions in photosynthesis, signal transduction, and regulation of transcription. Gene ontology analysis showed that cold-induced genes were increased in response to temperature stimulus, ROS and hormone stimulus in terms of biological processes. The functional annotation indicated that chilling stress significantly induced several genes included in calcium/calmodulin-mediated signal transduction, ABA homeostasis, transport and antioxidant defense systems. According to gene expression analysis, 27 genes related to electron transfer were significantly down-regulated indicating that the light reaction may be repressed by low temperature stress. Homologous *CBF2*, *CBF4*, and *CBF6* from *P. simonii* were significantly up-regulated as transcription factors responsive to cold stress. Beside these, *WRKY6*, *WRKY21*, *WRKY40*, *WRKY42*, and *WRKY48* genes were strongly induced by low temperatures. They also revealed ten AP2/ERF genes responsive to cold stress; six of them were up-regulated, while four MYB genes were down-regulated. *APX2* and *GPX2* were significantly up-regulated as an expression of antioxidant defense system-related genes in cold stress. All of these genes may play key roles in protecting poplars against low temperature stress.

1.4 Aims of the Study

Black poplars are widely distributed throughout Turkey due to their strong adaptabilities to environmental conditions. A diverse genetic variation lies in the natural stands of black poplars in Turkey which makes them ideal materials to indicate the mechanisms of cold tolerance.

The first aim of the present work was to determine whether the major families of antioxidant enzymes were quantitatively expressed by black poplars as a function of tolerance to low temperatures. The response of black poplar antioxidant system to low temperatures was determined by measuring the activities of glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and catalase (CAT).

Identification and characterization of differentially expressed genes in response to low temperatures were the second aim of this study. The comparison of cold-resistant and cold-sensitive genotypes in terms of differential gene expression by using microarray technology highlighted the candidate genes involved in cold response of black poplars.

We tried to determine the black poplar clones that indicated better resistance to cold damage and better growth performance. Low temperature related antioxidant enzyme activities and gene expression data could be effectively used in breeding programmes for the purpose of high productivity in low temperature areas. The profile of differentially expressed genes under low temperatures will be valuable for future studies on the molecular mechanisms of chilling tolerance in woody plants.

CHAPTER 2

MATERIALS AND METHODS

2.1 Investigation of Antioxidant Enzyme Activities during Low Temperature

2.1.1 Plant Material

As mentioned in previous section, black poplar individuals were selected from plantations or natural stands throughout the whole country within the scope of *ex situ* conservation. In the framework of this strategy, 297 black poplar clones were transferred to an experimental nursery in Ankara to establish a clone bank. In the spring of 2009, a field trial was established with the use of 297 black poplar clones represented with 5 ramets and 4 replications in Behiçbey Nursery (Figure 2.1) by the collaboration of Central Anatolia Forest Research Insititute and Poplar and Fast Growing Forest Trees Research Institute (PFGFTRI). Among this collection, 40 black poplar clones were selected to determine the low temperature effects as explained further below.

Figure 2.1 Black poplar field trial in Behiçbey Nursery, Ankara. A. Cuttings of selected trees were collected from natural stands or plantations throughout the whole country. B. 5 ramets in each clone were planted within a line in this field trial. To prepare the lines, identification tags on small stakes indicating the clone code number were placed at each planting spot. C. and D. Since the clones are planted in lines, it is possible to compare them with ease in regard to their growth behavior.

2.1.2 Selection of Black Poplar Individuals

The effects of low temperature on black poplars and their adaptation strategies to this stress condition were initially evaluated in the field trial. Total height and diameter of each tree were measured during growing season in 2010 and 2011 as growth parameters at first. We also determined the phenological time series of bud burst date and leaf abscission of black poplar clones. All of the phenological measurements were carried out during the growing seasons of 2010 and 2011 in Behiçbey Nursery,

Ankara. Bud flush (BF) was measured every week from the flushing of the first tree in the middle of April until all the trees flushed. Leaf abscission (LA) was also measured every week starting in mid-September and was continued until all the trees shed their leaves (Figure 2.2).

The researchers from PFGFTRI also carried out some experiments at different field trials to determine the low temperature effects in previous years. They identified the black poplar clones as cold-resistant or cold-sensitive according to their survival performance under cold temperatures. The results obtained from both our field trial observations and PFGFTRI were combined. According to these data, 40 black poplar individuals were selected among the 297 clones in Behiçbey Nursery to investigate the low temperature effects. Since black poplars are deciduous trees, it was decided to sample cambium tissues due to ease of accessibility in each month during the year. In this context, cambium tissues of 40 black poplar clones were sampled from 3 replications at monthly intervals.

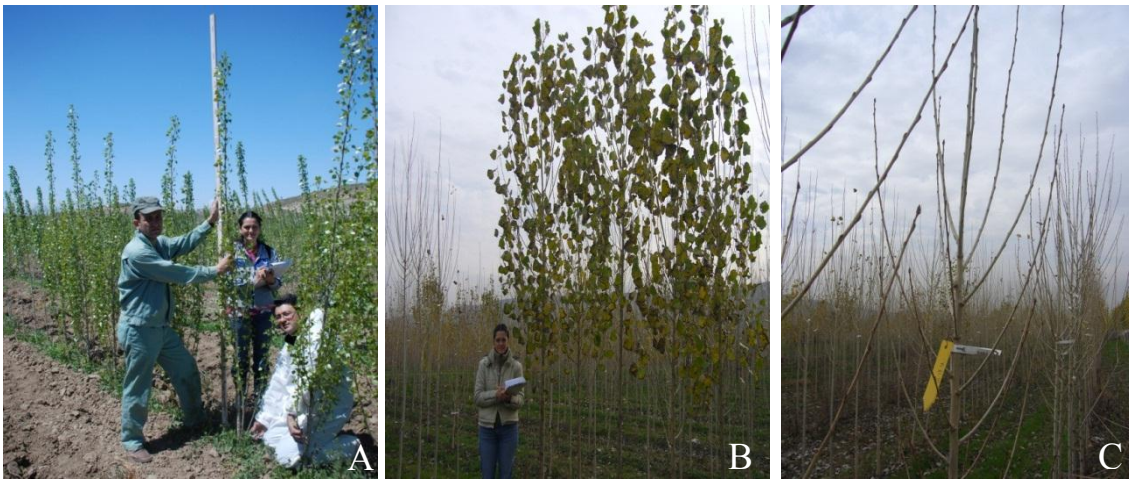


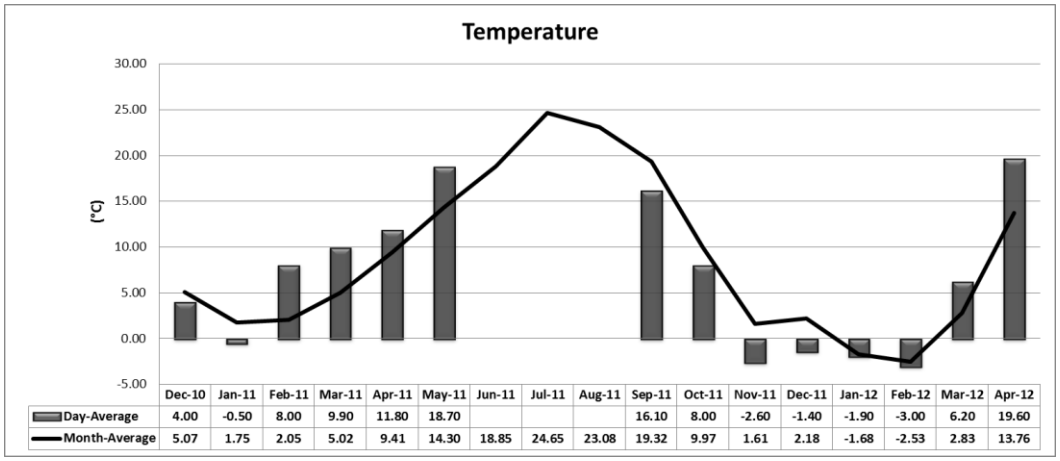
Figure 2.2 A. Growth parameter observations at Behiçbey Nursery, Ankara. B. and C. Phenological time series of bud burst date and leaf abscission of black poplar clones were also determined.

2.1.3 Tissue Sampling Time

About 15-cm-long branch sections of 40 black poplar clones were sampled during 14 months between 2010 and 2012 to measure antioxidant enzyme activities (Table 2.1). Data on air temperature (Figure 2.3) and total precipitation (Figure 2.4) for field condition were obtained from Turkish State Meteorological Service. The variation in antioxidant enzyme activities between 14 months were investigated to understand the low temperature effects on black poplar trees and their adaptation mechanisms to this stress condition.

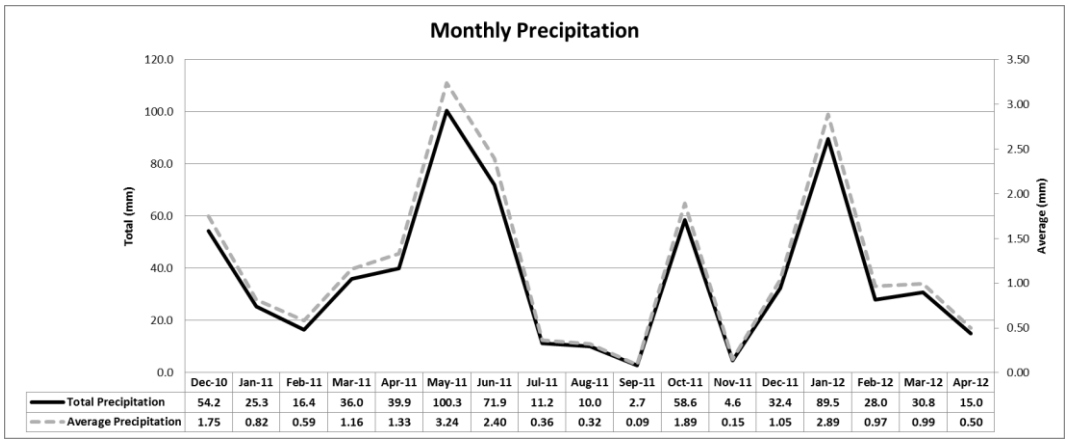
Table 2.1 Tissue sampling time and the measured antioxidant enzymes

Year	Months	Antioxidant enzymes
2010	December	GR, APX, DHAR and CAT
2011	January, February, March, April, May, September, October, November, December	GR, APX, DHAR and CAT
2012	January, February, March, April	GR, APX, DHAR and CAT



	Dec-10	Jan-11	Feb-11	Mar-11	Apr-11	May-11	Jun-11	Jul-11	Aug-11	Sep-11	Oct-11	Nov-11	Dec-11	Jan-12	Feb-12	Mar-12	Apr-12
Day-Average	4.00	-0.50	8.00	9.90	11.80	18.70				16.10	8.00	-2.60	-1.40	-1.90	-3.00	6.20	19.60
Month-Average	5.07	1.75	2.05	5.02	9.41	14.30	18.85	24.65	23.08	19.32	9.97	1.61	2.18	-1.68	-2.53	2.83	13.76

Figure 2.3 Average temperatures of months and sampling days from December 2010 to April 2012



	Dec-10	Jan-11	Feb-11	Mar-11	Apr-11	May-11	Jun-11	Jul-11	Aug-11	Sep-11	Oct-11	Nov-11	Dec-11	Jan-12	Feb-12	Mar-12	Apr-12
Total Precipitation	54.2	25.3	16.4	36.0	39.9	100.3	71.9	11.2	10.0	2.7	58.6	4.6	32.4	89.5	28.0	30.8	15.0
Average Precipitation	1.75	0.82	0.59	1.16	1.33	3.24	2.40	0.36	0.32	0.09	1.89	0.15	1.05	2.89	0.97	0.99	0.50

Figure 2.4 Total precipitation of months from December 2010 to April 2012

2.1.4 Protein Extraction

Twigs from about 2-year-old black poplars were harvested each month from December 2010 to April 2012 and stored at -80°C until use. The bark of 15-cm-long stem pieces were separated from wood and the outer layer was scraped off with a scalpel. Xylem was ground to a very fine powder with liquid nitrogen in a mortar with a pestle (Figure 2.5). The homogenized tissues were immediately frozen and stored at -80°C until use for protein extraction.

About 100 mg wood powder was homogenized in 1000 µl of 50mM phosphate buffer (KH_2PO_4/K_2HPO_4) at pH 7.8 containing 1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride, 12% (w/v) polyvinylpyrolydone (PVP-MW:10000) and 0.2% (v/v) Triton X-100. The homogenate was centrifuged at 15000 rpm for 20 min at 4°C. The supernatant obtained from this procedure was directly used as a crude extract for antioxidant enzyme assays.

Figure 2.5 Tissue sampling procedure. A. Twigs were harvested during 14 months between 2010 and 2012 from field trial. B. They were immediately frozen and stored at -80°C until protein extraction. C. Bark was peeled from the xylem with a scalpel. D. Xylem tissue was scrapped off with a scalpel and was ground to a very fine powder with liquid nitrogen.

Total soluble protein contents of the enzyme extracts were determined according to Bradford method (1976). This assay is based on the use of a dye, Coomassie Brilliant Blue G-250, to which protein binds and alters the light absorbance properties of the dye. The absorbance of Coomassie blue dye at 595 nm is proportional to the amount of protein bound, therefore it is necessary to establish a correspondence between absorbance values and known amounts of protein. For this purpose, bovine serum albumin (BSA) was used to prepare a series of protein standards.

2.1.5 Measurements of the Antioxidant Enzyme Activities

Ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities were determined according to Murshed *et al*., (2008), while catalase (CAT) activity was measured following to Aebi (1984) with minor modifications. All enzyme assays were performed within a 96 well UV- microplate at 25° C by using Epoch Microplate Spectrophotometer (BioTek, France). Samples were analysed in triplicate and blank corrections were carried out in the absence of the protein sample. The enzyme activities were expressed in μmol min^{-1} mg^{-1} protein.

APX activity was determined by monitoring the rate of hydrogen peroxide-dependent oxidation of ascorbate at 290 nm for 10 min at 25°C. The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0) and 0.25 mM ascorbate. One hundred and eighty-five μl reaction buffers were dispensed into each microplate wells and then15 μl crude enzyme extract was added. 5 μl of 200 mM H_2O_2 was added into wells and the reaction was initiated. APX activity was calculated with an extinction coefficient (ε) 2.8 $mM^{-1}cm^{-1}$ and expressed as μmol ascorbate oxidized min^{-1} mg^{-1} protein.

DHAR activity was assayed spectrophotometrically by monitoring the increase in absorbance at 265 nm for 10 min at 25°C. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA and 2.5 mM GSH (reduced glutathione). One hundred and eighty-five μl reaction buffer and 15 μl enzyme extract were dispensed into each microplate wells. 5 μl of 8 mM freshly prepared dehydroascorbic acid (DHA) was added and the reaction was initiated. DHAR activity was calculated by using the extinction coefficient 14 mM^{-1} cm^{-1} and expressed as μmol ascorbate formed per min mg^{-1} of protein.

GR activity in the samples is directly proportional to the oxidation of NADPH to $NADP^+$ which is accompanied by a decrease in absorbance at 340 nm. 190μl reaction mixture consisting of 0.2 M potassium phosphate buffer (pH 7.8), 3 mM EDTA, 0.2 mM NADPH and 0.5 mM GSSG (oxidized glutathione) was dispensed into each

microplate wells and then 15 µl of enzyme extract was added. The GR activity in the samples was calculated by using the extinction coefficient 6.22 $mM^{-1}cm^{-1}$ and expressed as µmol NADPH oxidized min^{-1} mg^{-1} protein.

CAT activity was determined by measuring the decrease rate of H_2O_2 disappearance at 240 nm at 25°C for 10 minutes. The reaction mixture contained 67 mM Tris buffer (pH 7.4), 15 mM H_2O_2 and enzyme extract. One hundred and eighty µl of Tris buffer and 4 µl of enzyme sample were dispensed into each microplate wells. The reaction was initiated by addition of 8 µl of H_2O_2 into wells. CAT activity was calculated by using the extinction coefficient 0.00394 L/mmole mm and expressed as µmol H_2O_2 reduced min^{-1} mg^{-1} protein.

2.1.6 Statistical Analysis

To understand the low temperature responses of 40 black poplar individuals, three biological replications were used for antioxidant enzyme activity measurements and each replicate was assayed in triplicate throughout the experiment. All of the statistical analyses for antioxidant enzyme activities were carried out using the SAS statistical package (SAS Inst. 9.1.3, 2002-2003). Analysis of variance (ANOVA) with General Linear Model (GLM) was applied in order to test differences between response variables (APX, DHAR, GR and CAT activities). The differences were calculated at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ significance levels and expressed as (*), (**) and (***), respectively. Non-significant difference was represented as (ns). In addition, mean and standard error (±SE) values for all dependent variables were given in graphical forms by using Microsoft Office Excel 2010.

2.2 Differential Expression of Low Temperature Related Genes in Black Poplar Clones

2.2.1 Plant Materials and Experimental Design for Microarray Studies

According to the results obtained from antioxidant enzyme activity assays and growth parameters, two black poplar clones (genotypes) were selected to use in microarray studies (Table 2.2). Cold resistant (CR) genotype coded with N.62.191 constituted the first genotypic form. This tree exhibited the highest growth rate at the end of each growing season. The second genotypic form was cold sensitive (CS) clone coded with N.03.368.A which had lower growth values, i.e., short height and narrow diameter.

As mentioned previously, about 15-cm-long stem sections of 2-years-old trees were harvested during 14 months from 2010 to 2012. Among them, four important time points which had significant effects on the clones were selected to identify the low-temperature-induced genes in black poplars. These temperature or time points comprised of September 2011 (control point: 19.32°C), November 2011 (before cold: 1.61°C), February 2012 (during cold: -2.53°C) and April 2012 (after cold: 13.76°C). Stem sections from 2 genotypes (CR and CS) sampled at these 4 time points were immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA extraction. The stem sections collected on September 2011 (SEP) was accepted as control point. The effects of temperatures on gene expressions were examined on November 2011 (NOV), February 2012 (FEB) and April 2012 (APR) and then transcriptional changes were compared with control point.

Three GeneChip arrays were used for each point as technical replicates. In this manner, microarray experiments were conducted with 24 GeneChip Poplar Genome Arrays for two genotypes, i.e. cold-resistant and cold-sensitive clones (2 genotypes × 4 treatments × 3 replicates = 24 arrays). The GeneChip Poplar Genome Arrays (Affymetrix) were used to compare the differential expression of low-temperature-induced genes between two genotypes in this study. The Poplar Genome Array

includes more than 61,000 probe sets which represents over 56,000 transcripts and gene predictions. The array is designed specifically to monitor gene expression in *Populus* species.

2.2.2 RNA Extraction

It is important to maintain RNase (ribonuclease)-free environment when working with RNA. For this purpose, all the solutions and reagents were prepared with diethyl pyrocarbonate (DEPC)-treated water. All materials used for RNA isolation were also treated with DEPC-water and autoclaved.

Total RNA from black poplar stem samples was extracted according to the method described by Xu *et al*. (2009) with minor modifications. The stem sections were grounded into a fine powder with liquid nitrogen by using mortar and pestle at first. Previously heated 900 µl CTAB (Cetyl trimethylammonium bromide) extraction buffer including 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB (w/v), 2% PVP (polyvinylpyrrolidone, w/v) and 2% β-mercaptoethanol (v/v, just prior to use) was added to 120 mg xylem tissue. This suspension was incubated at 65 °C for 10-15 min with shaking vigorously every 2 min. After adding chloroform-isoamyl alcohol (24:1, v/v), the mixture was vortexed and centrifuged at 6000 *g* for 20 min at 4 °C. The upper aqueous phase was transferred to a new tube and extraction was repeated with chloroform-isoamyl alcohol under same conditions once again. The upper phase was recovered and 0.33 volume of 8 M LiCl (lithium chloride) was added for overnight precipitation. Then, the solution was centrifuged at 10000 *g* for 30 min at 4 °C. After discarding the supernatant, pellet was resuspended in 600 µl of prewarmed SDS extraction buffer including 1 M NaCl, 0.5% SDS (w/v), 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The suspension was transferred to a new eppendorf tube and it was centrifuged at 9200 *g* for 10 min at 4 °C after putting 600 µl of chloroform-isoamyl alcohol. Two volume of precooled absolute ethanol was added to the supernatant and it was incubated at -70 °C for 2-3 hours for final precipitation. The RNA pellet was recovered by centrifugation at 15500 *g* at 4 °C for

30 min and then it was washed twice with 1 ml of precooled 70% ethanol by centrifuging at 9200 *g* and 4 °C for 2 min. The pellet was dried and resuspended in 25 µl of DEPC-water. Total RNA concentration was measured by using a Nanodrop spectrophotometer (Nanodrop Tech., Wilmington, USA). The absorbance should be checked at 260 and 280 nm to determine sample concentration and purity. The A_{260}/A_{280} ratio should be approximately 2.0 for pure RNA. Even the ratios between 1.8 and 2.0 are sufficiently pure for use in most applications which was also accepted in our study. Finally, total RNA samples were analyzed on 1% agarose gel which was stained with ethidium bromide to check the integrity and quality of rRNA bands. The quality of the RNA is essential to overall success of the analysis. The samples showing no smears below 18S and 28S bands confirm the good quality and integrity of RNA extracts of black poplars.

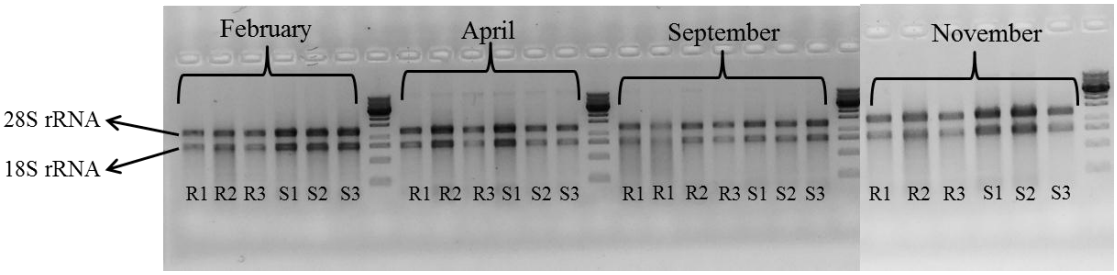


Figure 2.6 Agarose gel electrophoresis of total RNA isolated from xylem tissues of black poplars. The arrows indicate the 28S and 18S units of rRNA which are clearly visible in the intact RNA samples. The numbers indicate the codes for selected individuals. R1: Resistant genotype- 1st replication, R2: Resistant genotype- 2nd replication, R3: Resistant genotype- 3rd replication, S1: Sensitive genotype- 1st replication, S2: Sensitive genotype- 2nd replication and S3: Sensitive genotype- 3rd replication.

2.2.3 Target Preparation and Affymetrix GeneChip Hybridization

Target preparation and hybridization to the GeneChip Poplar Genome Arrays were done according to the manufacturer's protocol (Affymetrix, 2005-2009) and experimental outline is represented in Figure 2.7. 10 µg of total RNA isolated from xylem tissues was reverse transcribed with a T7- Oligo(dT) Promoter Primer in the reaction of first-strand cDNA synthesis. After RNase H-mediated second-strand cDNA synthesis, purification of the double-stranded cDNA was carried out. This cDNA serves as a template to incorporate biotin labelled nucleotides into a newly synthesized RNA molecule during *in vitro* transcription (IVT) reaction. The biotin-labeled complementary RNA (cRNA) targets were cleaned up and fragmented into 35–200 bases strands by metal-induced hydrolysis as described in the manufacturer's protocol. Hybridization to GeneChip expression arrays was carried out at 45°C with rotation for 16 h in Affymetrix GeneChip Hybridization Oven model 640. Following hybridization, washing and staining procedures were carried out. After 16 hours of hybridization, the probe arrays were filled completely with the appropriate volume of washing buffer. Then they were stained with streptavidin-phycoerythrin (SAPE) using Affymetrix Fluidics Station model 450. Finally, the probe arrays were scanned with a GeneChip Scanner 3000 using a laser.

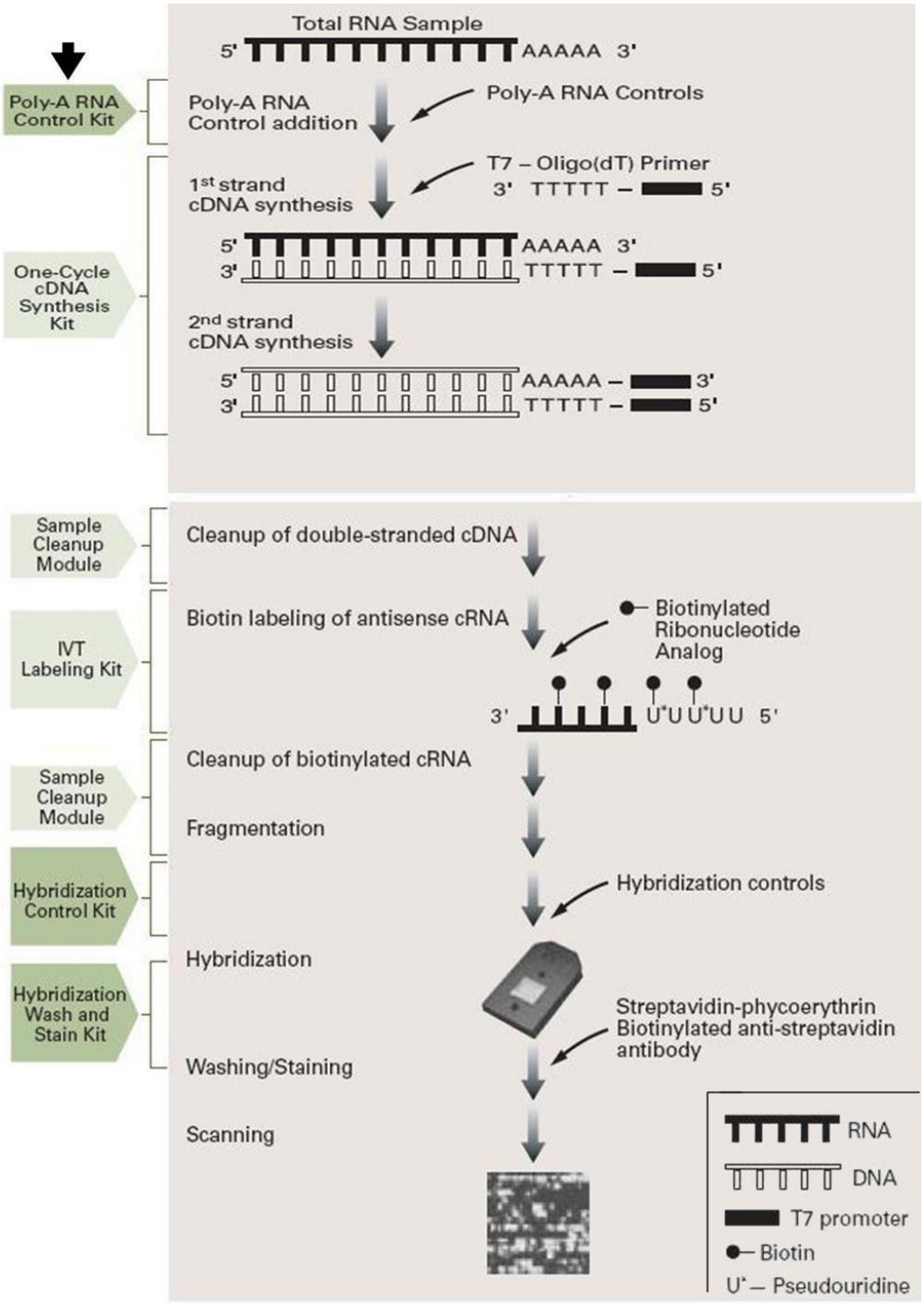


Figure 2.7 GeneChip Labeling Assays for Expression Analysis (Affymetrix, 2005-2009).

2.2.4 Microarray Data Analysis

The microarray analysis may require several different steps according to type of the data being assayed to find out the differentially expresses genes. However, reading raw data, quality assessment, removing bad spots, preprocessing the data and finding differential expression by statistical analysis are general applications followed by all data types. Afterwards, gene annotation can be carried out to capture data about a gene product.

2.2.4.1 Preprocessing of Microarray Data

Preprocessing is an inevitable step that converts each array used in the experiment into applicable data which are necessary to identify differentially expressed genes. There are several steps that must be considered before any kind of microarray data analyzed for differential gene expression. Quality assessment must be done for raw data to ensure its integrity. There is no chance to identify differentially expressed genes reliably without good quality data. Unprocessed raw data are always exposed to some types of technical variation; therefore it must be preprocessed to obtain the highest attainable level of accuracy. Several different methods may be used for preprocessing the data.

Background correction generally constitutes the first step. There are many sources, such as non-specific binding of labelled sample to the array surface, residues from washing or optical noise from the scanner that give rise to unwanted background fluorescence. There is always some level of background noise. Different algorithms using different methods exist for background correction. Normalization is the next stage by which data are adjusted for technical variation in contrast to biological differences between the samples. There is always slight inconsistency between the hybridization processes for each array leading to differences between overall fluorescence intensity levels of arrays. The RNA quantity in a sample, the amount of time for hybridizing, the volume of a sample, physical differences between arrays or

between the scanners can cause significant variances. These artifacts should be removed by applying proper normalization algorithms for the quality of the experiment. Data preprocessing procedures were accomplished by Robust Multi-Array Analysis (RMA) method in the current study. This algorithm converts probe level data to gene expression measures.

2.2.4.2 Statistical Analysis of Microarray Data

The gene expression values were imported into GeneSpring GX 12.5 (Agilent Technologies, Santa Clara CA) software for processing and data analysis in our study. GeneSpring GX software is widely used for expression data analysis and enables researchers to understand microarray data within a biological context. The software indicates whether gene expression was 'present' or 'absent' (detection call) and whether the fold change value represented a real change in expression. Initial probe set filtering procedure was applied with respect to percentile of raw signal intensities. RMA normalized data were used for the filtering process to produce the detection call for each probe set. Probe sets having a signal intensity value below 20% were assumed as absent and discarded from further analysis. Probe sets which provided these criteria were selected for further statistical analysis. After these procedures, moderate t-test was applied to each black poplar genotypes. Different time points were compared with the control group as means of FEB vs SEP, APR vs SEP and NOV vs SEP to identify differentially expressed genes under low temperatures. *P* value and fold changes were used to determine the differential expression of genes. If the *p* value was smaller than 0.05 and fold change was larger than +5, that gene was accepted as differentially expressed. By this way, two black poplar genotypes were compared to identify candidate genes responsible for cold tolerance.

The expression of thousands of genes is measured simultaneously in a typical microarray experiment. A multi-dimensional matrix is observed due to complexity of the data that becomes impossible to make a visual inspection of the relationship between genes or conditions. Therefore, the dimensionality of the data should be

reduced by simplifying complex data set. Principal Components Analysis (PCA) is one of the most widely used technique that reduces the data into lower dimensions by performing a covariance analysis between factors. PCA can determine the key features of high-dimensional data sets and identify predominant gene expression patterns. It allows to compare the expression profiles of samples and to explore correlations between these samples or conditions. Samples with the same experimental condition should be more similar to each other than to samples with distinct condition. Therefore, they can group closer together in a PCA plot. In the current study, PCA was applied to reduce the dimensionality with regard to different treatments (time/temperature points, i.e., FEB, APR, NOV and SEP). By this way, the variation of the data set was explained in terms of low temperature applications.

2.2.4.3 Bioinformatical Tools for Gene Expression Data

Microarray studies produce a list of thousands of genes at once. Achieving biological conclusions from these large gene lists is a challenging task for researchers. Therefore, several bioinformatical tools for interpretation of genes have been developed. The biological knowledge obtained from different experiments has been accumulated in public databases that allow researchers to systematically examine their large gene lists. Gene Ontology (GO) project is one of these projects which is a collaborative effort to provide descriptions of gene products in numerious databases. The GO project has three structured vocabularies (ontologies) that describe gene products in the way of biological processes, cellular components and molecular functions in any organism. Another aspect of this effort is annotation of gene products which allows getting associations between the ontologies, the genes and gene products in the collaborating databases (Gene Ontology, 1999-2013). GO annotations of gene products describe biological function from molecular to organism level. This approach is successful especially for differentially expressed genes that are identified by microarrays. This annotation database (GO) is also very suitable for high-throughput bioinformatics scanning for the enrichment analysis. This tool also statistically examines the enrichment of gene members for each of the

annotation terms. The gene-annotation enrichment analyses contribute significant insights to researchers for understanding the biological themes behind the large gene list.

In the current study, Singular Enrichment Analysis (SEA) option of agriGo bioinformatic website (http://bioinfo.cau.edu.cn/agriGO/analysis.php) was used as an enrichment tool. SEA is a very efficient method to obtain the major biological meaning behind the large gene lists. SEA analysis has been designed to identify enriched Gene Ontology (GO) terms in a list of microarray probe sets. Finding these terms corresponds to finding enriched biological facts. Enrichment level is evaluated by comparing query list to a background population from which the query list is derived. *Populus* Affymetrix Genome array was used as a background list during the enrichment analysis of up- and down-regulated genes in our study. Enrichment scores for GO terms were calculated based upon a set of entered gene list. Finally, enrichment scores and FDR corrected p-values ($p< 0.01$) were used to filter the biological, functional and cellular regulations.

Genes with differentially regulated under low temperatures were also annotated by using PLEXdb (Plant Expression Database) which is another bioinformatic tool (http://www.plexdb.org/). PLEXdb is a combined gene expression resource for plants. The integrated tools of this website allow researchers to utilize commonalities in plant biology for a comparative approach to functional genomics through use of expression data sets. Gene annotation analysis depending on sequence similarity with a known protein or EST in another organism provides significant informations for the selection of low temperature related genes. The best homology implies a true orthologous in the gene annotations. Therefore, the highest homology values (e-values) were considered to make certain the gene function. In our study, mostly *Arabidopsis* gene model was used as an annotation template to describe poplar genes.

CHAPTER 3

RESULTS

3.1 Changes in the Antioxidant Enzyme Activities of Black Poplar Clones under Different Temperatures

As mentioned previously, cambium tissues sampled from 40 black poplar clones at monthly intervals were used to compare the seasonal changes in the antioxidant enzyme activities. The responses of black poplar antioxidant system to low temperature were determined as the activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and catalase (CAT).

3.1.1 Ascorbate Peroxidase (APX) Activities

ANOVA results indicated that different low temperature values induced distinct enzyme activities and there were significant differences among the 14 months (Table 3.1). It was observed that December 2010, February 2011, April 2011, January 2012 and February 2012 had significant effect at $p \leq 0.001$ level on black poplar clones. May 2011 also induced significant differences through the samples in terms of APX activities at $p \leq 0.01$ level. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

Table 3.1 Analysis of variance (ANOVA) on APX activities in black poplar clones. (Numbers indicate mean square values, df: degrees of freedom)

APX	**Replication** *(df = 2)*	**Clone** *(df = 39)*	**Error** *(df = 67)*
December 2010	0.00132	0.00096***	0.00039
January 2011	0.00514	0.00131^{ns}	0.00109
February 2011	0.00724	0.00092***	0.00031
March 2011	0.00217	0.00123^{ns}	0.00097
April 2011	0.00144	0.00162***	0.00059
May 2011	0.00076	0.00079**	0.00038
September 2011	0.00956	0.00057^{ns}	0.00119
October 2011	0.00200	0.00130^{ns}	0.00086
November 2011	0.01168	0.00123^{ns}	0.00083
December 2011	0.00112	0.00089^{ns}	0.00113
January 2012	0.00014	0.00136***	0.00054
February 2012	0.00115	0.00299***	0.00115
March 2012	0.00028	0.00076^{ns}	0.00067
April 2012	0.00959	0.00122^{ns}	0.00115

** Significant at $p \leq 0.01$, *** Significant at $p \leq 0.001$, ns: non-significant

To indicate the differences between sampling times in terms of APX activity, the mean values were plotted and the results are given in Figure 3.1. APX activity was high on December 2010, January 2011 and February 2011 due to the low air temperatures. The activities decreased simultaneously with increasing temperatures on March, April and May 2011. We found that the earliest dates of bud burst occurred on March 15, 2010 and on April 1, 2011 for the black poplar clones in Behiçbey Nursery, Ankara. The latest bud burst days were also determined as on May 1, 2010 and on May 1, 2011. During the metabolic changing at budding stage, the enzyme activities began to decrease. This might also be due to the mild environmental conditions that black poplar clones were not required to antioxidant enzymes for defense purposes. The enzyme activities increased once again on September, October and November 2011. Our findings revealed that the metabolic

and structural changes related with acclimation purposes for wintering of black poplar clones in Behiçbey Nursery began to occur in September. The growth of tissues almost ceased in October and then the leaves shed by early November which made them ready for wintering. Increased enzyme activities in autumn have been suggested to be important in the acclimation of trees to winter conditions. As in the previous winter, the APX activities increased with decreasing air temperatures on December 2011, January 2012 and February 2012. These activities were higher than previous winter activities due to lower temperature values. When the temperature began to rise in spring, the activities decreased on March 2012 and April 2012. The highest APX activity (0.1718 μmol min^{-1} mg^{-1} protein) was recorded on February 2012 which was the coldest month (average temperature=-2.53°C and sampling day temperature= -3.00°C). According to these results, APX activity in black poplar clones gradually increased with a decrease in air temperatures and gradually decreased with increasing temperatures. It was also found that, there was a significant (at $p \leq 0.01$) negative correlation with correlation coefficient of -0.67 between APX activity and sampling day temperature.

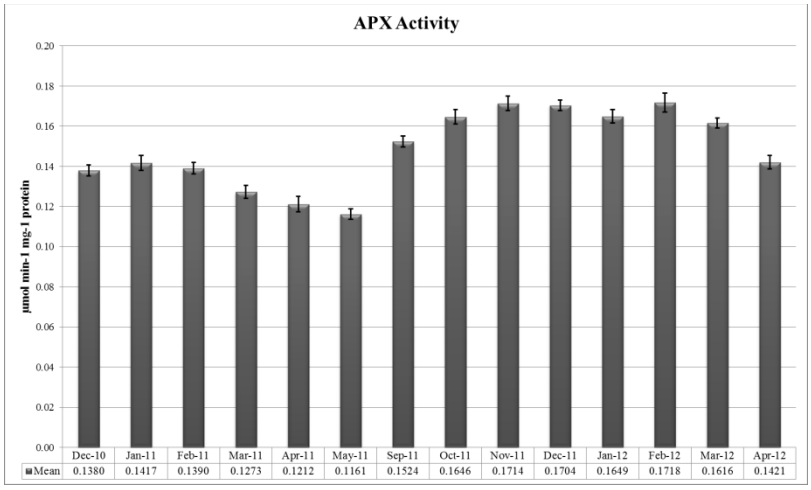


Figure 3.1 Monthly changes of APX activities in the 40 black poplar clones exposed to different temperatures. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

In another viewpoint, seasonal variations in APX activities were also analyzed and results are given in Figure 3.2. Activity levels of APX were high during the winter period (2010-2011) due to low air temperatures. APX activity decreased when metabolic transition occurred during the growing period in spring 2011. In fall 2011, when the growth began to cease, the activity increased once again. This might be important for the acclimation of trees. During winter 2011-2012, APX activity increased by about 21% compared with that of the winter 2010-2011 period. This was related with the air temperature of those times, i.e., average temperature was 2.96°C in the first winter although it was -0.68°C in the second winter. The activity in spring 2012 was higher than spring 2011 with an increase level of 25%. This might be related with the increased metabolic activity as the plants aged. Correlation coefficient was also found as -0.63 which indicated a negative correlation between seasonal APX activities and air temperature.

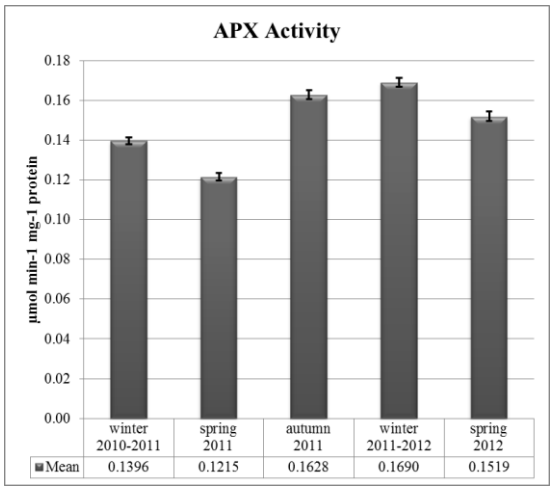


Figure 3.2 Seasonal variations in APX activities in 40 black poplar clones. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

As mentioned earlier, the researchers from PFGFTRI carried out some experiments with black poplar individuals to determine the low temperature effects at different field trials in previous years. They identified the black poplar individuals as cold resistant or cold sensitive according to their survival performance under cold temperatures. According to those data, half of our 40 black poplar trees were cold resistant and the other half was cold sensitive clones. During the analysis, we also wanted to comprehend if any differences occurred between these groups. For this purpose, we compared cold resistant and cold sensitive clones by means of enzyme activities. ANOVA results (Table 3.2) indicated that there were significant differences among cold resistant and cold sensitive groups and between the black poplar clones which belongs to these groups.

Table 3.2 Analysis of variance (ANOVA) on APX activities among cold resistant and cold sensitive groups. (Numbers indicate mean square values, Coldrs: cold resistant-sensitive groups, df: degrees of freedom)

APX	**Replication** ***(df = 2)***	**Coldrs** ***(df = 1)***	**Clone (coldrs)** ***(df = 38)***	**Error** ***(df = 67)***
December 2010	0.00132	0.00497***	0.00081**	0.00039
January 2011	0.00514	0.01015**	0.00104^{ns}	0.00109
February 2011	0.00724	0.01231***	0.00056*	0.00031
March 2011	0.00217	0.00211^{ns}	0.00120^{ns}	0.00097
April 2011	0.00144	0.02539***	0.00100*	0.00059
May 2011	0.00076	0.00140^{ns}	0.00078**	0.00038
September 2011	0.00956	0.00024^{ns}	0.00057^{ns}	0.00119
October 2011	0.00200	0.01442***	0.00087^{ns}	0.00086
November 2011	0.01168	0.00016^{ns}	0.00126^{ns}	0.00083
December 2011	0.00112	0.00093^{ns}	0.00090^{ns}	0.00113
January 2012	0.00014	0.00823***	0.00116**	0.00054
February 2012	0.00115	0.00437^{ns}	0.00294***	0.00115
March 2012	0.00028	0.00098^{ns}	0.00076^{ns}	0.00067
April 2012	0.00959	0.01337**	0.00086^{ns}	0.00115

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$ and *** Significant at $p \leq 0.001$, ns: non-significant

It was observed that different temperatures induced distinct enzyme activities among the groups and clones. The minimum, maximum, mean and standard deviation (SD) values of these groups are given in Appendix A.

Seasonal changes in APX activities of cold resistant and cold sensitive groups are given in Figure 3.3. APX activities in both groups were high during wintering periods. Then, the activities decreased with increasing temperatures in the springs. In fall, when the growth of trees began to cease, the APX activity raised. It was observed that cold sensitive groups had higher APX activities than cold resistant groups in all seasons. The highest APX activities were seen during winter 2011-2012 which was the coldest season in sampling times. In this period, the activity of cold resistant groups was 0.1636 µmol min^{-1} mg^{-1} protein, while it was 0.1745 µmol min^{-1} mg^{-1} protein in cold sensitive groups. The enzyme activity and air temperature were negatively correlated and correlation coefficients were -0.56 and -0.69 for cold resistant and cold sensitive groups, respectively.

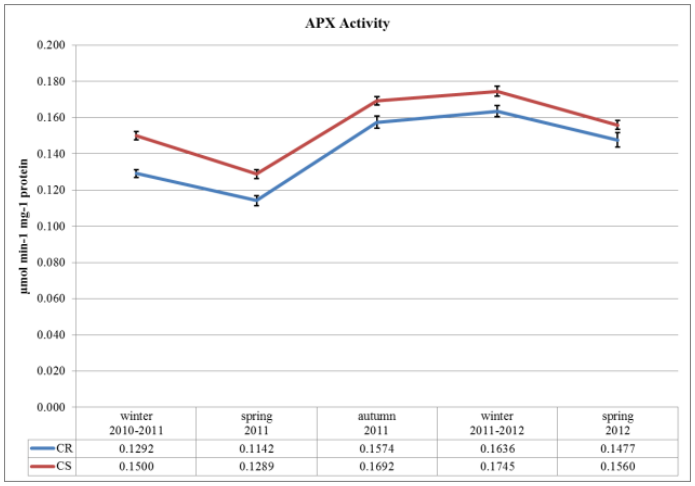


Figure 3.3 Seasonal variations in APX activities of the cold resistant and cold sensitive groups. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=60)

3.1.2 Dehydroascorbate Reductase (DHAR) Activities

ANOVA results revealed significant differences through the black poplar clones in terms of DHAR activities under various temperature conditions (Table 3.3). The significant effects were detected at $p \leq 0.05$ level on October 2011, February 2012 and April 2012, while it was at $p \leq 0.01$ level on January 2011, February 2011, April 2011, May 2011and March 2012. The most significant impact was seen on January 2012 at $p \leq 0.001$ level. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

Table 3.3 Analysis of variance (ANOVA) on DHAR activities in black poplar clones. (Numbers indicate mean square values, df: degrees of freedom)

DHAR	**Replication** ***(df = 2)***	**Clone** ***(df = 39)***	**Error** ***(df = 67)***
December 2010	0.00254	0.00135^{ns}	0.00100
January 2011	0.00849	0.00117**	0.00058
February 2011	0.02458	0.00165**	0.00084
March 2011	0.00779	0.00084^{ns}	0.00078
April 2011	0.00031	0.00040**	0.00018
May 2011	0.01136	0.00189**	0.00096
September 2011	0.03302	0.00463^{ns}	0.00404
October 2011	0.00716	0.00210*	0.00133
November 2011	0.05846	0.00496^{ns}	0.00317
December 2011	0.00222	0.00572^{ns}	0.00419
January 2012	0.02220	0.00812***	0.00262
February 2012	0.00239	0.00303*	0.00176
March 2012	0.05068	0.00576**	0.00276
April 2012	0.02252	0.00167*	0.00099

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$ and *** Significant at $p \leq 0.001$, ns: non-significant

Monthly changes of DHAR activities given in the Figure 3.4 were irregular when compared with APX activities. Although December 2010 and February 2011 activities were relatively high, it was low on January 2011. Before visible signs of growth appeared in spring, there was a sharp decrease on March 2011. The activities on April 2011 and May 2011 remained low with increasing temperatures. This was followed by a steep increase in DHAR activity on September 2011 (0.1232 µmol min^{-1} mg^{-1} protein) which was the maximum level within 14 months. Then the activities began to decrease on October 2011 and November 2011. The activities increased when the black poplar clones were exposed to low temperatures on December 2011, January 2012 and February 2012. The increase appeared to continue throughout winter due to decreasing temperatures. The activities on March 2012 and April 2012 decreased with increasing temperatures. Although DHAR activities in black poplar clones were not steady as much as APX activity, there was still negative correlation (-0.21) with sampling day temperature.

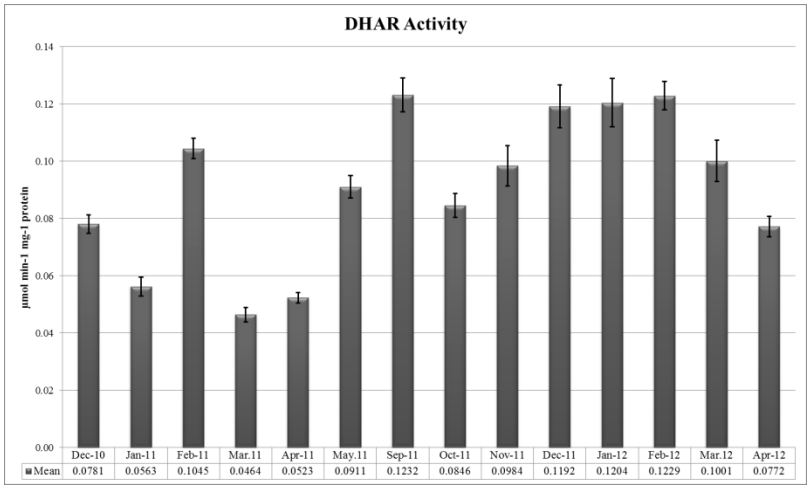


Figure 3.4 Monthly changes of DHAR activities in 40 black poplar iclones exposed to different temperatures. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

Seasonal variations in DHAR activity were given in Figure 3.5. DHAR activity varied seasonally in the similar general pattern as APX. It remained at higher levels in winter period (2010-2011) due to low air temperatures. The activity of the enzyme began to decrease when the growth signs were seen in spring 2011. There was appreciable increase in the activity on September 2011 and it continued throughout autumn 2011. During this time, growth began to cease and plants prepared themselves for winter conditions. The activity reached a highest level (0.1208 μmol min^{-1} mg^{-1} protein) during winter 2011-2012 period. There were higher activities on this time than on winter 2010-2011. DHAR activity increased by about 52% compared with that of first winter. When metabolic reactions and growth began, the enzyme activity decreased in spring 2012. Negative correlation was found between seasonal enzyme activities and temperatures with correlation coefficient of -0.75.

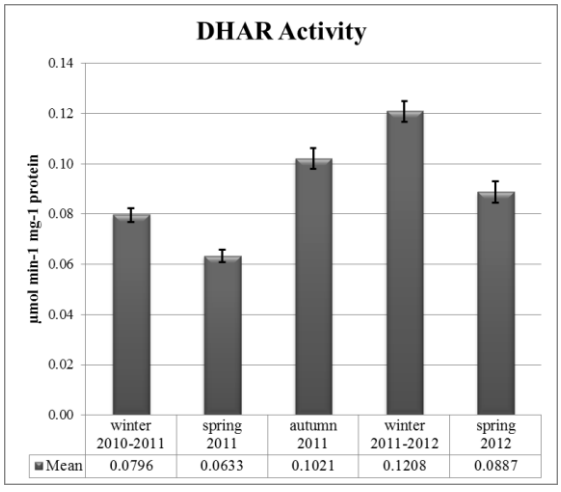


Figure 3.5 Seasonal variations in APX activities of 40 black poplar clones. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

When the analyses were carried out between cold resistant and cold sensitive groups, ANOVA results revealed that activities changed significantly in response to temperatures. According to ANOVA, the temperatures on May, October, November, December 2011 and on January, March, April 2012 induced significant differences at $p \leq 0.001$ level between cold resistant and cold sensitive groups. In addition, January, February, April 2011 and January, February 2012 had significant effects on clones belonging to these groups at $p \leq 0.01$ and $p \leq 0.05$ levels, respectively. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

Table 3.4 Analysis of variance (ANOVA) on DHAR activities among cold resistant and cold sensitive groups. (Numbers indicate mean square values, Coldrs: cold resistant-sensitive groups, df: degrees of freedom)

DHAR	**Replication** ***(df = 2)***	**Coldrs** ***(df = 1)***	**Clone (coldrs)** ***(df = 38)***	**Error** ***(df = 67)***
December 2010	0.00254	0.00013^{ns}	0.00138^{ns}	0.00100
January 2011	0.00849	0.00144^{ns}	0.00115**	0.00058
February 2011	0.02458	0.00176^{ns}	0.00167**	0.00084
March 2011	0.00779	0.00004^{ns}	0.00087^{ns}	0.00078
April 2011	0.00031	0.00008^{ns}	0.00041**	0.00018
May 2011	0.01136	0.02678***	0.00126^{ns}	0.00096
September 2011	0.03302	0.00009^{ns}	0.00474^{ns}	0.00404
October 2011	0.00716	0.01861***	0.00152^{ns}	0.00133
November 2011	0.05846	0.10716***	0.00200^{ns}	0.00317
December 2011	0.00222	0.08460***	0.00346^{ns}	0.00419
January 2012	0.02220	0.14054***	0.00451*	0.00262
February 2012	0.00239	0.00294^{ns}	0.00301*	0.00176
March 2012	0.05068	0.12965***	0.00217^{ns}	0.00276
April 2012	0.02252	0.01441***	0.00137^{ns}	0.00099

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$ and ***** Significant at $p \leq 0.001$, ns: non-significant

Seasonal changes in DHAR activities of cold resistant and cold sensitive groups are also given in Figure 3.6. DHAR activity of cold resistant group was higher than cold sensitive group in winter 2010-2011. The activities in both groups began to decrease with increasing temperatures during spring 2011. In fall, the activities increased due to acclimation purposes of black poplar clones. Although both groups increased their activities during winter 2011-2012, it was very sharp in cold sensitive clones which had the highest activity (0.1443 µmol min^{-1} mg^{-1} protein). With increasing temperatures and growth signs in spring 2012, the activities decreased once again in both groups. It was realized that, cold sensitive groups indicated higher activities than cold resistant groups except first winter period. However, the negative correlation between temperatures and seasonal enzyme activities was quite high in cold resistant clones (-0.90, significant at $p \leq 0.05$) according to cold sensitive ones (-0.55).

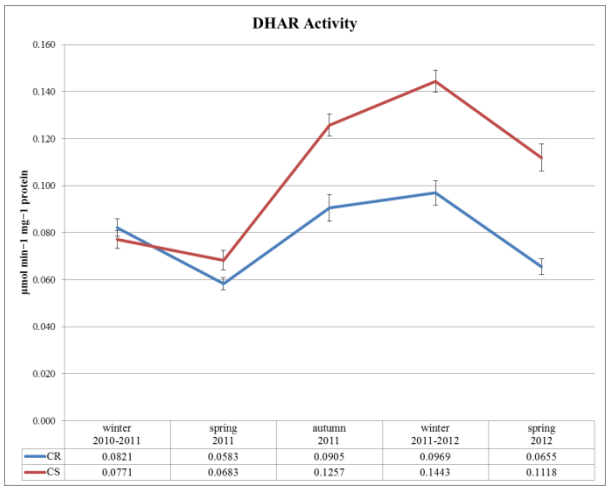


Figure 3.6 Seasonal variations in DHAR activities of the cold resistant and cold sensitive goups. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=60)

3.1.3 Glutathione Reductase (GR) Activities

The significant effects of temperatures on GR activities were also detected for black poplar clones. According to ANOVA results given in Table 3.5, the significant differences were observed at $p \leq 0.01$ level on May 2011 and at $p \leq 0.001$ level on January, February, March, April, October, November and December 2011 and January, February, March 2012. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

Table 3.5 Analysis of variance (ANOVA) on GR activities in black poplar clones. (Numbers indicate mean square values, df: degrees of freedom)

GR	**Replication** ***(df = 2)***	**Clone** ***(df = 39)***	**Error** ***(df = 67)***
December 2010	0.00530	0.00005^{ns}	0.00015
January 2011	0.00004	0.00014***	0.00004
February 2011	0.00006	0.00010***	0.00004
March 2011	0.00007	0.00010***	0.00002
April 2011	0.00036	0.00011***	0.00004
May 2011	0.00007	0.00005**	0.00002
September 2011	0.00067	0.00005^{ns}	0.00003
October 2011	0.00024	0.00011***	0.00004
November 2011	0.00024	0.00011***	0.00004
December 2011	0.00010	0.00033***	0.00005
January 2012	0.00022	0.00018***	0.00004
February 2012	0.00036	0.00013***	0.00005
March 2012	0.00026	0.00010***	0.00003
April 2012	0.00102	0.00003^{ns}	0.00003

** Significant at $p \leq 0.01$ and *** Significant at $p \leq 0.001$, ns: non-significant

Monthly changes of GR activities of black poplar clones are given in Figure 3.7. GR activity was gradually increased with decreasing air temperatures on December 2010,

January 2011 and February 2011. Then it declined simultaneously with increase of air temperature at the end of March 2011. Although the activity on April 2011 was a little bit higher, it decreased again on May 2011. The lowest activity was present on September 2011 (0.0352 μmol min^{-1} mg^{-1} protein). However, progressive increases were observed on October 2011 and November 2011 when trees prepared themselves for harsh winter conditions. The enzyme activities were higher on December 2011, January 2012 and February 2012. GR level peaked on February 2012 with 0.0647 μmol min^{-1} mg^{-1} protein activity. These activities were greater than in the previous winter. Finally it declined once again on March 2012 and April 2012 due to suitable temperature conditions. According to these results, GR activity in black poplar clones gradually increased with decreasing air temperatures and gradually decreased with increasing temperatures. For this reason, significant negative correlation with correlation coefficient of -0.75 (at $p \leq 0.01$) was found between GR activity and sampling day temperature.

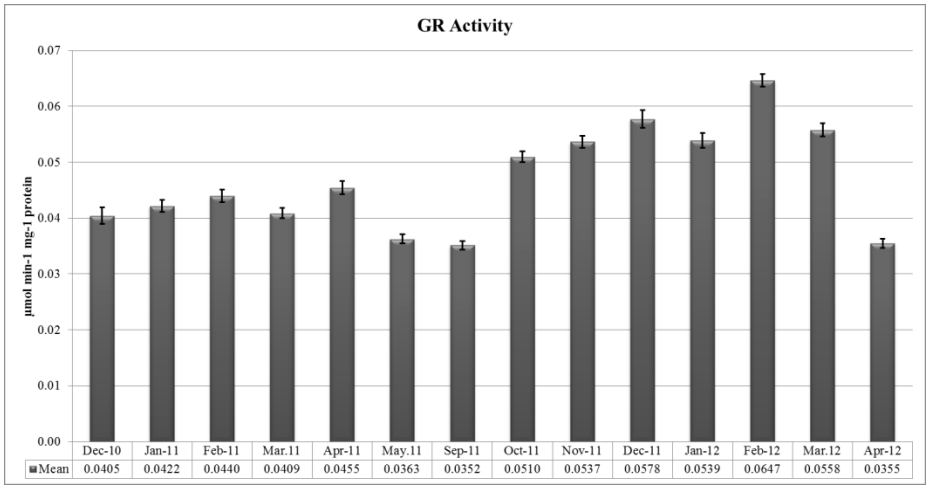


Figure 3.7 Monthly changes of GR activities of 40 black poplar clones exposed to different temperatures. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

GR activity followed a similar seasonal pattern as APX and DHAR activities (Figure 3.8). The highest activities were present in the winter conditions and the lowest activities in springs. As mentioned earlier, plants protected themselves from low temperatures by increasing the antioxidant enzyme activities. Temperature conditions are suitable in spring and trigger the growth initiation. Metabolic changes during this time decrease the antioxidant enzymes activities. In fall, GR activity increased once again to acclimation purposes. The highest seasonal activity was 0.0588 μmol min^{-1} mg^{-1} protein observed in winter 2011-2012. Negative correlation was determined between seasonal GR activities and temperatures with correlation coefficient of -0.76.

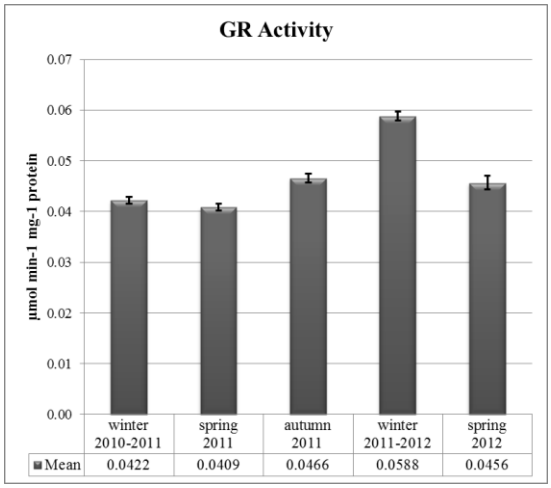


Figure 3.8 Seasonal variations in GR activities of 40 black poplar clones. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

The differences between cold resistant and cold sensitive groups in terms of GR activity were also detected. ANOVA results given in Table 3.6 indicated that different temperature values induced significant effects among cold resistant and

cold sensitive groups and between the black poplar clones belonging to these groups at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels. The minimum, maximum, mean and standard deviation (SD) values of these groups are given in Appendix A.

Table 3.6 Analysis of variance (ANOVA) on GR activities among cold resistant and cold sensitive groups. (Numbers indicate mean square values, Coldrs: cold resistant-sensitive groups, df: degrees of freedom)

GR	**Replication** ***(df = 2)***	**Coldrs** ***(df = 1)***	**Clone (coldrs)** ***(df = 38)***	**Error** ***(df = 67)***
December 2010	0.00530	0.00013^{ns}	0.00005^{ns}	0.00015
January 2011	0.00004	0.00030**	0.00013***	0.00004
February 2011	0.00006	0.00123***	0.00008**	0.00004
March 2011	0.00007	0.00037***	0.00009***	0.00002
April 2011	0.00036	0.00051***	0.00009***	0.00004
May 2011	0.00007	0.00039***	0.00004*	0.00002
September 2011	0.00067	0.00003^{ns}	0.00005^{ns}	0.00003
October 2011	0.00024	0.00119***	0.00009**	0.00004
November 2011	0.00024	0.00002^{ns}	0.00011***	0.00004
December 2011	0.00010	0.00306***	0.00024***	0.00005
January 2012	0.00022	0.00278***	0.00010**	0.00004
February 2012	0.00036	0.00046**	0.00012**	0.00005
March 2012	0.00026	0.00062***	0.00008***	0.00003
April 2012	0.00102	0.00021*	0.00003^{ns}	0.00003

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$ and *** Significant at $p \leq 0.001$, ns: non-significant

Seasonal variations in GR activities of the cold resistant and cold sensitive groups were also in the similar pattern as APX and DHAR. GR activities in both groups were high in winter 2010-2011. Then, the activities decreased with increasing temperatures in spring. When the growth of black poplar clones began to cease in

autumn, the activity increased with the aim of preparing themselves to winter conditions. The highest activities for both groups were recorded in winter 2011-2012 due to lowest temperature values. As shown in Figure 3.9, the individuals belonging to cold sensitive groups had higher GR activities than cold resistant group for each season. The GR activity and air temperature were negatively correlated again and correlation coefficients were -0.70 and -0.79 for cold resistant and cold sensitive groups, respectively.

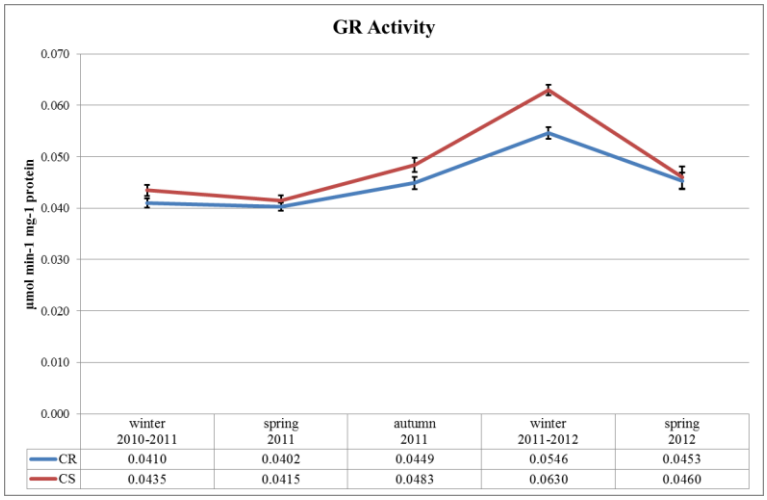


Figure 3.9 Seasonal variations in GR activities of the cold resistant and cold sensitive goups. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=60)

3.1.4 Catalase (CAT) Activities

ANOVA results indicated that temperature values induce significant differences between black poplar clones in terms of CAT activities (Table 3.7). Significant effects were detected on March 2011 and May 2011 at $p \leq 0.05$ level and on January

2011 at $p \leq 0.01$ level. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

Table 3.7 Analysis of variance (ANOVA) on CAT activities in black poplar clones. (Numbers indicate mean square values, df: degrees of freedom)

CAT	**Replication** ***(df = 2)***	**Clone** ***(df = 39)***	**Error** ***(df = 67)***
December 2010	121.677	40.333^{ns}	31.051
January 2011	7.292	12.753**	6.007
February 2011	202.735	13.679^{ns}	9.605
March 2011	56.540	20.696*	12.691
April 2011	81.572	66.064^{ns}	57.486
May 2011	227.911	21.034*	12.899
September 2011	219.745	30.684^{ns}	39.658
October 2011	304.483	34.670^{ns}	28.020
November 2011	27.200	25.996^{ns}	16.545
December 2011	153.222	43.473^{ns}	46.785
January 2012	21.948	20.827^{ns}	17.648
February 2012	34.215	9.137^{ns}	9.664
March 2012	94.853	9.988^{ns}	11.070
April 2012	405.470	112.465^{ns}	102.562

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$, ns: non-significant

The relationships between temperature and CAT activity were different from APX, DHAR and GR activities. Monthly changes of CAT activities given in Figure 3.10 indicated that the lowest activities were present in the winter months and the highest activities in the spring months. CAT activity on December 2010 was higher than the activity on January and February 2011. The monthly average temperature was 5.07°C and the temperature of sampling date was 4°C on December 2010. Before visible signs of growth appeared, the CAT activity began to increase in March 2011 and raised subsequently. Although it exhibited high levels during the metabolic

changes on April 2011, the activity decreased on May 2011 which closed to March 2011. There was appreciable increase in the activity on September 2011 but it was followed by decreases on October and November 2011. It gradually decreased in parallel to declining temperatures on December 2011, January 2012 and February 2012. On March 2011, the CAT activity began to rise again with increasing temperatures and it reached a maximum level in April 2012. According to these results, CAT activities were significantly increased during enlargement and early stage of dormancy periods. However, steep decreases were observed under low temperatures. It was found that, there was a significant positive correlation with correlation coefficient of 0.66 (at $p \leq 0.05$) between CAT activity and temperature.

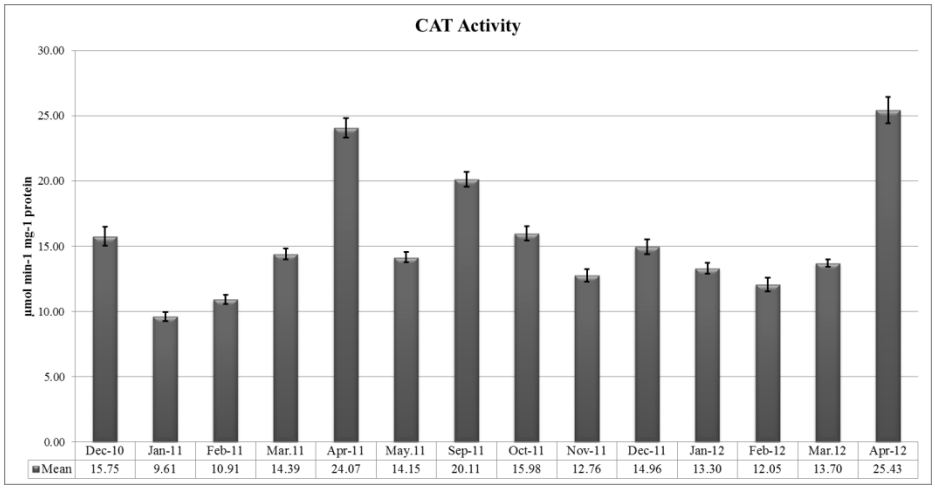


Figure 3.10 Monthly changes of CAT activities of 40 black poplar clones exposed to different temperatures. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

Seasonal changes in CAT activity (Figure 3.11) showed that the lowest activity appeared in winter 2010-2011. The samples exhibited low levels of CAT activity during winter and this situation remained same until spring. In contrast to the APX,

DHAR and GR activities, CAT activity began to increase when the growth and enlargement progressed. During the growing stage in spring 2011, the enzyme activities began to increase to about 45% of the winter activity. Decreases in activities were observed in autumn 2011 and winter 2011-2012 periods. The activities of CAT began to rise subsequently and it reached maximum level in spring 2012. CAT activities significantly increased at the stage of enlargement and it remained relatively high during dormancy period. However, it decreased under low temperature conditions during winter seasons. The correlation coefficient was found as 0.85 which indicated positive correlation between CAT activity and temperature.

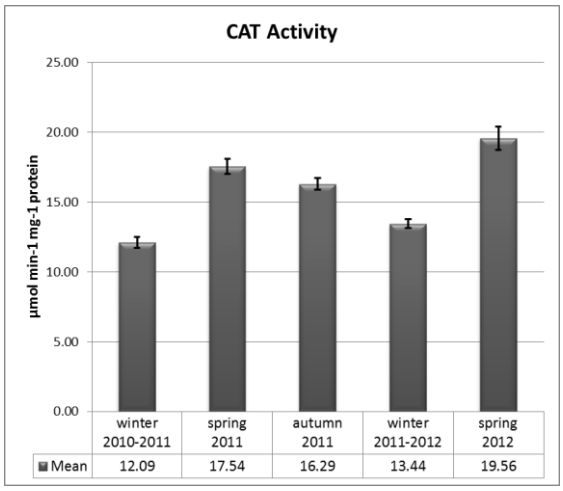


Figure 3.11 Seasonal variations in GR activities of 40 black poplar clones. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=120)

When cold resistant and cold sensitive groups were compared for CAT activities, ANOVA results revealed that activities changed significantly in response to temperatures (Table 3.8). Significant differences between these groups were observed on December 2010 (at $p \leq 0.05$), on February, March 2012 (at $p \leq 0.01$)

and on May, October, November 2012 (at $p \leq 0.001$). The only significant effect between black poplar clones which belonged to these groups were seen on January 2011 at $p \leq 0.01$ level. The minimum, maximum, mean and standard deviation (SD) values of 14 months are given in Appendix A.

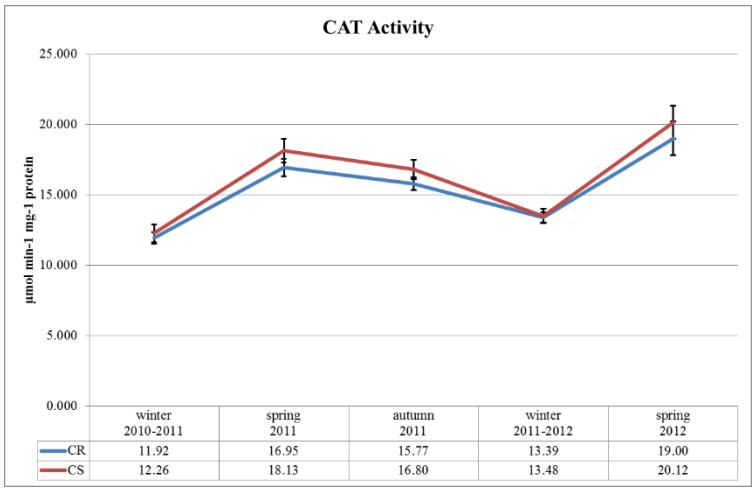
Table 3.8 Analysis of variance (ANOVA) on CAT activities among cold resistant and cold sensitive groups. (Numbers indicate mean square values, Coldrs: cold resistant-sensitive groups, df: degrees of freedom)

CAT	**Replication** ***(df = 2)***	**Coldrs** ***(df = 1)***	**Clone (coldrs)** ***(df = 38)***	**Error** ***(df = 67)***
December 2010	121.67661	163.62565*	35.95901^{ns}	31.05087
January 2011	7.29221	0.02450^{ns}	13.08506**	6.00714
February 2011	202.73452	93.42567**	11.44467^{ns}	9.60548
March 2011	56.54013	141.19579**	16.79888^{ns}	12.69127
April 2011	81.57225	174.21984^{ns}	60.90682^{ns}	57.48568
May 2011	227.91091	156.36159***	16.49830^{ns}	12.89879
September 2011	219.74454	80.51473^{ns}	29.06704^{ns}	39.65771
October 2011	304.48260	465.43973***	20.86861^{ns}	28.02016
November 2011	27.20049	345.82528***	16.66189^{ns}	16.54477
December 2011	153.22194	22.56910^{ns}	44.24536^{ns}	46.78524
January 2012	21.94813	1.21381^{ns}	21.37217^{ns}	17.64777
February 2012	34.21473	0.09543^{ns}	9.37171^{ns}	9.66410
March 2012	94.85315	9.42894^{ns}	9.75515^{ns}	11.06963
April 2012	405.46959	263.72419^{ns}	110.28611^{ns}	102.56175

* Significant at $p \leq 0.05$, ** Significant at $p \leq 0.01$ and *** Significant at $p \leq 0.001$, ns: non-significant

Seasonal CAT changes of cold resistant and cold sensitive groups are shown in Figure 3.12. Although CAT activities were not followed a similar seasonal pattern, cold sensitive groups had higher activities than cold resistant group in all seasons as

other enzymes. As mentioned earlier, CAT activity decrease with decreasing temperatures in winter conditions. It subsequently increased with the growth initiation which was triggered off increased temperatures in springs. The activities of both groups declined in autumn. Positive correlation between CAT activity and temperature was also found for cold resistant and cold sensitive groups with correlation coefficients of 0.83 and 0.86, respectively.



	winter 2010-2011	spring 2011	autumn 2011	winter 2011-2012	spring 2012
CR	11.92	16.95	15.77	13.39	19.00
CS	12.26	18.13	16.80	13.48	20.12

Figure 3.12 Seasonal variations in CAT activities of the cold resistant and cold sensitive groups. The data presented in the graph are means±SE. (Vertical lines on the bar graphs indicate the standard errors (SE). Sample size, N=60)

3.1.5 Identification of Cold Resistant and Cold Sensitive Clones

To investigate the variation among black poplar clones in terms of antioxidant enzyme activities under different temperature conditions was one of the objectives in this study. We tried to determine the best clones that indicated better resistance to frost damages and better growth performance under low temperatures. The phenological measurements and growth performance data collected during field

experiments were combined with antioxidant enzyme activities. Bud flush (BF), leaf abscission (LA), height and diameter values were recorded during growing seasons. BF and LA rates shown in tables were expressed as the number of days elapsed since January 1, 2011. Height and diameter data represented the September 2011 values which combined the means of 5 ramets in 4 replications for each individual. Likewise, antioxidant enzyme activities in tables are the mean values of 14 months for each clone. According to this, cold resistant clones indicated better tolerance to low temperatures and better growth performance were determined and given in Table 3.9. Cold sensitive clones were also identified as shown in Table 3.10 to further understand cold acclimation phenomena and adaptation strategies in black poplar.

Table 3.9 Cold resistant black poplar clones and concerning data in terms of growth, phenology and antioxidant enzyme activities

Clone	Height	Diameter	LA	BF	GR	APX	DHAR	CAT
N.62.191	636.7	49.4	346	102	0.0423	0.1430	0.0841	13.98
N.64.014	465.0	35.8	346	102	0.0434	0.1225	0.0682	14.55
N.87.001	546.7	45.7	346	102	0.0441	0.1324	0.0736	15.21
N.92.219	466.7	33.8	346	121	0.0392	0.1357	0.0594	15.50
N.90.036	426.7	42.5	341	121	0.0429	0.1562	0.0672	15.33
N.92.179	458.3	37.8	346	121	0.0445	0.1313	0.0590	13.51
KOCABEY	571.7	47.8	346	102	0.0440	0.1345	0.0757	15.66
ATA-1	521.7	48.3	346	102	0.0435	0.1286	0.0735	15.75
N.92.142	536.7	38.8	341	109	0.0435	0.1186	0.0703	17.26
N.92.218	556.7	43.7	341	102	0.0419	0.1309	0.0767	16.58
Average	**518.7**	**42.4**	**344.5**	**108.4**	**0.0429**	**0.1334**	**0.0708**	**15.33**

Table 3.10 Cold sensitive black poplar clones and concerning data in terms of growth, phenology and antioxidant enzyme activities

Clone	Height	Diameter	LA	BF	GR	APX	DHAR	CAT
N.82.4	480.0	32.5	336	95	0.0498	0.1677	0.1083	15.58
N.83.12	533.3	38.7	336	95	0.0452	0.1766	0.1088	15.94
N.83.2	455.0	27.2	336	95	0.0485	0.1618	0.1154	17.18
N.91.052	430.0	25.6	336	95	0.0492	0.1459	0.1105	16.02
N.96.325	481.7	31.5	341	95	0.0496	0.1613	0.1261	17.92
N.92.243	398.7	28.1	346	95	0.0527	0.1606	0.1037	14.54
N.03.366	421.7	27.8	311	66	0.0665	0.1568	0.1156	14.05
N.93.306	450.0	27.3	336	95	0.0485	0.1675	0.1047	18.15
N.90.010	417.3	36.0	336	95	0.0511	0.1625	0.0875	15.56
N.91.119	491.7	29.4	336	102	0.0516	0.1706	0.1219	14.87
Average	**455.9**	**30.4**	**335.0**	**92.8**	**0.0513**	**0.1631**	**0.1103**	**15.98**

According to values represented in tables, cold resistant clones indicated better growth performances since they were taller and wider. Leaf abscission and bud flush occurred lately when comparing to cold sensitive clones. However, cold resistant clones had lower antioxidant enzyme activities. We also identified the moderately cold resistant black poplar clones, i.e., Çubuk-1, Gazi, Anadolu, Geyve and N.85.016.

In general, cold sensitive black poplar clones were shorter and thinner than resistant ones. Time series of leaf abscission and bud flush also set earlier. Cold sensitive black poplar clones had higher APX, DHAR, GR and CAT activities than cold resistant ones. It may be suggested that cold sensitive clones had increased need of ROS removal under low temperatures. By this way, they became less sensitive to low temperature induced oxidation. In addition, the black poplars coded as N.03.368.A, N.83.5, N.88.4, N.91.088, N.91.109 and N.96.322 were identified as moderately cold sensitive clones.

3.2 Identification of Differentially Expressed Genes in Response to Low Temperatures

As mentioned previously, four temperature treatments which had significant effects on the clones were selected to identify the low temperature induced genes in cold resistant and cold sensitive clones (genotypes). The stem sections collected on September 2011 (19.32°C) was accepted as control point. The effects of temperatures on genes were examined on November 2011 (before cold: 1.61°C), February 2012 (during cold: -2.53°C) and April 2012 (after cold: 13.76°C) and then transcriptional changes were compared with control point. Stem sections from 2 genotypes (N.62.191 and N.03.368.A) sampled at these 4 time points were used for antioxidant enzyme activity assays and microarray experiments.

3.2.1 Antioxidant Enzyme Activities of Cold Resistant and Cold Sensitive Genotypes

According to the results obtained from antioxidant enzyme activity assays and growth parameters, the first genotypic form was cold resistant clone coded with N.62.191 which exhibited the highest growth rate at the end of each growing season. The second genotypic form was cold sensitive clone coded with N.03.368.A which had lower growth rates, i.e., short height and narrow diameter. Height, diameter and antioxidant enzyme activity values of these two genotypes are given in Table 3.11. Height and diameter data in the table represented the September 2011 values which combined the means of 5 ramets in 4 replications for each clone. Antioxidant enzyme activities were the mean values of 14 months for each clone.

Table 3.11 Cold resistant and cold sensitive genotypes selected for microarray studies

Clone	Height	Diameter	GR Activity	APX Activity	DHAR Activity	CAT Activity
N.62.191	636.7	49.4	0.0423	0.1443	0.0845	11.86
N.03.368.A	415	37.3	0.0393	0.1436	0.0642	14.74

It was previously mentioned that cold resistant clones had lower antioxidant enzyme activities than cold sensitive ones. However, when the clones selected for microarray experiment were analyzed separately according to 14 months, it was realized that N.62.191 (cold resistant) had higher APX, DHAR and GR activities than N.03.368.A (cold sensitive). When the effects of four temperature treatments selected for microarray experiment were observed, the antioxidant enzyme activities of N.62.191 were still higher than N.03.368.A except on February 2012 (Figure 3.13). Under low temperatures, cold sensitive black poplar clone still indicated higher enzyme activities. This finding attributed to the increased level of reactive oxygen species under low temperatures in especially cold sensitive genotype. It may be suggested that cold sensitive individuals had increased need of ROS removal under low temperatures; therefore they increased their antioxidant enzyme activities on February 2012. By this way, they became less sensitive to low temperature induced oxidation.

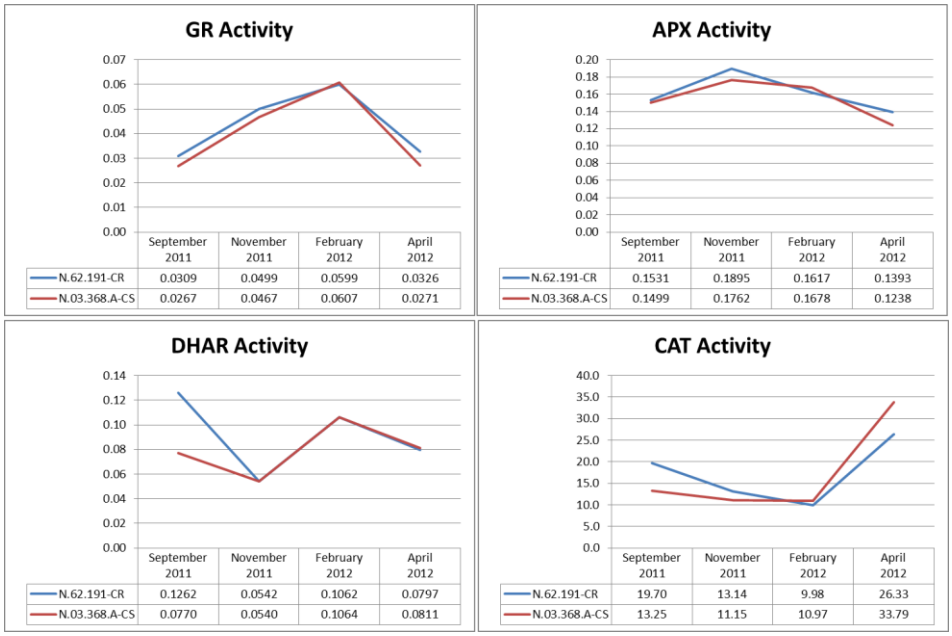


Figure 3.13 APX, DHAR, GR and CAT activities of N.62.191 and N.03.368.A on September 2011, November 2011, February 2012 and April 2012 (CR: Cold resistant, CS: Cold sensitive)

3.2.2 Expression Profile of Low Temperature Induced Genes in Black Poplar

It is a known fact that poplar is a fast-responsive and chilling-tolerant plant species. Cold acclimation is a multigenic process which triggers a response network with respect to stress perception and direct or indirect effects of low temperatures. Broad variation in the degree of freezing tolerance among poplar species occur due to this process. Gene expression during four different temperature treatments were compared in 2 genotypes, N.62.191 and N.03.368.A, to indicate the profile of differentially expressed genes under low temperatures in black poplars. Microarray analysis which is a powerful and rapid approach to discover the differential gene expression may require several different steps according to type of the data being assayed to find out the differentially expresses genes.

3.2.2.1 Principal Components Analysis of Microarray Data

The expression of thousands of genes is measured simultaneously in a typical microarray experiment. Due to complexity of the data, a multi-dimensional matrix is observed which renders impossible to make a visual inspection of the relationship between genes or conditions. Principal Components Analysis (PCA) is one of the most widely used technique that reduces the dimensionality of data sets to create 2 or 3 D plots that reflects relatedness of objects within clusters. Principal components are vectors that capture the most variance through the whole data. In the current study, PCA was used to assess the major source of variation in the expression data. It was applied to reduce the dimensionality with regard to different treatments (time/temperature points, i.e., September 2011 (SEP), November 2011 (NOV), February 2012 (FEB) and April 2012 (APR)). By this way, the variation of the data set was explained in terms of different treatments which were important source of variation represented along y axis in PCA scatter plot (Figure 3.14). Statistical evaluation by PCA of the expression data indicated that each treatment segregates as a distinct pool and the biological replicates within each comparison clustered together as expected. The figure stands for the projection of the 24 arrays on the first three Principal Components, and 53.5% of the total variance is corresponded by the sum of these three axes. According to PCA analysis, the largest source of variability was due to treatments which were indicated by different colored ellipsoids, accounting for 29.9% of the variance in the data. Each treatment could be seen as distinct groups in PCA scatter plot.

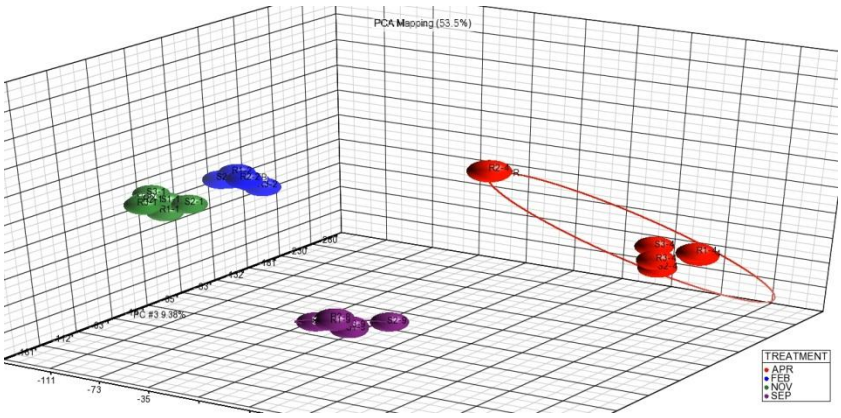


Figure 3.14 Principle component analysis (PCA) of the four different temperature treatments of microarray experiments. On the scatter plot, each colour represents one treatment, i.e., Purple: September 2011 (control), Green: November 2011 (before cold), Blue: February 2012 (during cold) and Red: April 2012 (after cold). Each dots indicate the genechips used in low temperature related expression profiling of the samples.

3.2.2.2 Identification of Transcriptional Profiles in Cold Resistant Genotypes

Moderate t-test was applied to each sample of two black poplar genotypes to indicate the differential expression of genes in this study. Different time points were compared with control group (SEP) as a means of FEB vs SEP, APR vs SEP and NOV vs SEP to identify differentially expressed genes under different treatments. A statistical threshold for the microarray data analysis detected a total of 3983 genes significantly expressed (Fold Change (FC) $\geq$ 5, p value $\leq$ 0.01) in cold resistant individuals. The comparison of gene expression between NOV and SEP showed that 1509 genes were differentially expressed. As the temperature values began to drop, 1649 genes were significantly expressed in FEB condition. During growing season, the number of differentially expressed genes was increased up to 2101 genes in APR. Among the differentially expressed probe sets, 497, 569 and 1765 genes were found to be significantly expressed only in NOV, FEB and APR, respectively. In addition,

124 genes were identified as common in all treatments among the differentially expressed genes (Figure 3.15).

A B

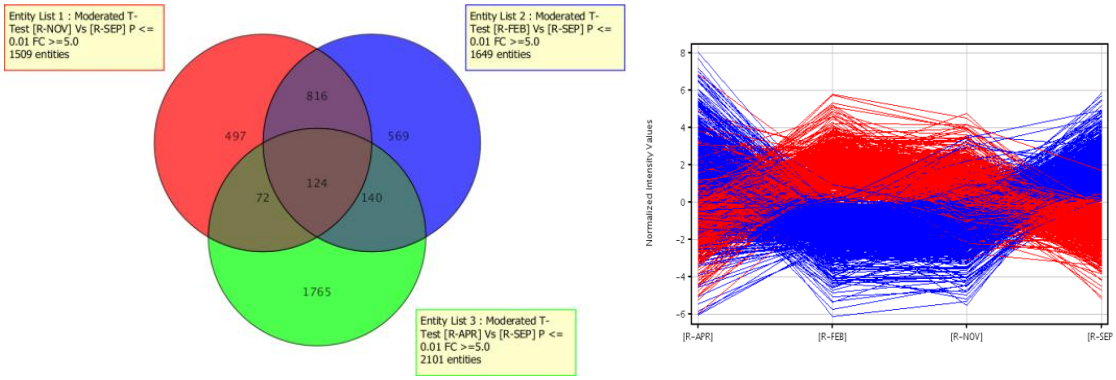


Figure 3.15 The number of differentially expressed genes (FC ≥ 5, p value ≤ 0.01) and their profile plot in cold resistant black poplar genotype (N.62.191) under different temperature treatments. A. Venn diagram shows the number of differentially expressed genes in each treatment. B. The profile plot indicated the general trend of gene regulations under different temperatures. (R: Resistant, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

Up and down regulation of these genes were also determined to identify the differential expression under different treatments (Table 3.12). In November 2011 and February 2012, the transcriptional profiles of the resistant genotype were in a down-regulated direction. In NOV conditions, the number of up-regulated genes was 532, whereas 977 genes were down-regulated. Under low temperature conditions (FEB vs SEP), 645 genes were up-regulated and 1004 were down-regulated in cold resistant genotype. Finally, the comparison between APR vs SEP revealed 1542 up-regulated genes and 559 down-regulated genes. According to these result, it can be

concluded that the main transcriptional response of the resistant genotype occurred after cold conditions (APR).

Table 3.12 The number of differentially expressed genes (FC ≥ 5, p value ≤ 0.01) and their up and down regulations in cold resistant genotype. (R: Resistant, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

Transcriptional comparison	**Differential Regulation of Cold Related Genes (FC ≥ 5, p value ≤ 0.01)**		
	UP	**DOWN**	**TOTAL**
R-NOV vs R-SEP	532	977	1509
R-FEB vs R-SEP	645	1004	1649
R-APR vs R-SEP	1542	559	2101

3.2.2.3 Identification of Transcriptional Profiles in Cold Sensitive Genotypes

A statistical threshold for the microarray data analysis of cold sensitive genotype indicated a total of 3732 differentially expressed genes (FC ≥ 5, p value ≤ 0.01). The comparison between NOV and SEP showed that 1514 genes were differentially expressed. Under low temperature conditions, 1817 genes were significantly expressed in FEB condition. In contrast to differentially altered genes on February 2012 for the resistant genotype, low temperature had more impact on the transcriptome of the sensitive genotype. This finding might attribute to the increased effects of low temperature stress conditions in especially cold sensitive genotype. A pairwise transcriptional comparison between FEB and SEP clearly confirmed the differential cold responses between genotypes. During growing season, the number of differentially expressed genes was determined as 1839 entities which were nearly

same with FEB values. Among the differentially expressed probe sets, 383, 594 and 1482 genes were found to be significantly expressed only in NOV, FEB and APR, respectively. In addition, 165 genes were identified as common in all treatments among the differentially expressed genes (Figure 3.16).

A B

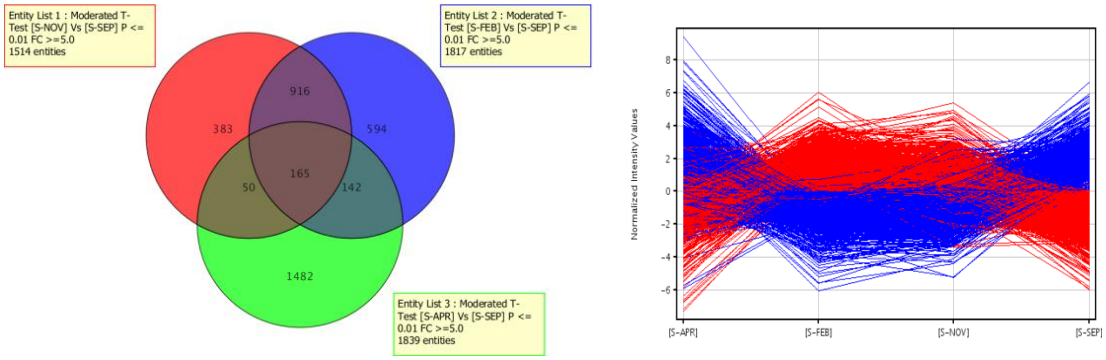


Figure 3.16 The number of differentially expressed genes (FC ≥ 5, p value ≤ 0.01) and their profile plot in cold sensitive black poplar genotype (N.03.368.A) under different temperature treatments. A. Venn diagram shows the number of differentially expressed genes in each treatment. B. The profile plot indicated the general trend of gene distribution under different temperatures. (S: Sensitive, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

According to up and down regulation of these genes (Table 3.13), the response of the sensitive genotype to low temperature stress began much earlier than the resistant clone. The up-regulated genes were detected as 618 in NOV condition which was higher than cold resistant genotypes. This might be related with the preparing themselves to winter conditions. Under low temperature conditions (FEB vs SEP), 767 genes were up-regulated and 1050 were down-regulated in cold sensitive genotype. Finally, the comparison between APR vs SEP revealed 1305 up-regulated genes and 534 down-regulated genes. These results highlighted that the main

transcriptional response of the sensitive genotype began under low temperatures and continued during growing season.

Table 3.13 The number of differentially expressed genes (FC ≥ 5, p value ≤ 0.01) and their up and down regulations in cold sensitive genotype. (S: Sensitive, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

Transcriptional comparison	**Differential Regulation of Cold Related Genes (FC ≥ 5, p value ≤ 0.01)**		
	UP	**DOWN**	**TOTAL**
S-NOV vs S-SEP	618	896	1514
S-FEB vs S-SEP	767	1050	1817
S-APR vs S-SEP	1305	534	1839

3.2.2.4 Gene Ontology Annotation of Cold Resistant and Cold Sensitive Genotypes in Response to Different Temperatures

After determining transcriptional profile of cold resistant and cold sensitive genotypes, these gene lists were sorted into functional categories by Gene Ontology (GO) annotation using agriGO online tool. The gene annotation enrichment analyses contribute significant insights to researchers for understanding the biological themes behind the large gene list. In the current study, Singular Enrichment Analysis (SEA) was employed to evaluate the enrichment of GO terms in a query list of genes in comparison to the prevalence of the same GO terms in a reference list (Populus Affymetrix Genome Array). SEA analysis allowed to annotation of the most expressed sequences according to the main Gene Ontology (GO) vocabularies, i.e., biological processes, cellular components and molecular functions. In this study,

SEA was conducted independently on the up and down-regulated genes that differentially expressed in NOV, FEB and APR conditions. Enrichment scores and p-values ($p \leq 0.01$) were used to determine the significantly enriched GO vocabularies in terms of biological processes and molecular functions.

3.2.2.4.1 SEA of Differentially Expressed Genes in Both Genotypes at NOV Conditions

Annotations of genes according to biological processes and molecular functions were determined for cold sensitive and cold resistant genotypes for NOV (before cold) conditions at first. In up-regulated biological GO terms, the most represented enrichment was metabolic process, i.e., glucan metabolic process (GO:0044042), cellular polysaccharide metabolic process (GO:0044264), starch metabolic process (GO:0005982), disaccharide metabolic process (GO:0005984), sucrose metabolic process (GO:0005985), polysaccharide metabolic process (GO:0005976), oligosaccharide metabolic process (GO:0009311) and glycoside metabolic process (GO:0016137) in both genotypes. According to these findings, carbohydrate metabolism seems to be important for black poplar clones for preparing themselves to winter conditions. The significantly enriched biological term specific for cold resistant genotype was related with photosynthesis. For cold sensitive genotype, the most significant biological enrichments were determined as carbohydrate metabolic process and response mechanisms (oxidative stress, stimulus and chemical stimulus). Annotation of genes according to the molecular functions in cold sensitive genotype led to the identification of significantly up-regulated GO terms, such as oxidoreductase activity and binding mechanisms, i.e., ion binding and cation binding.

GO term annotations to biological process and molecular functions were also applied to down-regulated genes of both genotypes. The significantly enriched common biological process terms included cellular nitrogen compound metabolic process (GO:0034641) and transport mechanisms, i.e., oligopeptide transport (GO:0006857), peptide transport (GO:0015833) and lipid transport (GO:0006869) in cold sensitive

and cold resistant genotypes. Annotation of genes according to the molecular functions led to the identification of common terms as protein dimerization activity (GO:0046983), oxidoreductase activity (GO:0016491), monooxygenase activity (GO:0004497) and iron ion binding (GO:0005506). The most significant specific enrichment was related with cellular amino acid and derivative metabolic process in cold resistant genotype for biological GO term. However, more significant terms were detected specific to cold sensitive genotype including response mechanisms (response to stimulus, response to hormone stimulus, response to auxin stimulus, response to chemical stimulus, response to light stimulus and response to other organism); regulation processes (regulation of transcription, regulation of nitrogen compound metabolic process, regulation of biosynthetic process and regulation of macromolecule biosynthetic process) and transcription. Among the molecular function genes category, those genes with transferase activities and transporter activity were significantly down-regulated in cold resistant genotype. Binding mechanism (tetrapyrrole and heme) and serine-type peptidase activity related genes were determined in only cold sensitive genotype. The gene annotation enrichment analyses of significantly up and down-regulated groups for both genotypes at NOV conditions are given in Appendices B.

3.2.2.4.2 SEA of Differentially Expressed Genes in Both Genotypes at FEB Conditions

The distribution of enriched GO terms for up and down-regulated probe sets under low temperatures indicated several important findings. According to the biological processes vocabulary of ontological annotation, the most represented categories in up-regulated libraries for both genotypes were those of cellular nitrogen compound metabolic process (GO:0034641), disaccharide metabolic process (GO:0005984), oligosaccharide metabolic process (GO:0009311), polysaccharide metabolic process (GO:0005976), response to stimulus (GO:0050896), transport (GO:0006810) and response to stress (GO:0006950). Annotation according to the molecular function identified common up-regulated groups for both genotypes as protein kinase activity

(GO:0004672), transporter activity (GO:0005215), protein serine/threonine kinase activity (GO:0004674) and phosphotransferase activity (GO:0016773). The specific up-regulated annotation terms for cold resistant and cold sensitive genotypes were also determined. According to these findings, cellular amino acid metabolic process, amine metabolic process, cellular carbohydrate metabolic process, anion transport, peptide transport and oligopeptide transport were some of the up-regulated biological process vocabularities specific to cold resistant genotype. Oxidoreductase activity and substrate-specific transporter activity constituted the molecular functions found in this genotype. Annotation of modulated genes in terms of biological processes specific for sensitive genotype were those of response to water deprivation, response to abscisic acid stimulus, response to water, response to oxidative stress, response to abiotic stimulus, protein amino acid phosphorylation and response to osmotic stress. The only significant term for molecular function was determined as receptor activity in this genotype. Although most of the terms were related with carbohydrate metabolism and transport activities in resistant genotype, response to stress conditions were found especially in cold sensitive genotypes.

The significantly enriched GO terms were also identified for down regulated probe sets of both genotypes. Among the biological process genes, those involved in aromatic compound biosynthetic process (GO:0019438), secondary metabolic process (GO:0019748), phenylpropanoid biosynthetic process (GO:0009699), glycolipid metabolic process (GO:0006664), stilbene metabolic process (GO:0009810), L-ascorbic acid metabolic process (GO:0019852), coumarin biosynthetic process (GO:0009805) and polyketide metabolic process (GO:0030638) were significantly down-regulated in both genotypes. Most of the down-regulated genes were related with metabolic process of organic compounds, such as secondary metabolites, phenylpropanoids, stilbenes, polyketides and coumarin. Another group of genes related with glycolipids and ascorbate metabolism were also significantly reduced in both genotypes. Transferase activities including transferring glycosyl groups (GO:0016757) and transferring hexosyl groups (GO:0016758), iron ion binding (GO:0005506), cation binding (GO:0043169), ion binding (GO:0043167),

heme binding (GO:0020037), metal ion binding (GO:0046872) and catalytic activity (GO:0003824) constituted significantly enriched molecular function terms. In terms of the specific down-regulated GO annotation vocabularities for cold resistant genotypes, low temperatures led to expression arrest of secondary metabolism (terpene and flavonoid biosynthesis) and amino acid metabolism (β-alanine and glutamate). In addition, transferase activities (UDP-glycosyltransferase, O-methyltransferase and glucosyltransferase) were also reduced in terms of molecular function. On the other hand, several down-regulated probe sets were mainly related with protein dimerization activity, serine-type peptidase activity, serine proteases (subtilases) and transmethylation reactions in cold sensitive genotypes. The gene annotation enrichment analyses of significantly up and down-regulated groups for both genotypes at FEB conditions are given in Appendices B.

3.2.2.4.3 SEA of Differentially Expressed Genes in Both Genotypes at APR Conditions

The eventual gene annotation enrichment analyses were conducted independently on the up and down-regulated genes that differentially expressed in APR (after cold) conditions. Among the biological process genes category, those genes with polysaccharide metabolic process (GO:0005976), carbohydrate metabolic process (GO:0005975), starch metabolic process (GO:0005982), disaccharide metabolic process (GO:0005984), cellular nitrogen compound metabolic process (GO:0034641), transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169), electron transport (GO:0006118) and response to organic substance (GO:0010033) were significantly up-regulated in both genotypes. Annotation of genes according to the molecular function identified transferase activity (transferring hexosyl groups-GO:0016758, transferring glycosyl groups GO:0016757), hydrolase activity (GO:0016787), cation binding (GO:0043169), ion binding (GO:0043167), copper ion binding (GO:0005507) and oxidoreductase activity, acting on diphenols and related substances as donors (GO:0016679) in both genotypes. The specific up-regulated annotation terms for cold resistant and cold sensitive genotypes were also

detected. Among the biological process genes category, protein amino acid phosphorylation, cell surface receptor linked signaling pathway, post-translational protein modification, protein modification process, macromolecule modification, serine family amino acid metabolic process, phosphorylation, phosphate metabolic process and phosphorus metabolic process were significantly up-regulated in cold resistant genotype. Protein serine/threonine kinase activity, protein kinase activity and phosphotransferase activity constituted the most significant up-regulated molecular functions. In cold sensitive genotype, phenylpropanoid biosynthetic process, aromatic compound biosynthetic process, cellular amino acid derivative biosynthetic process, response to chemical stimulus and response to stimulus were the major biological processes. Among the molecular functions, transition metal ion binding and metal ion binding were significantly up-regulated gene groups in this genotype.

The distribution of enriched GO terms for down-regulated probe sets demonstrated less groups than up-regulated ones. Annotation of genes according to the biological process identified starch catabolic process (GO:0005983), glucan catabolic process (GO:0009251), cellular polysaccharide catabolic process (GO:0044247) and carbohydrate catabolic process (GO:0016052) as common groups in both genotypes. Among the molecular function genes category, UDP-glycosyltransferase activity (GO:0008194), galactosyltransferase activity (GO:0008378), oxidoreductase activity (GO:0016491), metal ion binding (GO:0046872) and iron ion binding (GO:0005506) were significantly down-regulated in both genotypes. According to specific down-regulated GO annotation vocabularities for cold resistant genotype, most of the down-regulated genes were related with secondary metabolisms, such as phenylpropanoids, flavonoid and aromatic compounds. Transferase activities, i.e., glucosyltransferases and acyltransferases constituted the most significant molecular function in this genotype. With regard to cold sensitive genotype, those genes with glycolipid metabolic process, sulfur compound biosynthetic process, membrane lipid metabolic process and lipid biosynthetic process were significantly down-regulated biological processes. The gene annotation enrichment analyses of significantly up

and down-regulated groups for both genotypes at APR conditions are given in Appendices B.

3.2.2.5 Genotype Specific Transcriptional Profiles and Their Gene Ontology Analysis

The comparison of cold resistant and cold sensitive genotypes in terms of differential gene expression by using microarray technology highlights the candidate genes involved in cold response of black poplars. Differentially expressed genes may be suitable targets for biotechnological manipulation on the purpose of improving cold tolerance in poplars. These findings enable us to understand the molecular mechanisms of cold tolerance in woody plants. By this way, genetic markers might be developed and they can be used for selection of cold tolerant trees in breeding programmes.

To find out the differentially and co-expressed genes between cold resistant and cold sensitive genotypes, a statistical threshold was applied to the microarray data ($FC \geq 5$, p value ≤ 0.05). As mentioned previously, different time points were compared with control group (SEP) as a means of NOV vs SEP, FEB vs SEP and APR vs SEP in both genotypes to identify differentially expressed genes under different treatments. Beside this, cold resistant and cold sensitive genotypes were also contrasted to find out the differences between transcriptional profiles at FEB condition (during cold). According to this, a total of 4421 genes significantly expressed in cold resistant genotype, while 4187 genes were in cold sensitive genotype. All of these genes were clustered in a Venn diagram to indicate the differentially and co-expressed genes between two genotypes (Figure 3.17). The numbers of differentially expressed genes were determined as 1246 and 1012 in cold resistant and cold sensitive genotypes, respectively. In addition, 3175 genes were identified as co-expressed genes between two genotypes.

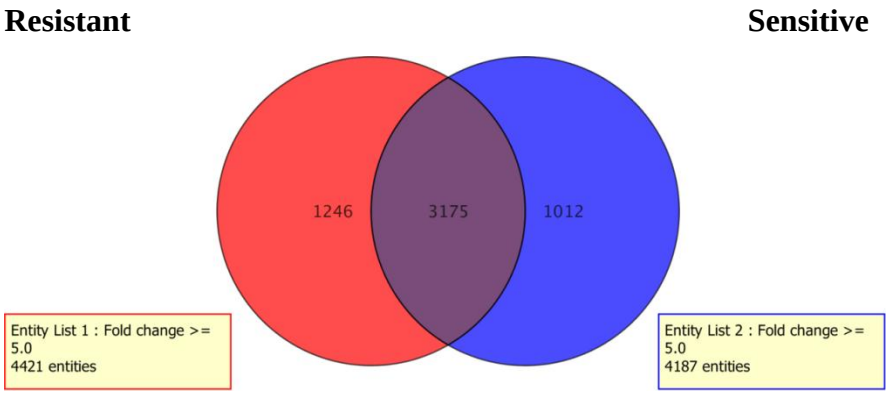


Figure 3.17 The number of differentially and co-expressed genes between cold resistant and cold sensitive genotypes under low temperature conditions (FC ≥ 5, p value ≤ 0.05)

The distribution of enriched GO terms for cold resistant and cold sensitive probe sets under different temperatures indicated several important findings. According to the biological processes vocabulary of ontological annotation, the most representative categories in cold resistant genotype were those of transport mechanisms, i.e., water transport (GO:0006833), fluid transport (GO:0042044), amine transport (GO:0015837) and amino acid transport (GO:0006865); metabolic processes, i.e., cellular amino acid and derivative metabolic process (GO:0006519), methionine metabolic process (GO:0006555), cell wall polysaccharide metabolic process (GO:0010383) and response mechanims including response to freezing (GO:0050826), response to gibberellin stimulus (GO:0009739) and cellular response to hormone stimulus (GO:0032870). Annotation of genes according to the molecular function identified mainly transporter activity (GO:0005215) and oxidoreductase activity (GO:0016706) in cold resistant genotype. In cold sensitive genotype, it was found that response to stress (GO:0006950), response to external stimulus (GO:0009605) and response to wounding (GO:0009611) were the most represented biological terms. In terms of molecular functions, oxidoreductase activity (GO:0016701) and protein dimerization activity (GO:0046983) were significantly activated terms in cold sensitive genotype. Enrichment of co-expressed genes were

also analyzed and it was found that response to cold (GO:0009409), response to abiotic stimulus (GO:0009628), response to oxidative stress (GO:0006979), response to osmotic stress (GO:0006970), response to water deprivation (GO:0009414), carbohydrate metabolic process (GO:0005975) and cellular nitrogen compound metabolic process (GO:0034641) were most represented biological process terms in both genotype. In terms of molecular function, oxidoreductase activity (GO:0016491), transferase activity, transferring glycosyl groups (GO:0016757), protein kinase activity (GO:0004672), protein serine/threonine kinase activity (GO:0004674) and catalytic activity (GO:0003824) were also significantly expressed in both genotypes. The gene annotation enrichment analyses with regard to differentially expressed GO terms in cold resistant and cold sensitive genotypes are given in Table 3.14 and Table 3.15, respectively.

Table 3.14 Significantly enriched GO terms responsive to low temperatures in cold resistant genotype. P value (≤ 0.01) was used to test significance level in annotation terms.

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0050826	Response to freezing	8	1.90E-04
GO:0015800	Acidic amino acid transport	9	4.20E-04
GO:0006575	Cellular amino acid derivative metabolic process	80	4.70E-04
GO:0006664	Glycolipid metabolic process	13	2.30E-03
GO:0009739	Response to gibberellin stimulus	15	2.40E-03
GO:0006555	Methionine metabolic process	17	2.80E-03
GO:0010383	Cell wall polysaccharide metabolic process	6	3.00E-03
GO:0006519	Cellular amino acid and derivative metabolic process	202	3.20E-03
GO:0045491	Xylan metabolic process	5	3.20E-03
GO:0006833	Water transport	7	3.90E-03
GO:0042044	Fluid transport	7	3.90E-03
GO:0015837	Amine transport	19	4.00E-03
GO:0006083	Acetate metabolic process	13	4.20E-03
GO:0030636	Acetate derivative biosynthetic process	13	4.20E-03
GO:0030639	Polyketide biosynthetic process	13	4.20E-03
GO:0030638	Polyketide metabolic process	13	4.20E-03
GO:0030635	Acetate derivative metabolic process	13	4.20E-03
GO:0015701	Bicarbonate transport	5	4.40E-03
GO:0042181	Ketone biosynthetic process	13	4.70E-03
GO:0009765	Photosynthesis, light harvesting	10	4.90E-03
GO:0009755	Hormone-mediated signaling pathway	35	6.30E-03
GO:0006865	Amino acid transport	17	6.30E-03
GO:0007017	Microtubule-based process	33	6.30E-03
GO:0016102	Diterpenoid biosynthetic process	7	6.90E-03
GO:0032870	Cellular response to hormone stimulus	35	7.00E-03
GO:0010410	Hemicellulose metabolic process	5	7.60E-03
GO:0006825	Copper ion transport	9	8.90E-03
Molecular Function			
GO:0046527	Glucosyltransferase activity	26	3.50E-05
GO:0004289	Subtilase activity	9	1.30E-03
GO:0005516	Calmodulin binding	29	1.40E-03
GO:0015291	Secondary active transmembrane transporter activity	41	4.20E-03
GO:0008017	Microtubule binding	9	5.90E-03
GO:0016717	Oxidoreductase activity	8	7.00E-03
GO:0016706	Oxidoreductase activity	15	7.80E-03
GO:0030508	Thiol-disulfide exchange intermediate activity	9	8.90E-03
GO:0005215	Transporter activity	155	9.50E-03

Table 3.15 Significantly enriched GO terms responsive to low temperatures in cold sensitive genotype. P value (≤ 0.01) was used to test significance level in annotation terms.

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0009835	Ripening	11	2.50E-04
GO:0009611	Response to wounding	20	9.10E-04
GO:0009605	Response to external stimulus	43	1.60E-03
GO:0006950	Response to stress	200	2.40E-03
GO:0000038	Very-long-chain fatty acid metabolic process	6	2.50E-03
GO:0006012	Galactose metabolic process	22	3.20E-03
GO:0032271	Regulation of protein polymerization	7	4.70E-03
GO:0043254	Regulation of protein complex assembly	7	4.70E-03
GO:0009620	Response to fungus	19	6.30E-03
GO:0044087	Regulation of cellular component biogenesis	7	6.80E-03
GO:0042726	Riboflavin and derivative metabolic process	12	7.50E-03
GO:0006771	Riboflavin metabolic process	12	7.50E-03
GO:0009651	Response to salt stress	33	7.80E-03
GO:0009225	Nucleotide-sugar metabolic process	12	8.30E-03
GO:0042545	Cell wall modification	12	8.30E-03
GO:0009914	Hormone transport	13	8.60E-03
GO:0009926	Auxin polar transport	13	8.60E-03
GO:0060918	Auxin transport	13	8.60E-03
GO:0051493	Regulation of cytoskeleton organization	7	9.40E-03
GO:0009312	Oligosaccharide biosynthetic process	11	9.80E-03
Molecular Function			
GO:0016701	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	21	4.80E-05
GO:0051213	Dioxygenase activity	20	6.80E-05
GO:0016702	Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	20	6.80E-05
GO:0004568	Chitinase activity	11	1.10E-03
GO:0008378	Galactosyltransferase activity	11	1.30E-03
GO:0046983	Protein dimerization activity	31	1.50E-03
GO:0015103	Inorganic anion transmembrane transporter activity	13	2.20E-03
GO:0004805	Trehalose-phosphatase activity	5	2.70E-03
GO:0004564	Beta-fructofuranosidase activity	6	9.40E-03

3.2.2.6 Candidate Genes Responsive to Low Temperatures in Cold Resistant and Cold Sensitive Genotypes

In this study, 4421 genes in cold resistant genotype and 4187 genes in cold sensitive genotype were identified as significantly expressed (FC $\geq$ 5, p value $\leq$ 0.05). The numbers of differentially expressed genes were determined as 1246 and 1012 in cold resistant and cold sensitive genotypes, respectively and 3175 genes were detected as co-expressed genes in two genotypes. In the following tables, typical genes for both genotypes are listed. Due to the large number of gene lists, only the genes having relatively high fold change values are given. Consequently, 10 candidate up-regulated and 10 candidate down-regulated genes for cold resistant and cold sensitive genotypes are listed in Table 3.16 and Table 3.17, respectively.

According to these findings, especially the genes related with carbohydrate metabolic processes and sugar transport activities, i.e., Beta-galactosidase, Beta-1,3-glucanases, sugar transporters particularly monosaccharide transporters were up-regulated in cold resistant genotype. As defense related genes, glutathione peroxidases and gibberellin 20-oxidase were significantly expressed for oxidation-reduction processes. Apart from that, the genes such as receptor kinases and Leucine-rich repeat receptor like kinases (LRR-RLKs) which are responsible for protein phosphorylation were also significantly expressed. IAA7 like protein and vacuolar-type H^+-translocating inorganic pyrophosphatase (V-PPase) were other up-regulated genes. On the other hand, sulfate transporter protein, metallothionein 2b and ABC transporter genes constituted some of the most significantly down-regulated genes in cold resistant genotype.

In cold sensitive genotype, especially transcription factors including AP2 (APETALA2) domain containing protein, WRKY-type DNA binding protein, DREB2A (dehydration responsive element binding) proteins and myb-like (myeloblastosis) protein were significantly up-regulated. Upregulations of the transcription factors of many cold responsive genes confer freezing tolerance to cold sensitive black poplar clones. Genes involved in scavenging of ROS, i.e.,

Cytochromes P450, 5-Oxoprolinase-like protein and metallothioneins (MTs) were also higly expressed in cold sensitive genotype. Serine/threonine protein kinase and ACC (1-Aminocy- clopropane-1-carboxylate) oxidase were other up-regulated genes. Serine/threonine protein kinases are a large protein family in plants and they have key functions in many signal transduction processes including stress conditions such as cold, salt, drought and temperature. On the other hand, peroxidase, serine carboxypeptidase II and inorganic pyrophosphatase constituted some of the down-regulated genes in cold sensitive genotype.

Table 3.16 List of genes induced by low temperatures in cold resistant genotype (FC ≥ 5, p value ≤ 0.05). The fold change values for each gene were relative to control samples. (R: Resistant, S: Sensitive, FC: Fold change, FEB: February 2012 (during cold) and SEP: September 2011 (control))

Probe Set ID	Gene Annotation	FC ([R-FEB] vs [R-SEP])	FC ([S-FEB] vs [S-SEP])
UP			
Ptp.8053.1.S1_at	receptor like protein kinase	331.1	185.2
PtpAffx.7639.1.A1_at	IAA7 like protein	314.5	59.7
PtpAffx.11727.1.A1_at	vacuolar-type H+-translocating inorganic pyrophosphatase	87.1	17.8
PtpAffx.204209.1.S1_at	sugar transport	67.5	7.5
PtpAffx.54771.1.A1_at	gibberellin 20-oxidase	56.2	26.7
PtpAffx.213309.1.S1_at	leucine rich repeat-like protein	53.4	21.9
PtpAffx.215090.1.S1_s_at	putative sugar transporter	42.5	6.5
PtpAffx.4462.1.S1_at	beta-1,3-glucanase 2	20.7	5.1
Ptp.5821.1.S1_at	putative glutathione peroxidase	17.2	3.2
Ptp.2657.1.S1_s_at	beta-galactosidase precursor - like protein	6.8	-1.3
DOWN			
PtpAffx.208164.1.S1_at	sulfate transporter protein	-277.3	-60.4
PtpAffx.140261.1.A1_at	ABC transporter	-115.1	-56.8
PtpAffx.141148.1.A1_at	putative protein sulfate transporter	-70.2	-9
PtpAffx.618.2.S1_a_at	metallothionein 2b	-59.7	-3.3
Ptp.1970.1.S1_s_at	putative protein polygalacturonase	-59.7	-17
PtpAffx.84732.1.S1_s_at	putative indole-3-acetate beta-glucosyltransferase	-47.4	-12.5
Ptp.378.1.A1_at	subtilisin-like serine protease	-42.9	-24.3
PtpAffx.21151.2.A1_a_at	subtilase	-36.7	-11
Ptp.858.1.S1_at	putative myo-inositol 1-phosphate synthase	-34.3	-4.3
PtpAffx.1041.1.S1_at	putative xyloglucan endotransglycosylase	-28.1	-8.2

Table 3.17 List of genes induced by low temperatures in cold sensitive genotype (FC ≥ 5, p value ≤ 0.05). The fold change values for each gene were relative to control samples. (R: Resistant, S: Sensitive, FC: Fold change, FEB: February 2012 (during cold) and SEP: September 2011 (control))

Probe Set ID	Gene Annotation	FC ([S-FEB] vs [S-SEP])	FC ([R-FEB] vs [R-SEP])
UP			
PtpAffx.71066.3.A1_a_at	ACC oxidase	209.6	110.5
PtpAffx.160139.1.A1_at	putative serine/threonine protein kinase	189.7	50.8
Ptp.6995.1.S1_at	cytochrome P450	183.7	31.4
PtpAffx.17234.1.S1_at	5-oxoprolinase -like protein	174.7	42.9
PtpAffx.80967.1.S1_at	putative protein AP2 domain containing protein	81.5	10.3
PtpAffx.618.1.S1_x_at	metallothionein-like protein	69.9	3.2
Ptp.6928.2.S1_s_at	putative protein thiamin pyrophosphokinase	61.1	23.6
PtpAffx.213667.1.S1_at	DREB2A	52.6	28.1
Ptp.2164.1.S1_s_at	putative WRKY-type DNA binding protein	43.3	6.6
PtpAffx.57050.1.A1_at	myb-like protein	19.4	1.5
DOWN			
PtpAffx.25548.1.S1_a_at	peroxidase	-131.0	-11.2
PtpAffx.13979.1.S1_at	histone deacetylase -like protein	-129.8	-67.4
PtpAffx.1405.1.S1_x_at	putative protein predicted proteins	-123	-29.9
PtpAffx.134527.1.A1_at	serine carboxypeptidase II	-57.3	-25.3
PtpAffx.1398.8.A1_at	inorganic pyrophosphatase	-50.6	-38.6
PtpAffx.73927.1.S1_at	endo-beta-1,4-D-glucanase	-25.4	-7.8
PtpAffx.220036.1.S1_at	peptide transporter PTR2-B	-24.7	-6.2
PtpAffx.127311.1.A1_at	inositol 1,3,4-Trisphosphate 5/6 kinase	-17.4	-5.2
PtpAffx.25139.1.A1_s_at	arabinogalactan protein	-9.4	-2.3
PtpAffx.216874.1.S1_s_at	oligopeptide transporter,	-6.6	-1.7

Hierarchical clustering algorithm (HCA) can be used to group all of these entities and conditions with regard to similarities of their expression profiles. By this way, most similar profiles are joined together into a group. Then, these groups are further joined in a tree structure until all data forms a single group. Due to large numbers of genotype specific genes, a statistical threshold was increased and the genes which had fold change higher than ±50 were determined. Consequently, it was found that 247 genes were significantly expressed in cold resistant genotype, while 229 genes were in cold sensitive genotype during cold (FEB). Within these genes, 10 for each genotype were selected and labeled on heat maps which give summaries about low temperature dependent transcriptional regulation in cold resistant and cold sensitive genotype in Figure 3.18 and Figure 3.19, respectively.

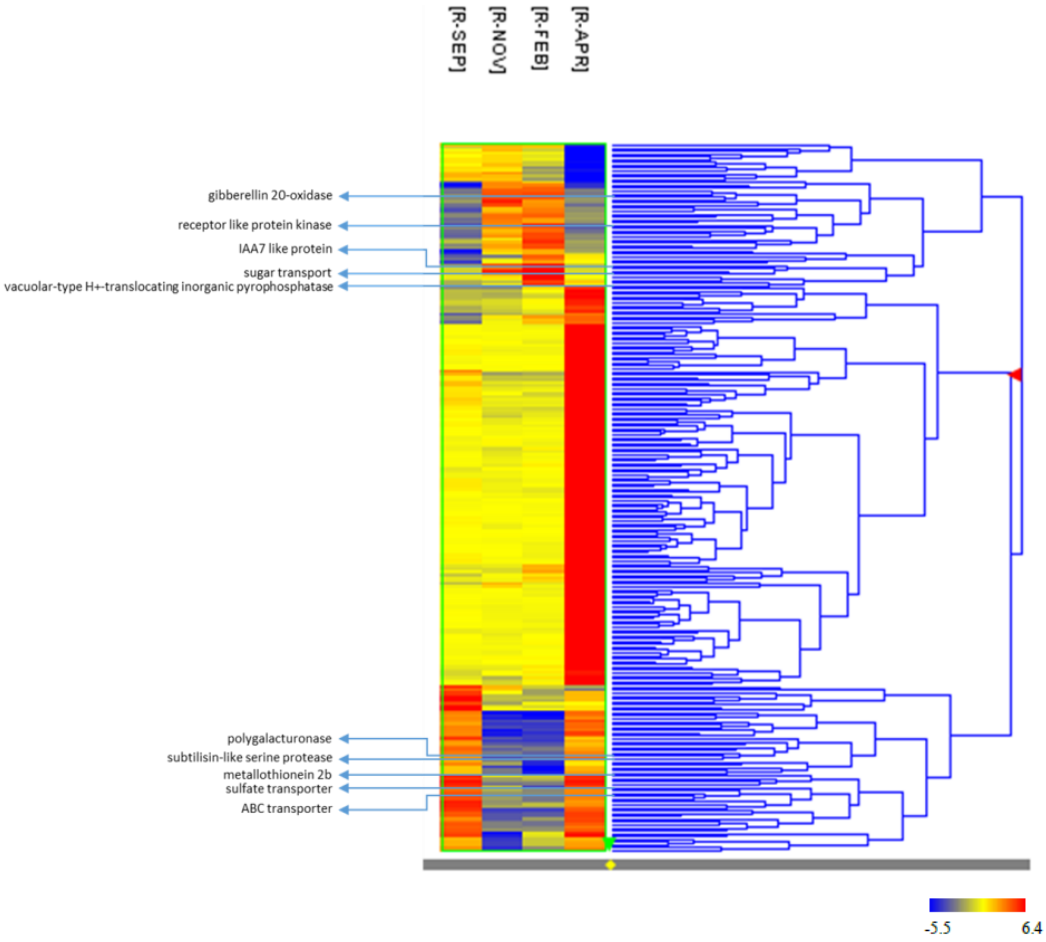


Figure 3.18 Hierarchical clustering among the 247 genes of cold resistant genotype identified by microarray analysis. Genotype specific heat maps indicate the differentially expressed genes in response to low temperatures. Higher and lower expression values in FEB condition (during cold) than those of the control are indicated in r*ed* and *blue*, respectively. Expression values approximately equal between the cold and the control are shown in *yellow*. (R: Resistant, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

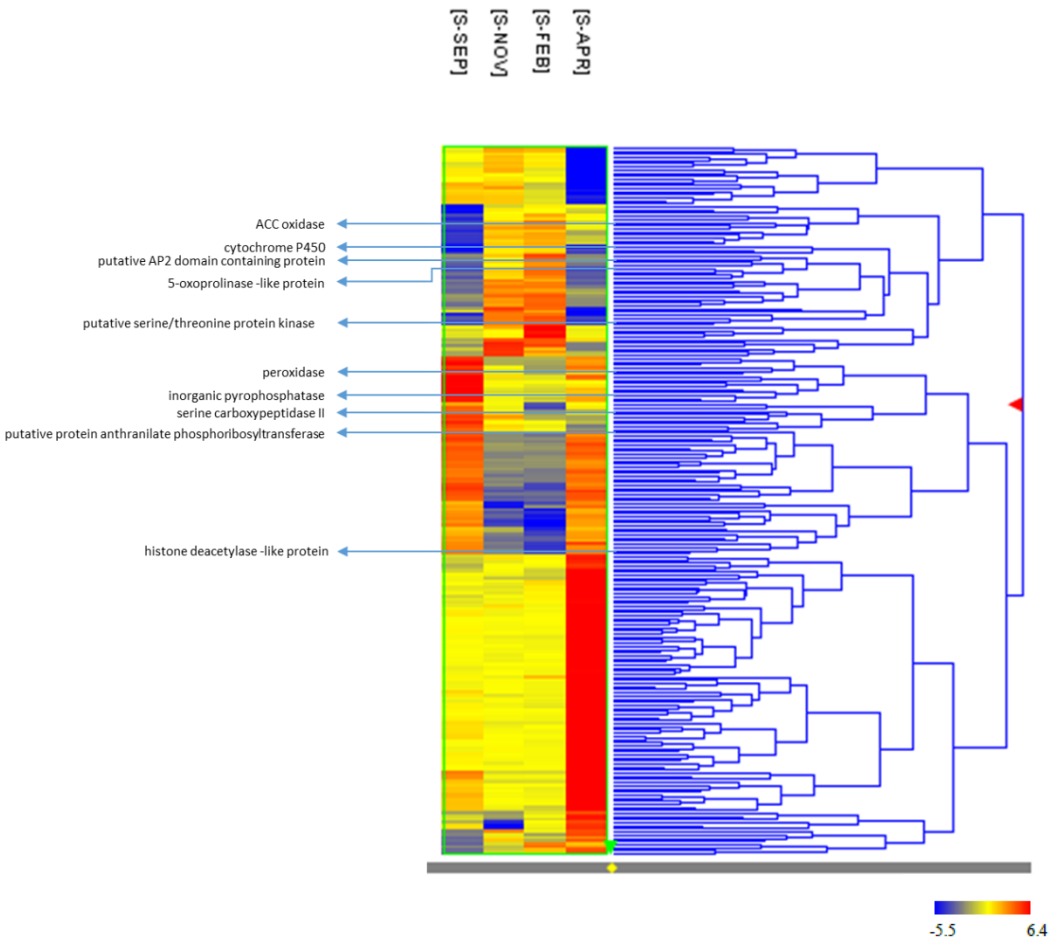


Figure 3.19 Hierarchical clustering among the 229 genes of cold sensitive genotype identified by microarray analysis. Genotype specific heat maps indicate the differentially expressed genes in response to low temperatures. Higher and lower expression values in FEB condition (during cold) than those of the control are indicated in red and blue, respectively.Expression values approximately equal between the cold and the control are shown in yellow. (S: Sensitive, SEP: September 2011 (control), NOV: November 2011 (before cold), FEB: February 2012 (during cold) and APR: April 2012 (after cold)).

CHAPTER 4

DISCUSSION

4.1 Antioxidant Defence System of Black Poplar Clones Under Low Temperatures

Plants encounter different environmental changes throughout their lifespan. Although they are sessile organisms, they have developed natural mechanisms to overcome different types of abiotic and biotic stresses. Plants quickly recognize these adverse alterations in their environment and numerious responses are expressed. Among the environmental stresses, low temperature is one of the major abiotic factors affecting plants life. It is a known fact that low temperature as an environmental stress increases the production of ROS due to disruption of cellular homeostasis (Mittler, 2002; Sharma *et al*., 2012). High levels of ROS are very harmful to plants and eventually lead to oxidative stress (Mittler, 2002; Suzuki and Mittler, 2006). The equilibrium between production and detoxification is important for plant cells to escape from oxidative damage. In order to avoid the oxidative injury, scavenging of excess ROS is carried out by antioxidant defense systems composed of nonenzymatic and enzymatic components. In the current study, we investigated the low temperature effects on black poplar clones by means of antioxidant enzyme activities including ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and catalase (CAT).

APX, DHAR and GR are three important components of the ascorbate-glutathione (ASA-GSH) cycle which founds in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes. The finding of the ASA-GSH cycle in almost all cellular compartments plays a crucial role in detoxifying ROS under stressful conditions (Mittler, 2002). On the contrary, CAT only exists in peroxisomes, but it is still one of the major ROS-

scavenging mechanisms in plants and is indispensible for ROS detoxification (Mittler, 2002). CAT does not require a supply of reducing equivalents for function properly, therefore it might not be sensitive to redox status of plant cells and its function might not be impressed under low temperatures (Mittler, 2002). It was also indicated that redundancy has exist among the ROS-scavenging mechanisms. According to this, plants with suppressed CAT production may induce APX or other enzymes (Mittler, 2002). APX is widely distributed antioxidant enzymes in plants and has higher affinity for H_2O_2 than CAT. Therefore, APX is more effective for H_2O_2 detoxification under stress conditions (Sharma *et al*., 2012). GR is another ubiquitous enzyme in wintering perennials (Sagisaka, 1982).

In this study, it was found that the cambium tissue of black poplar clones contained high levels of APX, DHAR and GR under low temperatures. These enzymes followed a similar seasonal pattern and their highest activities were present in winter conditions while the lowest activities were in springs. As mentioned previously, plants protected themselves from low temperatures by increasing their antioxidant enzyme activities. When the growth signs and accompanying metabolic changes were appeared in spring, the antioxidant enzyme activities began to decrease. In autumn, the activities increased once again for acclimation purposes. During this time, growth began to cease and plants prepared themselves for winter conditions. The profile of CAT activity was different from these three enzymes. Seasonal changes in CAT activity demonstrated that the lowest activities appeared in winter periods. In contrast to the APX, DHAR and GR activities, CAT activity began to increase when the growth and enlargement progressed in spring and it remained relatively high during early dormancy period in autumn.

The growth phenomenon of perennial plants in the temperate areas is made up of an alternating periods of growth and dormancy. Plants begin to prepare themselves for winter conditions after an active growth period in spring and in early summer. Winter dormancy is the strategy for woody perennial plants growing in the temperate zone to live under adverse environmental conditions. There are numerious metabolic changes during leaf senescence, abscission, nutrient recycling, bud formation,

acclimation to cold and dormancy acquisition. ROS formation also accompanies to these processes as in the signaling pathways or metabolic degredation. As a result, increased autumn activities of black poplar antioxidant enzymes in our experiments could be related with the high level of ROS production during all of these processes. Our findings revealed that the metabolic and structural changes related with acclimation purposes for wintering of black poplar clones in Behiçbey Nursery began to occur in September. The growth of tissues almost ceased in October and then the leaves shed by early November which made them ready for wintering. In addition, the precipitation level was low during September and November 2011 as shown in Figure 2.4. Water stress is also induces the ROS production in plant species. The increased activities of ASA-GSH cycle and CAT activity in autumn may also be associated with low water levels. The profile of CAT activities showed different pattern from GR, APX and DHAR activities in black poplar clones under low temperatures. It began to increase in March and made a peak in April during bud burst and active growth. Until October, the cells can actively synthesize organelles and proteins. During this time, CAT activities were also relatively high. ROS formation also takes place under optimal growth conditions. It may be concluded that deleterious ROS produced during growing period in spring is decomposed by CAT instead of ASA-GSH cycle enzymes in black poplar clones.

In general, most antioxidant enzyme activities increase in cold resistant species under low temperatures, whereas decreased levels appear in cold sensitive cultivars (Gossett *et al*., 1994; Guo *et al*., 2007). However, we found that cold sensitive black poplar clones had higher activities of APX, DHAR, GR and CAT during all seasons. Excess level of antioxidant enzyme activities in sensitive lines under chilling temperatures was also reported in the literature (Hodges *et al*., 1997). It may be suggested that cold sensitive individuals had increased need of ROS removal under low temperatures. By this way, they became less sensitive to low temperature induced oxidation. Late bud set and early bud flush are also associated with increased risks of frost damage. We found that bud flush occured earlier in cold sensitive trees which made them more vulnerable to frost damage. Because of this,

they increased antioxidant enzyme activities for ROS scavenging. Leaf abscission and bud flush occured lately in cold resistant clones when comparing to cold sensitive ones. Late bud flush decreases the risk of late spring frost damage which might cause the decreased antioxidant enzyme activities in cold resistant clones. On the other hand, trees with early bud set or late bud flush have a shortened growing season which decreases competitive ability for resources. However, cold resistant trees delayed their leaf abscission and accordingly bud set formation. Thus, there was no problem related with the span of growing season. Cold resistant clones indicated better growth performances as being taller and wider than cold sensitive clones. Relatively lower levels of antioxidant enzyme activities in cold resistant clones might also be explained with other mechanisms which make them already resistant to low temperatures, such as accumulation of some carbohydrates, pigments, polyamines or proteins.

In recent years, considerable efforts have been made toward the studies of changing antioxidant enzyme activities as a result of cold acclimation. It is well documented that the increased activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) constituted the ASA-GSH cycle and other antioxidant enzymes are necessary to reduce the effects of low temperature stress (Krivosheeva *et al*., 1996; Tao *et al*., 1998; Jin *et al*., 2003). These types of responses have also been well documented for many poplar species.

Song *et al*. (2014) indicated sex-specific floral developmental responses to seasonal temperature transitions in *Populus tomentosa*. They suggested that female floral buds had better adaptation mechanisms than do males. SOD, POD and CAT activities in female floral buds were significantly increased at the later stage of enlargement and later stage of dormancy under maximum and minimum values of climatic air temperature. It was also showed that the enzymes activities changed significantly according to temperature. Under chilling conditions, CAT activity was unchanged in female floral buds, whereas it decreased in male floral buds.

Jiang *et al*. (2011) indicated the effects of different low temperatures (cold and freezing) on cuttings of three hybrid clones of *P. ussuriensis* × *P. deltoids*. They found that the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) variously increased at 10, 5, and 0°C. However, the activities decreased relatively under lower temperatures (-5, -10, and -15°C) treatment. They also searched the effect of consecutive low temperature (5°C) treatment during which activities of CAT, APX and GR increased immediately or after 3 h, however, SOD and POD activities slightly decreased at the beginning of the treatment; then increased quickly, and finally declined.

Zhang *et al*. (2011) showed sex-related differences in *Populus cathayana* Rehd. under chilling (4°C) conditions. According to this study, 4°C temperature induced a chilling stress and females suffered more negatively than males. Although APX activity decreased in both sexes at chilling temperature, GR and POD activities decreased only in females. Males showed higher GR levels under chilling stress. They suggested that sex-related differences to chilling stress are important and *Populus cathayana* males had a better protection mechanism than do females.

Luo *et al*. (2007) also investigated the activities of some critical enzymes of the ASA-GSH cycle in *Populus suaveolens* cuttings. They found that cold acclimation increased the SOD, POD, CAT, MDAR, APX, DHAR and GR activities. Besides, APX, DHAR, MDAR and GR levels were higher than SOD, POD and CAT during freezing acclimation. They concluded that higher levels of the ASA-GSH cycle was necessary for H_2O_2 detoxification, growth and development of cuttings and it played an important role on cold resistance of cuttings.

The effects of cold exposure on microcuttings of *Populus tremula* L. were indicated by Jouve *et al*. (2000). It was found that CAT, APX and MDHAR activities were not affected when plants were cultured at 10°C. However, DHAR and GR activities were temporarily higher than control ones at this temperature.

It was demonstrated that a high activity of ASA-GSH cycle was necessary for a decline of ROS in *Populus* cutting during freezing acclimation. In a study conducted by Nakagawara and Sagisaka (1984), APX, DHAR and CAT activities in the twigs of poplar, *Populus gelrica,* were measured throughout a year. APX and DHAR activities were high during the winter period of life cycle. It was revealed that the changes in these enzyme activities occured simultaneously. When the growth began to cease in fall, the activities increased. In contrast to APX and DHAR activities, CAT activity began to increase when the growth progressed and decrease by early November. According to these results, they suggested that deleterious H_2O_2 was decomposed by catalase in the growing period.

Metabolic transition in September was also reported and it was demonstrated that the activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) suddenly increased in the living bark and xylem of poplar twigs in September to form NADPH (Sagisaka, 1974; Sagisaka and Asada, 1981). ASA-GSH cycle needs NADPH in order to function properly. According to this, it may be deduced that some enzymes in the NADPH oxidation reaction associated with ascorbate and glutathione metabolism are appeared in the same time with the increased activities of G6PDH and 6PGDH. By this way, dehydroascorbate and ascorbate free radicals in the cell could be reduced back to ascorbate right away (Nakagawara and Sagisaka, 1984). According to this, black poplar cuttings had increased activity of ASA-GSH cycle during cold acclimation as the way of detoxification of ROS.

Most of the studies indicated increased levels of CAT activity, whereas decreased CAT activity under different stress conditions in *Populus* species were also explained. Zhao *et al.* (2009) revealed sex-related differences of *Populus cathayana* Rehd. to photoperiods. They found that although males had higher activities, short day shift significantly decreased POD, CAT and SOD activities in both male and female seedlings. On the other side, long day shift significantly increased POD, CAT and SOD activities in females, but they significantly decreased in males. In another

study conducted by Stobrawa and Lorenc-Plucińska (2007), the effects of heavy-metal-polluted environment to the antioxidant enzyme activity of black poplar (*Populus nigra* L.) and cottonwood (*Populus deltoides* Bartr. ex Marsch) were investigated. They found that catalase was highly sensitive to the heavy metals, whereas APX and GR activities were not affected by heavy metals. *Populus nigra* showed a clear decrease in CAT activity independent of the season. In case of *Populus deltoides*, a decrease in autumn and an increase in spring were observed for CAT activity. Decreased CAT activity under chilling temperatures were also reported in other plant species, such as in cucumber seedlings (Omran, 1980), in grapewine (*Vitis vinifera* L.) buds (Nir *et al*., 1986), in *Nicotiana plumbaginifolia* L. (Willekens *et al*., 1994), in squash (Wang, 1995), in young sal *(Shorea robusta)* seedlings (Keshavkant and Naithani, 2001) and in tobacco (Gechev *et al*., 2003).

Under chilling or freezing temperatures, increases of various antioxidant enzyme activities have been showed in literature as mentioned previously, while decreases in their activities have also been indicated. Such inconsistent responses may be attributed to temperature duration and/or values, species differences and field conditions. Field experiments suffer from continuously changing biotic and abiotic environmental factors such as wind, air humidity, water status and soil which might affect the final result of antioxidant enzymes. It is quite impossible to control all of these environmental factors; therefore drawing unambiguous conclusions from field experiment is difficult. However, based on our experiments we proposed that ASA-GSH cycle plays a crucial role in freezing resistance of black poplar clones under low temperatures, while deleterious ROS produced during growing period in spring is decomposed by CAT.

4.2 Differentially Expressed Genes in Cold Resistant and Cold Sensitive Genotypes under Low Temperatures

Poplar is a fast-responsive and chilling-tolerant species. Poplars have a broad diversity of proteomic, biochemical and physiological mechanisms to cope with low temperature effects. Besides inducing different types of responses, exposed to low

temperature triggers many pathways that causes differential gene and protein expression (Renaut *et al*., 2005). In this study, comparison of gene expression profiling of cold resistant and cold sensitive genotype revealed important genes and mechanisms which can be used for further understanding of cold tolerance in black poplars.

4.2.1 Transcriptional Profile of Cold Resistant Genotype under Low Temperatures

It was indicated that IAA7 like protein was one of the most differentially expressed gene in cold resistant genotype in our study. Auxin is one of the plant hormone which have various roles in cellular and developmental responses such as cell division, cell elongation, cell growth, differentiation, apical dominance and gravitropism (Friml, 2003). Aux/IAA is one of the major classes of early auxin response genes through which auxin exerts its effect by regulating expression of numerous auxin-responsive genes at the molecular level. Aux/IAA gene family is well characterized and it was indicated that these genes play a central role in auxin signaling (Reed, 2001). Although auxin influences many growth and developmental processes by altering gene expression in plants, some recent studies suggest that it is also involved in stress or defense responses, especially Aux/IAA gene family (Ghanashyam and Jain, 2009; Jain and Khurana, 2009; Wang *et al*., 2010). This gene family includes many DNA elements thought to be responsive to ABA, SA, drought, light, cytokinin, and pathogens. Therefore, it was suggested that these genes may be related with phytohormone signals, abiotic and biotic stresses. Moreover, many auxin responsive genes have been included in abiotic stress responses which indicate crosstalk between stress and auxin signaling. Wang *et al*. (2007) hypothesized that repression of the auxin pathway is an important aspect of the defense response. Differential expression of Aux ⁄ IAA genes during cold acclimation in Arabidopsis was also indicated by Hannah *et al*. (2005).

In our study, increased expression level of H^+-translocating inorganic pyrophosphatase (V-PPase) in cold resistant genotype may also be related to defense

mechanisms against low temperatures. Meanwhile, this might be accomplished by the accumulation and transport of sugars for osmotic pressure. Vacuoles occupy a large part of the plant cells which accumulate many soluble substances including inorganic ions, organic acids, enzymes and secondary metabolites. They are acidic organelles and crucial to detoxification and general cell homeostais (Taiz, 1992; Martinoia *et al*., 2007). The acidic environment is essential for the hydrolytic enzymes, active transport and accumulation of substances. There are two distinct proton transport enzymes in the plant vacuolar membrane, i.e., H^+-translocating inorganic pyrophosphatase (V-PPase) and H^+-ATPase (V-ATPase, vacuolar-type H^+-ATPase). V-PPase is different in terms of an energy source that uses inorganic pyrophosphate (Ppi, diphosphate). The proton pumps generate electrochemical potential gradient of protons across the vacuole membrane and transports many metabolites. They are key elements for vacuolar pH regulation and many transport systems. Plant cells should maintain a constant turgor pressure by changing the osmotic pressure of the cytosol and vacuole. Transport of sugars, ions and other metabolites across the vacuolar membrane and the plasma membrane regulates the osmotic pressure. Plant vacuoles also contain some cytotoxic substances for defense against biotic and abiotic stress conditions. Recent studies indicated that expression of V-PPase genes is related to abiotic stress response. Zhang *et al*. (2011) reported that overexpression of OVP1 (vacuolar H^+-translocating inorganic pyrophosphatase) enhanced cold tolerance in rice which was associated with osmolite accumulation (proline) and increaing membrane integrity. Expression of V-PPase was also induced in grapevine and maize by cold stress (Venter *et al*., 2006; Yue *et al*., 2008).

4.2.1.1 Carbohydrate Metabolic Processes and Sugar Transport Activities May Be Crucial in the Adaptation of Cold Resistant Genotype to Low Temperatures

Carbohydrates are one of the most essential structures for plant cells in which energy is stored, transported and used for metabolism. Plants have various mechanisms related with storing and recycling nutrients that can be used during growth period. The carbohydrate content can be regarded as a part of sources and sinks. The stored

carbohydrates might be hydrolyzed and used for growth and respiration. Although there are many changes during cold acclimation, soluble sugars play important roles based on the fact that they are usually detected in various plant species. Accumulation of sugars is correlated with development of freezing tolerance during cold acclimation (Ristic and Ashworth, 1993). Trees need to store reserves to get a chance for survival during winter. Some carbohydrate reserves, such as starch and soluble sugars, are essential to live while overwintering or after stress. The enzymes involved in the conversion of starch to the sugar are also induced by low temperatures. By this way, starch accumulated in reserve tissues during the preceding summer can be converted to sucrose during winter conditions. The level of soluble sugars increases during the onset of winter when plants are subjected to low temperatures. Beside these, sugars can also act as cryoprotectants for specific enzymes (Carpenter *et al*., 1986), as molecules for promoting membrane stability (Lineberger and Steponkus, 1980; Anchordoguy *et al*., 1987) and as osmolytes to inhibit dehydration (Steponkus, 1984) to protect cells from freezing injury. It is also indicated that total soluble sugar accumulation lower ice nucleation (Reyes-Díaz *et al*., 2006). As a result, carbohydrates are essential for the winter survival of trees in diverse roles and sugar accumulation correlates with an increase in freezing tolerance in woody plants (Sauter *et al*., 1996). Moreover, carbohydrates may also act as scavengers of ROS and promote to increased membrane stabilization (Bohnert and Sheveleva, 1998). Sugar signalling is also related to hormone signalling and stress responses in plants (Zeng *et al*., 2011).

Populus stems, branches and roots are important storage organs for starch, sugars, lipids, and protein. Concentrations of these substances change in response to cold hardiness (Nelson and Dickson, 1981; Fege and Brown, 1984; Bonicel *et al*., 1987; Sauter 1988). Carbohydrate in poplar stem wood is stored as starch and then converted to soluble sugars in late fall during low temperatures (Nelson and Dickson, 1981; Fege and Brown, 1984; Bonicel *et al*., 1987). Conversion of starch to sugar is a well known fact of cold acclimation (Levitt, 1980). The accumulation of soluble sugars and proteins are required for osmotic adjustment which is also important for

freezing tolerance and cold acclimation. Low temperature treatments cause cellular perturbations, therefore the level of sucrose and its galactosides increase in poplar leaves. It was also found that raffinose and trehalose level raise with the increase in freezing tolerance. Sucrose and trehalose can stabilise membranes and proteins in the case of dehydration under freezing conditions. Membrane stabilisation is provided by the insertion of these disaccharides in the membranes (Oliver *et al.,* 1998). As a result, sucrose, glucose, fructose, trehalose and raffinose accumulated under chilling temperatures and these carbohydrates have putative roles in cold acclimation of poplar (Renaut *et al*., 2004).

In this study, numerious genes involved in carbohydrate metabolic process and sugar transport were significantly up-regulated in cold resistant genotype. Beta-galactosidase is one of these genes which are encoded by a multigene family in several plant species. Glycoside hydrolases (GHs) are widely distributed enzymes which hydrolyse the glycosidic bonds. β-galactosidase belonging to GH Family 35 catalyse the hydrolysis of terminal β-galactosyl residues from carbohydrates, galactolipids and glycoproteins. It is considered that these enzymes function in diverse biological processes such as modification of cell wall components during fruit ripening (Carey *et al*., 1995; Smith *et al*., 2002), loosening of the cell wall during growth (Dopico *et al*., 1989) and galactolipid turnover (Bhalla and Dalling, 1984). It was also indicated that sevaral β-galactosidase genes are regulated by biotic and abiotic stresses. Oono *et al*. (2006) indicated that β-galactosidase genes involved in carbohydrate metabolism were up-regulated during deacclimation in *Arabidopsis*. Increased level of β-galactosidase genes as a drought response in *Populus* was also indicated by Street *et al*. (2006).

According to GO biological process, Beta-1,3-glucanases are also involved in carbohydrate_metabolic process. Beta-1,3-glucanases are a group of hydrolytic enzymes which catalyze the cleavage of 1,3-β-D-glucosidic linkages in β-1,3-glucans. They have diverse gene families in plants and are involved in numerious physiological and developmental processes, such as cell division, pollen formation,

seed germination, response to ozone and defense against pathogenic fungi (Vogeli-Lange *et al*., 1994; Schrauder *et al*., 1996; Mauch and Staehelin, 1989). Beside these, Hincha *et al*. (1997) indicated that class I β-1,3-glucanase had cryoprotective activity and they were accumulated in spinach and cabbage leaves during cold acclimation. A tobacco class I β-1,3-glucanase protects cells from freeze-thaw injury by reducing solute influx across membranes and alleviating osmotic stress. The accumulation of β-1,3-glucanases during chilling was demonstrated in birch (*Betula pendula*) by Rinne *et al*. (2001). It was also reported that 10 putative cell wall β-1,3-glucanases genes were identified in *Populus* that could turn over β-1,3-glucans at pores and plasmodesmata. Those chilling induced β-1,3-glucanases reopened signal pathway and release dormancy in *Populus* (Rinne *et al*., 2011). According to this, β-1,3-glucanase which were upregulated in cold resistant genotype in our study migh have cryoprotective activity which allow black poplar clones to live under low temperature conditions.

Sugar transport activities are also thought to be important phenomenon for freezing tolerance or the winter survival of trees. There are several sugar transport proteins which have crucial roles in the cell-to-cell and long distance distribution throughout the plants. The transportes have various functions including nutritional role, sugar supply to cells for development, signaling and generating osmotic gradients (Williams *et al*., 2000). Higher plants contain two main types of sugar transporter, i.e., disaccharide transporters (catalyse sucrose transport) and monosaccharide transporters (transport of a variable range of monosaccharides). Monosaccharide transporters are integral membrane proteins and constitute a large gene family. In most plant species, soluble sugars are mainly present in the forms of glucose, fructose, and sucrose.The disaccharide sucrose is the major form of long distance transport of sugars in the phloem sieve cells. However, the principal monosaccharide sugar forms are glucose and fructose in many plants. Monosaccharide transport have important role in intercellular exchanges both in source and sink tissues. In sink organs, sucrose is frequently hydrolyzed, and the resulting monosaccharides are stored or utilized in parenchyma cells. Plant species have many of these transporters

for sugar transport in different tissues under numerious environmental conditions. The expression of genes for sugar transporters are induced by abiotic stress conditions and sugars are transported throughout the plants by this way (Maruyama *et al*., 2004, Wormit *et al*., 2006).

In this study, especially monosaccharide transporters were up-regulated in cold resistant genotype under low temperature conditions as a means to increase stress tolerance. It was demonstrated that *Arabidopsis thaliana* tonoplast monosaccharide transporter (AtTMT) and vacuolar glucose transporter (AtVGT) were significantly up-regulated by environmental stresses such as salt, drought and cold (Wormit *et al*., 2006). Cold adaptation of plants increased the uptake of glucose into mesophyll vacuoles. The researchers reported that energy was necessary for transport and same carrier taken up both glucose and fructose. TMT1 was involved in vacuolar monosaccharide transport and had key roles in stress conditions.

4.2.1.2 Protein Phosphorylation May Be Key Event for Cold Resistant Genotype in response to Low Temperatures

Protein phosphorylation plays important roles in plant metabolism and signal transduction (Fallon and Trewavas, 1993). Phosphorylation mainly comprises on some amino acids including serine, threonine, and tyrosine. However, lysine, arginine, histidine, aspartic acid, glutamic acid, and cysteine can also be phosphorylated under some conditions. Protein kinases are responsible from phosphorylation events, but dephosphorylation requires protein phosphatases. Both of these events occur in plant species for cellular communication and growth regulation responsive to environmental conditions. There are many types of protein kinases one of which is receptor like kinases belonging to a serine/threonine class of protein kinases with differences in their extracellular domains.

Numerious stress conditions such as dehydration, cold and osmotic stress affect the protein phosphorylation (Holappa and Walker-Simmons, 1995; Hwang and Goodman, 1995; Mizoguchi *et al*., 1996). In our study, many receptor kinases were up-regulated in cold resistant genotype by which black poplar clones might increase

stress tolerance and live under low temperature conditions. Hong *et al*. (1997) also demonstrated that receptor like kinase gene was immediately expressed by various environmental stresses such as dehydration, high salt and low temperature.

Leucine-rich repeat receptor like kinases (LRR-RLKs) also constitute a large group of the receptor like kinase (RLK) superfamily in plant genome. These proteins are composed of different domains, i.e., an extracellular N-terminal LRR domain for signal perception, a single-pass transmembrane domain for protein anchoring and a C-terminal cytoplasmic serine/threonine (ser/thr) protein kinase domain for signal transduction (Zhang, 1998; Gou *et al*., 2010). LRR-RLKs play critical roles in plant growth, development, morphogenesis, organogenesis and hormone signaling. Beside these, they are defense-related gene family which respond to abiotic and biotic stress such as tolerance to oxidative stress (Pitorre *et al*., 2010) and salt stress. In our study, cold resistant genotype significantly expressed LRR-RLK under low temperature conditions. Hwang *et al*. (2011) evaluated the expression patterns of LRR RLK genes of both rice and Arabidopsis against abiotic stresses. They employed two microarray datasets composed of seven abiotic stress treatments, including drought, salt, cold, heat, anoxia, and chilling, as well as gamma ray irradiation, in both genomes and indicated differential expression of these gene family. PtLRR-RLK gene expression under many stress conditions such as drought, cold, hypoxia and nitrogen limitation was also investigated in *Populus* (Zan *et al*., 2013). They suggested that PtLRR-RLKs have key roles in growth and adaptation of plants.

4.2.1.3 Defence Related Genes in Cold Resistant Genotype

As mentioned previously, the ROS production is one of the most important phenomena in plants exposed to low temperatures as an environmental stress. Although ROS act as signaling molecules in regulation of many physiological processes, excessive levels create harmful effects to plant cells. As a result, there are numerious enzymatic and non-enzymatic systems against oxidative injury. Glutathione peroxidases (GPXs) are one of these enzymatic systems which protect

plant cells against oxidative damage due to ROS. They catalyze the reduction of H_2O_2, organic hydroperoxides and lipid peroxides by using glutathione as hydrogen donor (Ursini *et al*., 1995). In our study, cold resistant genotype increased the expression level of glutathione peroxidases under low temperatures to protect themselves against oxidative damage. According to GO biological process, GPX is involved in response to oxidative stress. Overexpression of GPX under cold stress was also reported in many plant species, such as Arabidopsis (Rodriguez Milla *et al*., 2003) and *N. tabacum* (Roxas *et al*., 1997). Differential transcript abundance and upregulation of GPX2 in response to chilling in *Populus simonii* was also reported (Song *et al*., 2013). Therefore, it can be concluded that elevated levels of GPXs may play important role in protecting plant cells against low temperature induced oxidative damage.

Gibberellin 20-oxidase was also one of the upregulated genes in cold resistant genotype involved in oxidation-reduction pathways with regard to GO biological process. Gibberellins (GA) are plant hormones which affect the plant development in many aspects such as seed germination, stem elongation and leaf expansion (Davies, 1995). They also induce the accumulation of protein and non-structural carbohydrate (Wasilewska *et al*., 1987), the expression calcium-dependent protein kinase gene (Yang *et al*., 2003) and affect the gene expression of vacuolar H^+ - inorganic pyrophosphatase (V-PPase) and H^+ -ATPase (Fukuda *et al*., 2006). GA 20-oxidase is considered as a regulatory enzyme which catalyzes the last step of GA biosynthetic pathway (Yamaguchi and Kamiya, 2000; Kusaba *et al*., 2001). GA 20-oxidase gene accumulates in seeds, leaves, actively growing shoots and internodes (Phillips, 1998; Xu *et al.*, 2002). Recent studies suggested that low temperature can affect the expression of genes included in distinct steps of GA biosynthetic pathway. Yamauchi *et al*., (2004) indicated the upregulation of GA 20-oxidase genes (GA20ox1 and GA20ox2) under low temperatures in Arabidopsis. In addition, the differential expression of ZjGA20 in different *Z. japonica* tissues induced by low temperature was investigated by Dai *et al*. (2012). These results demonstrated that expression of GA 20 was regulated by abiotic stresses especially under low temperatures.

4.2.2 Transcriptional Profile of Cold Sensitive Genotype under Low Temperatures

Serine/threonine protein kinase is one of the up-regulated genes induced by cold sensitive genotype in this study. Potein phosphorylation and dephosphorylation are catalyzed by protein kinases and protein phosphatases, respectively and they are recognized as major processes for regulating cellular functions. Protein kinases might be categorized as serine/ threonine or tyrosine kinases depending on the phosphorylated amino acid. Serine/threonine protein kinases are a large protein family in plants and they have key functions in many signal transduction processes including stress conditions such as salt, drought, cold and temperature (Stone and Walker, 1995). Protein serine/threonine kinases in plant cells accept input information from receptors sensing environmental conditions, phytohormones and other external factors. Then, they convert it into suitable outputs such as changes in metabolism, gene expression, cell growth and division (Hardie, 1999). The role of serine/threonine protein kinase in cold stress response was reported in peanut (Rudrabhatla and Rajasekharan, 2003) and transgenic Arabidopsis (Mao *et al*., 2010)

Chilling sensitive genotype significantly increased the level of ACC (1-Aminocy-clopropane-1-carboxylate) oxidase gene under low temperatures in our study. ACC oxidase is the terminal enzyme in ethylene biosynthetic pathway. Ethylene synthesis takes place in a two-step biosynthetic pathway. S-adenosylmethionine converts to ACC in a reaction catalyzed by ACC synthase at first. Then, ethylene is subsequently generated from ACC by ACC oxidase (Yang and Hoffman, 1984). It has been indicated that ACS and ACO are differentially up-regulated by various types of abiotic and biotic stresses in angiosperms (Bleecker and Kende, 2000). Ethylene is one of the most important plant hormone which has key roles in numerous processes including cell differention, growth, development, and response to environmental stress (Yang and Hoffman, 1984; Bleecker and Kende, 2000). Plants increase their hormones such as ethylene and ABA under biotic or abiotic stress conditions. Cell signaling pathway during stress begins with signal perception and then continues with second messenger generation such as ROS and inositol phosphates. These

second messengers regulate intracellular Ca^{2+} levels which often initiate a protein phosphorylation cascade. By this way, proteins directly involved in cellular protection or transcription factors related to stress-regulated genes are targeted. Finally, subsequent products may have roles in the production of regulatory molecules like the plant hormones ethylene and ABA (Xiong *et al*., 2002). These hormones may have roles in regulating stress signaling and plant stress tolerance. As a result, ACC oxidase and ethylene are necessary compounds for plant species to gain a stress resistance. The effects of cold on the stimulating of ACC oxidase activity were reported in the literature (Knee, 1987; Jobling *et al*., 1991; Larrigaudière and Vendrell, 1993). A study conducted by Andersson-Gunnerås *et al*. (2003) showed that ACC oxidase activity was important for asymmetric ethylene production control in poplar stems when tension wood was induced by gravitational stimulation. (Tension wood is the reaction wood formed on the top side of branches and leaning stems of woody angiosperms.) ACC oxidase gene was also up-regulated in cold resistant genotype in this study. However, when the fold changes are considered, cold sensitive genotype (209.6 fold change) had significantly higher expression value than cold resistant genotype (110.5 fold change).

4.2.2.1 Transcriptional Regulations May Be Crucial in the Adaptation of Cold Sensitive Genotype to Low Temperatures

Cold stress inhibits the expression of full genetic potential of plants via preveention of metabolic reactions. Low temperatures may trigger other stresses such as osmotic or oxidative stress. These stress conditions have also significant effects on the expression of genetic potential of plants. Cold acclimation includes precise regulation for expression of transcription factors and effector genes which are called cold-regulated (COR) genes (Thomashow, 1999, Viswanathan and Zhu, 2002). Differences in the expression of COR genes determine the physiological and biochemical changes during plant cold acclimation. Many of the COR genes include cis-elements in their promoters such as dehydration-responsive elements/C-repeat elements (DRE/CRT, A/GCCGAC), abscisic acid (ABA)-responsive element (ABRE, PyACGTGGC), myeloblastosis (MYB) (C/TAACNA/G) and

myelocytomatosis (MYC) recognition sequences (CANNTG) (Yamaguchi-Shinozaki and Shinozaki, 2005). The expression of COR genes is under the control of some important transcription factors (TFs). Thousands of genes encoding transcription factors involved in stress resistance mechanisms have been identified by genome-wide analyses. Environmental stresses might induce or repress these genes which affect the plant adaptation. Recently, many transcription factors regulating the expression of several genes related to stress have been discovered. The ethylene responsive element binding factors (ERF), basic-domain leucine-zipper (bZIP), MYC, MYB and WRKY binding (WRKY) transcription factors are some of the important families of stress responsive transcription factors.

Cold acclimation induces the expression of C-repeat (CRT) binding factor (CBF) family of transcriptional activators which is also known as dehydration-responsive-element-binding proteins (DREB). The transcription activation factors bind *cis*-elements found in the promoter region of various abiotic stress related genes. By this way, the expression of many genes are up-regulated that results in abiotic stresses tolerance. The CBF/DREB family of transcription factors binds to the promoters of many COR genes and activates cold-responsive genes. CBFs are upstream transcription factors in the APETALA2 (AP2)/Ethylene response factor (ERF) family that bind to the promoter cis-element which control the transcription of many cold responsive genes conferring freezing tolerance to plants (Thomashow, 1999). The *Arabidopsis* CBF/DREB1 family consists of six paralogues, however three of them, i.e., *CBF1* (*DREB1b*), *CBF2* (*DREB1c*) and *CBF3* (*DREB1a*) are cold-inducible (Sakuma *et al.,* 2002). It was indicated that the key role of the CBF family in cold acclimation of herbaceous annuals such as *Arabidopsis* has also been maintained in *Populus* species (Benedict *et al*., 2006). Although the CBF pathway is a major component of cold response, there are also CBF-independent pathways which might be included in the cold stress response (Zhu *et al*., 2004). Some cold regulated transcription factors, i.e., MYB-type transcription factors, do not participate in the CBF cold response pathway (Zhu *et al*., 2004). According to this, it

can be concluded that TFs have important roles in gene expression and in the regulation of cross talk between different signaling pathways.

In this study, some trancription factors including AP2 domain containing protein, WRKY-type DNA binding protein, DREB2A and myb-like protein were up-regulated in cold sensitive genotype under low temperature conditions. It was suggested that activation of these genes confer chilling or freezing tolerance to cold sensitive black poplar clones. Increased gene expression patterns of these transcription factors under cold conditions suggest that they may be involved in cold related gene regulation. All of these transcription factors were also expressed in cold resistant genotype. However, when the fold change values are considered, it is easily realized that they are more crucial for cold sensitive genotype. By this way, upregulations of the transcription factors of many cold responsive genes confer freezing tolerance to cold sensitive black poplar clones.

AP2/ERF class is one of the most important TF in plant cold responses. AP2/ERF regulates COR gene expressions by binding to the DRE/CRT cis-elements in the promoter of COR genes. It is a large family encoding transcriptional regulators which are involved in the numerios developmental and physiological processes. AP2/ERF family conserved in many plants is important in various stress responses (Shigyo *et al*., 2006). van Raemdonck *et al*. (2005) analyzed cDNAs encoding an AP2/ERF-domain transcription factor which was specific in aspen and concluded that they played important roles under stress conditions. Zhuang *et al*., (2008) described the poplar AP2/ERF gene family and identified 200 AP2/ERF genes in *Populus trichocarpa*. According to their number and function, 200 AP2/ERF genes were classified into four subfamilies named the AP2, DREB, ERF, RAV, and a soloist. They concluded that the AP2/ERF family genes and their function were very similar in woody (poplar) and herbaceous (Arabidopsis) plants. Song *et al*., (2013) reported that transcriptome profiling of *Populus simonii* showed differential transcript abundance in response to chilling stress. Their microarray data identified

ten AP2/ERF genes responsive to cold stress and six of them were up-regulated in poplar.

The WRKY proteins are also a family of transcription factors unique to plants. WRKY proteins include either one or two WRKY domains, a 60-amino acid region that highly conserved among the family members. The WRKY proteins are involved in numerious physiological processes and cellular defense against numerious abiotic and biotic stresses such as drought, wounding and heat which indicate their regulatory roles in signaling pathways (Eulgem *et al*., 2000; Chen *et al*., 2002; Robatztek and Somssich, 2001; Rizhsky *et al.*, 2002). Recent studies have been demonstrated that WRKY proteins are also involved in cold stress response. Different plant species induce distinct members of WRKY gene family. Poplars have 104 members of the WRKY gene family (Pandey and Somssich, 2009). Song *et al*., (2013) identified 10 differentially expressed WRKY genes under cold stress. Six of them, i.e., WRKY6, WRKY21, WRKY40, WRKY42, and WRKY48, were strongly induced by low temperatures. The researchers suggested that WRKY genes may have key roles in the response of poplar to cold stress.

The dehydration responsive element binding proteins (DREB) are another important transcription factors playing diverse roles in plants. They are responsible from plant defense as a means of stress tolerance such as cold, drought and salt (Dubouzet *et al*., 2003). There are many DREB genes identified from various plant species. The DREB transcription factors consist of two subclasses, i.e., DREB1 and DREB2 which are involved in signal transduction pathways of low temperature and dehydration, respectively. They are members of the ERF (ethylene responsive element binding factors) family of transcription factors and follow ABA-independent signal transduction pathway. A genome-wide analysis of Arabidopsis revealed eight DREB2 members. DREB2A and DREB2B are considered to be major transcription factors under drought and salt conditions but not under cold stress conditions (Yamaguchi-Shinozaki and Shinozaki, 2005). However, a DREB2 type transcription factor isolated from wheat induced by cold was reported by Shen *et al*. (2003).

Overexpression of PgDREB2A transcripts under cold, drought and salt stresses was also indicated by Agarwal *et al*. (2006). Chen *et al*. (2009) studied the expression and characterization of a DREB2-type gene from *Populus euphratica*. A novel DREB gene, i.e., PeDREB2, was isolated from *Populus euphratica*. They classified PeDREB2 as an A-2 group member of the DREB family according to multiple sequence alignment and phylogenetic characterization. The researchers indicated that expression of PeDREB2 was induced by cold, drought, and high salinity, but not by abscisic acid (ABA) treatment.

The MYB family of proteins is large and functionally diverse TFs. They possess a MYB domain, which consists of 1–4 imperfect tandem repeats (MYB repeat) located near the N-terminus. Until now, many MYB genes have been identified in different plant species, such as 204 members in *Arabidopsis* and 197 members in *Populus* (Wilkins *et al*., 2009). The MYB family was mostly induced late under cold stress in *Arabidopsis* (Fowler and Thomashow, 2002). The MYB proteins function in a variety of physiological and biochemical processes such as the regulation of primary and secondary metabolism, the control of cell development and cell cycle, defence response, hormone synthesis, signal transduction and response to various biotic and abiotic stresses (Stracke *et al*., 2001; Du *et al*., 2009; Dubos *et al*., 2010; Feller *et al*., 2011). Recent studies indicated that MYB transcription factor is involved in the cold regulation of plant species. Dai *et al*. (2007) reported that the overexpression of OsMYB3R-2 caused to increased tolerance to cold, dehydration, and salt stress in *Arabidopsis* transgenic plants. The expression of dehydration-responsive element-binding protein 2A, COR15a, and RCI2A was increased to a higher level in OsMYB3R-2-overexpressing plants. COR15a and DREB2A belong to the DRE/CRT class of transcription factors. COR15a was induced after cold stress in *Arabidopsis* and its overexpression led to increased freezing tolerance. Agarwal *et al*. (2006) also reported that the *Arabidopsis* MYB15 was involved in freezing tolerance. Wilkins *et al*. (2009) determined the role of R2R3-MYB family members in *Populus* genus. Maestrini *et al*. (2009) determined gene expression in white poplar (*Populus alba*)

plants under chilling conditions. They indicated the cold induced MYB-related protein putatively belonging to the CBF regulon in *P. alba.*

4.2.2.2 Genes Involved in Scavenging of ROS in Cold Sensitive Genotype

Cold sensitive black poplar genotype induced the up-regulation of cytochrome P450 gene under low temperatures in our study. Cytochromes P450 consist of a family of heme-containing monooxygenases which catalyze a variety of oxidative reactions (Schuler, 1996). Beside this, cytochromes P450 have key roles in biosynthesis of fatty acids, sterols, phenylpropanoids, terpenoids, phytoalexins, brassinolides and gibberellins in higher plants (Donaldson and Luster, 1991; Bolwell *et al*., 1994). The expression of cytochrome P450 genes, i.e., CYP710A1, CYP81D8, CYP71B6, CYP76C2, CYP83B1, CYP79B2, CYP71B22, CYP706A and CYP71B7 in *Arabidopsis* under low temperature, drought and salinity was also reported by Narusaka *et al*. (2004). It is known that these abiotic stresses also cause the production of ROS. Cytochrome P450 genes contain ACGT sequences and/or TGA-boxes in their promoters. These elements are considered as the core-sequences of *activation sequence-1* (TGACGT/C) (Lam *et al*., 1989).The *activation sequence-1* also acts as an oxidative stress responsive element. In ROS-responsive promoters, these sequences might function as cis-acting elements (Garreton *et al*., 2002). Therefore, cytochrome P450 genes might be responsible for ROS detoxification under low temperature conditions which makes cold sensitive individuals being tolerant.

5-Oxoprolinase-like protein is another up-regulated gene induced by cold sensitive genotype in this study. 5-Oxoprolinase is widely distributed enzyme in higher plants. This enzyme catalyzes the ATP-dependent hydrolysis of 5-oxo-L-proline (L-pyrollidone carboxylate, L-pyroglutamate) to glutamate. It has key functions during glutathione (GSH) turnover in plant cells where GSH is converted to 5-oxoproline and then to glutamate by the combined action of γ-glutamyl cyclotransferase and 5-oxoprolinase in the cytoplasm. Glutathione has many functions in plant development,

biosynthetic pathways, detoxification, antioxidant biochemistry and redox homeostasis. Reduced glutathione acts as antioxidant which is important in detoxification pathway. Recent studies also indicate the role of glutathione in signalling and cellular homeostasis. Glutathione can sense intracellular redox status. Disorders of glutathione reduction state are transduced into changes in gene expression. This central role requires precise control of both the concentration and the reduction state of glutathione in different compartments (Foyer *et al*., 2001). As a result, increased gene expression patterns of 5-oxoprolinase under cold conditions induces the glutathione turnover which confer chilling tolerance to cold sensitive black poplar clones.

Metallothioneins (MTs) are cysteine-rich proteins which belong to a small multigene family. They can bind metal ions through the thiol groups of their cysteine residues. Thereby, it is suggested that MTs are involved in metal tolerance. In addition, they contribute to cell homeostasis in response to various stress conditions (Cobbett and Goldsbrough, 2002). Recent studies showed that MTs are also involved in the scavenging of ROS. For instance, transcript levels of type 2 metallothionein increased in watermelon under water stress conditions. It was suggested that increased level of type 2 metallothionein promote the detoxification of oxygen radicals (Akashi *et al*., 2004). Bogeat-Triboulo *et al.* (2007) reported that gradual soil water depletion resulted in gene expression changes in *Populus euphratica*. Although type 2b metallothionein reduced in leaves and in roots, type 2a and 3a metallothioneins highly increased in roots only. Increased level of MTs in a cDNA library derived from senescing *Populus* leaves was reported by Bhalerao *et al*. (2003). The induction by senescence might also be related to ROS removal or signalling instead of metal mobilisation. This is acceptable because the same Cys residues which bind metals are also capable of scavenging ROS species. It is also possible that increased MT expression during senescence can detoxify metals which are released during the breakdown of pigments and other proteins. Yang *et al.* (2009) demonstrated that overexpression of MT gene induced drought tolerance and higher antioxidant enzyme activities in rice. They suggested that OsMT1a was involved

directly or indirectly in the antioxidant system. There is no distinct interaction between metal binding and ROS scavenging. During detoxification of ROS, metals might be released when ROS species are bound to the Cys residues of the MTs. Some authors suggested that the released metals may be necessary inn a signalling cascade (Hassinen *et al*., 2011). As a result, increased level of metallothionein in cold sensitive black poplar genotype might be responsible for ROS scavenging induced under low temperatures.

CHAPTER 5

CONCLUSION

Among the limiting environmental conditions, low temperature or cold stress is one of the major abiotic factors affecting the distribution, growth, development and productivity of plants. It is a well known fact that low temperature as an environmental stress increases the production of reactive oxygen species (ROS) due to disruption of cellular homeostasis.

This study revealed that black poplar clones possessed high levels of ASA-GSH cycle enzymes, i.e., APX, DHAR and GR, during winter periods in order to keep ROS levels under control. In contrast to the these enzymes, CAT activity of black poplar clones decreased under low temperatures. It began to increase when the growth and enlargement progressed in spring and remained relatively high during early dormancy period in autumn. From this pattern, it may be concluded that the ASA-GSH cycle has a crucial role in enhancement of freezing resistance of black poplar. However, deleterious ROS produced during growing period in spring is decomposed by CAT instead of ASA-GSH cycle enzymes in black poplar clones.

It was also found that cold sensitive black poplar clones had higher APX, DHAR, GR and CAT activities than cold resistant clones. Excess level of antioxidant enzyme activities in cold sensitive clones may be associated with the increased need of ROS removal. By this way, they became less sensitive to low temperatures. Relatively lower levels of antioxidant enzyme activities in cold resistant clones might be explained with other mechanisms such as accumulation of some carbohydrates, pigments, polyamines or proteins.

With the present work, selection of the best clones that indicated better resistance to low temperature damages and better growth performance for planting in the cold regions of Turkey were also aimed. The black poplar clones coded with N.62.191, N.64.014, N.87.001, N.90.036, N.92.179, Kocabey, ATA-1, N.92.142 and N.92.218 were identified as cold resistant clones, while Çubuk-1, Gazi, Anadolu, Geyve and N.85.016 were found to be moderately cold resistant. The black poplar clones coded with N.82.4, N.83.12, N.83.2, N.91.052, N.96.325, N.92.243, N.03.366, N.93.306, N.90.010 and N.91.119 were found to be cold sensitive, while N.03.368.A, N.83.5, N.88.4, N.91.088, N.91.109 and N.96.322 were identified as cold sensitive clones.

In addition to antioxidant enzyme activities, exposure of clones to low temperature might trigger many pathways that causes differential gene and protein expression. In this study, comparison of gene expression profiling of cold resistant (N.62.191) and cold sensitive (N.03.368.A) genotype (clone) revealed important genes and mechanisms which can be used for further understanding of cold tolerance in black poplar. It was found that a total of 4421 genes significantly expressed in cold resistant genotype, while 4187 genes were induced in cold sensitive genotype (fold change ≥ 5, p value ≤ 0.05). The numbers of differentially expressed genes were determined as 1246 and 1012 in cold resistant and cold sensitive genotypes, respectively. Additionally, 3175 genes were identified as co-expressed genes in two genotypes.

The gene annotation analyses with regard to differentially expressed GO vocabularies in cold resistant genotypes indicated that most of the genes were enriched in transport mechanisms. Other biological process vocabularies included metabolic processes and response to freezing terms. Annotation of genes according to the molecular function identified mainly transporter activity and oxidoreductase activity in cold resistant genotype. In cold sensitive genotype, response to stress was one of the most represented biological terms. In terms of molecular functions, oxidoreductase activity and protein dimerization activity were significantly activated in cold sensitive genotype. Enrichment of co-expressed genes demonstrated that

response to cold, response to abiotic stimulus, response to oxidative stress, response to osmotic stress, response to water deprivation and carbohydrate metabolic process were most represented biological process terms in both genotype. Oxidoreductase activity, transferase activity, protein kinase activity and protein serine/threonine kinase activity were also significantly expressed in both genotypes with regard to molecular function.

This study also revealed that many isolated genes encoded different types of functional proteins in cold resistant and cold sensitive clones. For instance, especially the genes related with carbohydrate metabolic processes and sugar transport activities, i.e., beta-galactosidase, beta-1,3-glucanases, sugar transporters (monosaccharide transporters) were up-regulated in cold resistant genotype. As defense related genes, glutathione peroxidases and gibberellin 20-oxidase were significantly expressed for oxidation-reduction processes. Apart from that, the genes such as receptor kinases and leucine-rich repeat receptor like kinases which are responsible from protein phosphorylation were also significantly expressed. IAA7 like protein and vacuolar-type H^+-translocating inorganic pyrophosphatase (V-PPase) were other up-regulated genes. On the other hand, sulfate transporter protein, metallothionein 2b and ABC transporter genes constituted some of the most significantly down-regulated genes in cold resistant genotype.

In cold sensitive genotype, especially transcription factors including AP2 domain containing protein, WRKY-type DNA binding protein, DREB2A proteins and myb-like protein were significantly up-regulated. Genes involved in scavenging of ROS, i.e., cytochromes P450, 5-oxoprolinase-like protein and metallothioneins were also highly expressed. Serine/threonine protein kinase and ACC oxidase were the other up-regulated genes. Beside these, peroxidase, serine carboxypeptidase II and inorganic pyrophosphatase constituted some of the down-regulated genes in cold sensitive genotype.

The results obtained from this study can be used for further understanding of the molecular mechanisms of cold acclimation phenomenon and adaptation strategies in black poplar. Periodic changes in the basic antioxidant enzyme activities and differentially expressed genes may be suitable targets for biotechnological manipulation on the purpose of improving cold tolerance in black poplars.

The low temperature related antioxidant enzyme activities and gene expression data could be effectively used in breeding programmes which aim to combine high productivity in low temperature areas. These data could also be very valuable to select the potential cold resistant clones involved in breeding studies. However, the selected clones should be tested in a wide range of environmental stresses for verification of cold resistance and high productivity.

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APPENDIX A: MEAN, STANDARD DEVIATION, MINIMUM AND MAXIMUM VALUES OF ANTIOXIDANT ENZYME ACTIVITIES

A.1 Mean, Standard Deviation, Minimum and Maximum Values of APX in All Individuals

APX	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	120	0.1380	0.0241	0.0602	0.2473
January 2011	120	0.1417	0.0374	0.0604	0.3083
February 2011	120	0.1390	0.0265	0.0937	0.2199
March 2011	120	0.1273	0.0338	0.0436	0.2474
April 2011	120	0.1212	0.0323	0.0582	0.1964
May 2011	120	0.1161	0.0238	0.0595	0.2071
September 2011	119	0.1524	0.0339	0.0577	0.2490
October 2011	120	0.1646	0.0344	0.0712	0.3033
November 2011	120	0.1714	0.0368	0.1062	0.3147
December 2011	120	0.1704	0.0314	0.1075	0.2587
January 2012	120	0.1649	0.0292	0.0855	0.2490
February 2012	120	0.1718	0.0419	0.1078	0.3969
March 2012	120	0.1616	0.0258	0.1028	0.2416
April 2012	120	0.1421	0.0375	0.0756	0.2404

A.2 Mean, Standard Deviation, Minimum and Maximum Values of DHAR in All Individuals

DHAR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	120	0.0781	0.0336	0.0366	0.2099
January 2011	120	0.0563	0.0320	0.0151	0.1656
February 2011	120	0.1045	0.0396	0.0282	0.2012
March 2011	120	0.0464	0.0301	0.0126	0.1726
April 2011	120	0.0523	0.0159	0.0169	0.1023
May 2011	120	0.0911	0.0379	0.0109	0.2009
September 2011	119	0.1232	0.0678	0.0583	0.4239
October 2011	120	0.0846	0.0416	0.0184	0.2357
November 2011	120	0.0984	0.0719	0.0276	0.3108
December 2011	120	0.1192	0.0695	0.0166	0.2951
January 2012	120	0.1204	0.0728	0.0170	0.2978
February 2012	120	0.1229	0.0481	0.0239	0.2585
March 2012	120	0.1001	0.0706	0.0254	0.2809
April 2012	120	0.0772	0.0399	0.0188	0.1972

A.3 Mean, Standard Deviation, Minimum and Maximum Values of GR in All Individuals

GR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	120	0.0405	0.0093	0.0093	0.0838
January 2011	120	0.0422	0.0066	0.0293	0.0985
February 2011	120	0.0440	0.0072	0.0281	0.0649
March 2011	120	0.0409	0.0057	0.0261	0.0652
April 2011	120	0.0455	0.0074	0.0272	0.0737
May 2011	120	0.0363	0.0051	0.0245	0.0552
September 2011	119	0.0352	0.0049	0.0157	0.0629
October 2011	120	0.0510	0.0061	0.0318	0.0832
November 2011	120	0.0537	0.0068	0.0398	0.0856
December 2011	120	0.0578	0.0100	0.0053	0.1219
January 2012	120	0.0539	0.0085	0.0333	0.0847
February 2012	119	0.0647	0.0070	0.0390	0.0993
March 2012	120	0.0558	0.0075	0.0407	0.0834
April 2012	120	0.0355	0.0054	0.0228	0.0619

A.4 Mean, Standard Deviation, Minimum and Maximum Values of CAT in All Individuals

CAT	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	117	15.75	6.67	6.27	50.73
January 2011	119	9.61	2.97	4.30	18.44
February 2011	119	10.91	4.07	2.86	23.60
March 2011	120	14.39	3.97	4.70	24.48
April 2011	119	24.07	7.74	7.05	53.94
May 2011	120	14.15	4.41	6.94	32.03
September 2011	119	20.11	6.58	4.92	39.09
October 2011	120	15.98	6.00	6.83	39.69
November 2011	118	12.76	4.49	4.41	28.45
December 2011	119	14.96	6.72	4.76	64.64
January 2012	120	13.30	4.19	5.62	25.56
February 2012	120	12.05	5.75	4.55	64.61
March 2012	120	13.70	3.44	5.17	22.81
April 2012	120	25.43	10.86	9.97	68.04

A.5 Mean, Standard Deviation, Minimum and Maximum Values of APX in Cold Resistant Group

APX	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.1300	0.0225	0.0602	0.1872
January 2011	60	0.1310	0.0279	0.0807	0.2107
February 2011	60	0.1266	0.0208	0.0937	0.1977
March 2011	60	0.1240	0.0380	0.0436	0.2273
April 2011	60	0.1055	0.0301	0.0582	0.1834
May 2011	60	0.1130	0.0182	0.0760	0.1571
September 2011	59	0.1492	0.0427	0.0577	0.2490
October 2011	60	0.1501	0.0293	0.0712	0.2326
November 2011	60	0.1730	0.0408	0.1062	0.3147
December 2011	60	0.1684	0.0325	0.1075	0.2587
January 2012	60	0.1565	0.0298	0.0855	0.2490
February 2012	60	0.1658	0.0448	0.1103	0.3969
March 2012	60	0.1641	0.0277	0.1028	0.2416
April 2012	60	0.1313	0.0381	0.0756	0.2131

A.6 Mean, Standard Deviation, Minimum and Maximum Values of DHAR in Cold Resistant Group

DHAR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.0760	0.0316	0.0431	0.1739
January 2011	60	0.0620	0.0348	0.0172	0.1656
February 2011	60	0.1082	0.0337	0.0529	0.1794
March 2011	60	0.0465	0.0290	0.0126	0.1677
April 2011	60	0.0512	0.0141	0.0169	0.0945
May 2011	60	0.0772	0.0380	0.0109	0.1704
September 2011	59	0.1378	0.0711	0.0583	0.3925
October 2011	60	0.0706	0.0353	0.0184	0.1885
November 2011	60	0.0630	0.0301	0.0276	0.2230
December 2011	60	0.0897	0.0560	0.0166	0.2271
January 2012	60	0.0865	0.0628	0.0170	0.2500
February 2012	60	0.1145	0.0466	0.0239	0.2049
March 2012	60	0.0639	0.0462	0.0254	0.2224
April 2012	60	0.0671	0.0396	0.0188	0.1677

A.7 Mean, Standard Deviation, Minimum and Maximum Values of GR in Cold Resistant Group

GR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.0364	0.0052	0.0115	0.0608
January 2011	60	0.0399	0.0054	0.0293	0.0577
February 2011	60	0.0467	0.0071	0.0340	0.0631
March 2011	60	0.0389	0.0057	0.0261	0.0617
April 2011	60	0.0425	0.0071	0.0278	0.0582
May 2011	60	0.0394	0.0046	0.0245	0.0525
September 2011	59	0.0335	0.0035	0.0157	0.0593
October 2011	60	0.0485	0.0044	0.0318	0.0621
November 2011	60	0.0526	0.0047	0.0439	0.0772
December 2011	60	0.0525	0.0071	0.0053	0.0831
January 2012	60	0.0479	0.0057	0.0333	0.0716
February 2012	60	0.0634	0.0063	0.0390	0.0844
March 2012	60	0.0534	0.0065	0.0407	0.0707
April 2012	60	0.0372	0.0063	0.0268	0.0619

A.8 Mean, Standard Deviation, Minimum and Maximum Values of CAT in Cold Resistant Group

CAT	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	58	14.29	5.13	6.27	31.90
January 2011	60	9.64	3.10	4.62	18.44
February 2011	59	11.84	3.53	5.76	21.14
March 2011	60	15.58	3.52	9.29	24.48
April 2011	59	22.33	7.43	7.05	37.68
May 2011	60	12.93	2.80	6.94	22.25
September 2011	59	18.89	6.45	4.92	33.91
October 2011	60	13.69	4.00	6.83	24.79
November 2011	60	14.73	4.43	7.02	28.45
December 2011	59	15.28	4.31	7.21	32.69
January 2012	60	13.21	4.59	5.62	23.44
February 2012	60	11.70	2.74	6.64	22.78
March 2012	60	13.27	3.66	5.17	22.58
April 2012	60	24.73	11.45	9.97	68.04

A.9 Mean, Standard Deviation, Minimum and Maximum Values of APX in Cold Sensitive Group

APX	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.1460	0.0232	0.1120	0.2473
January 2011	60	0.1525	0.0425	0.0604	0.3083
February 2011	60	0.1515	0.0259	0.1000	0.2199
March 2011	60	0.1307	0.0289	0.0785	0.2474
April 2011	60	0.1369	0.0265	0.0631	0.1964
May 2011	60	0.1192	0.0281	0.0595	0.2071
September 2011	60	0.1556	0.0225	0.1002	0.2110
October 2011	60	0.1790	0.0333	0.1103	0.3033
November 2011	60	0.1730	0.0328	0.1069	0.2757
December 2011	60	0.1724	0.0305	0.1251	0.2504
January 2012	60	0.1733	0.0261	0.1148	0.2410
February 2012	60	0.1778	0.0381	0.1078	0.3194
March 2012	60	0.1590	0.0235	0.1109	0.2329
April 2012	60	0.1529	0.0338	0.0783	0.2404

A.10 Mean, Standard Deviation, Minimum and Maximum Values of DHAR in Cold Sensitive Group

DHAR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.0802	0.0356	0.0366	0.2099
January 2011	60	0.0505	0.0281	0.0151	0.1467
February 2011	60	0.1007	0.0446	0.0282	0.2012
March 2011	60	0.0463	0.0315	0.0158	0.1726
April 2011	60	0.0535	0.0175	0.0264	0.1023
May 2011	60	0.1050	0.0327	0.032	0.2009
September 2011	60	0.1445	0.0650	0.0611	0.4239
October 2011	60	0.0986	0.0430	0.0421	0.2357
November 2011	60	0.1339	0.0835	0.0335	0.3108
December 2011	60	0.1487	0.0696	0.0263	0.2951
January 2012	60	0.1544	0.0663	0.0179	0.2978
February 2012	60	0.1298	0.0487	0.0291	0.2585
March 2012	60	0.1364	0.0723	0.0254	0.2809
April 2012	60	0.0873	0.0379	0.0295	0.1972

A.11 Mean, Standard Deviation, Minimum and Maximum Values of GR in Cold Sensitive Group

GR	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	60	0.0445	0.0106	0.0093	0.0838
January 2011	60	0.0445	0.0069	0.0317	0.0985
February 2011	60	0.0413	0.0063	0.0281	0.0649
March 2011	60	0.0429	0.0050	0.0337	0.0652
April 2011	60	0.0484	0.0064	0.0272	0.0737
May 2011	60	0.0331	0.0036	0.0254	0.0552
September 2011	60	0.0368	0.0055	0.0255	0.0629
October 2011	60	0.0534	0.0067	0.039	0.0832
November 2011	60	0.0547	0.0082	0.0398	0.0856
December 2011	60	0.0630	0.0096	0.0474	0.1219
January 2012	60	0.0599	0.0064	0.0433	0.0847
February 2012	59	0.0659	0.0073	0.0497	0.0993
March 2012	60	0.0582	0.0077	0.0449	0.0834
April 2012	60	0.0338	0.0036	0.0228	0.0554

A.12 Mean, Standard Deviation, Minimum and Maximum Values of CAT in Cold Sensitive Group

CAT	Sample Size (N)	Mean	Standard Deviation	Minimum	Maximum
December 2010	59	17.21	7.61	8.62	50.73
January 2011	59	9.59	2.85	4.30	17.05
February 2011	60	9.98	4.37	2.86	23.60
March 2011	60	13.20	4.06	4.70	23.42
April 2011	60	25.82	7.71	10.93	53.94
May 2011	60	15.36	5.33	7.47	32.03
September 2011	60	21.34	6.54	11.01	39.09
October 2011	60	18.28	6.77	8.36	39.69
November 2011	58	10.79	3.61	4.41	21.13
December 2011	60	14.64	8.48	4.76	64.64
January 2012	60	13.40	3.78	6.61	25.56
February 2012	60	12.40	7.67	4.55	64.61
March 2012	60	14.12	3.17	8.59	22.81
April 2012	60	26.12	10.27	11.26	55.17

APPENDIX B: THE GENE ANNOTATION ENRICHMENT ANALYSES

B.1 Significantly enriched GO terms in the up-regulated groups of cold resistant genotype at NOV conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0006073	Cellular glucan metabolic process	15	1.10E-04
GO:0044042	Glucan metabolic process	15	1.10E-04
GO:0044264	Cellular polysaccharide metabolic process	15	2.30E-04
GO:0015979	Photosynthesis	10	7.80E-04
GO:0034641	Cellular nitrogen compound metabolic process	21	5.50E-04
GO:0005982	Starch metabolic process	12	8.20E-04
GO:0005984	Disaccharide metabolic process	12	8.30E-04
GO:0005985	Sucrose metabolic process	12	6.60E-04
GO:0019684	Photosynthesis, light reaction	7	4.80E-04
GO:0005976	Polysaccharide metabolic process	16	5.00E-04
GO:0009311	Oligosaccharide metabolic process	12	1.00E-03
GO:0016137	Glycoside metabolic process	12	1.40E-03
GO:0009607	Response to biotic stimulus	12	4.00E-03
GO:0006857	Oligopeptide transport	5	4.90E-03
GO:0015833	Peptide transport	5	5.30E-03
Molecular Function			
GO:0016798	Hydrolase activity, acting on glycosyl bonds	12	5.90E-03

B.2 Significantly enriched GO terms in the down-regulated groups of cold resistant genotype at NOV conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0034641	Cellular nitrogen compound metabolic process	38	2.10E-06
GO:0009877	Nodulation	5	8.80E-05
GO:0006857	Oligopeptide transport	9	1.60E-04
GO:0015833	Peptide transport	9	1.80E-04
GO:0006869	Lipid transport	8	3.70E-04
GO:0044419	Interspecies interaction between organisms	5	5.60E-04
GO:0044403	Symbiosis, encompassing mutualism through parasitism	5	5.60E-04
GO:0015698	Inorganic anion transport	7	8.60E-04
GO:0006575	Cellular amino acid derivative metabolic process	22	3.60E-03
GO:0042398	Cellular amino acid derivative biosynthetic process	16	5.10E-03
GO:0006519	Cellular amino acid and derivative metabolic process	51	7.90E-03
Molecular Function			
GO:0046524	Sucrose-phosphate synthase activity	6	4.60E-07
GO:0015380	Anion exchanger activity	5	1.20E-05
GO:0015301	Anion:anion antiporter activity	5	2.00E-05
GO:0016157	Sucrose synthase activity	5	5.00E-05
GO:0008171	O-methyltransferase activity	6	5.70E-04
GO:0046983	Protein dimerization activity	12	5.60E-04
GO:0008509	Anion transmembrane transporter activity	8	7.20E-04
GO:0016757	Transferase activity, transferring glycosyl groups	24	1.20E-03
GO:0035251	UDP-glucosyltransferase activity	8	1.00E-03
GO:0008194	UDP-glycosyltransferase activity	13	1.10E-03
GO:0016706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	7	1.10E-03
GO:0046527	Glucosyltransferase activity	8	1.80E-03
GO:0015300	Solute:solute antiporter activity	5	3.40E-03
GO:0016491	Oxidoreductase activity	54	3.20E-03
GO:0016758	Transferase activity, transferring hexosyl groups	16	7.30E-03
GO:0005215	Transporter activity	41	8.30E-03
GO:0004497	Monooxygenase activity	12	9.20E-03
GO:0005506	Iron ion binding	21	9.50E-03

B.3 Significantly enriched GO terms in the up-regulated groups of cold sensitive genotype at NOV conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0006073	Cellular glucan metabolic process	23	3.20E-08
GO:0044042	Glucan metabolic process	23	3.40E-08
GO:0044264	Cellular polysaccharide metabolic process	23	1.00E-07
GO:0005985	Sucrose metabolic process	20	1.10E-07
GO:0005982	Starch metabolic process	20	1.60E-07
GO:0005984	Disaccharide metabolic process	20	1.60E-07
GO:0009311	Oligosaccharide metabolic process	20	2.40E-07
GO:0016137	Glycoside metabolic process	20	4.40E-07
GO:0005976	Polysaccharide metabolic process	24	6.10E-07
GO:0009835	Ripening	5	9.20E-05
GO:0044262	Cellular carbohydrate metabolic process	26	2.50E-04
GO:0042221	Response to chemical stimulus	37	4.20E-04
GO:0005975	Carbohydrate metabolic process	35	3.90E-04
GO:0046351	Disaccharide biosynthetic process	5	5.00E-04
GO:0009312	Oligosaccharide biosynthetic process	5	9.10E-04
GO:0010035	Response to inorganic substance	8	1.10E-03
GO:0006820	Anion transport	7	2.10E-03
GO:0015698	Inorganic anion transport	5	3.90E-03
GO:0006979	Response to oxidative stress	11	4.50E-03
GO:0009620	Response to fungus	6	4.50E-03
GO:0034641	Cellular nitrogen compound metabolic process	21	4.90E-03
GO:0016138	Glycoside biosynthetic process	5	6.30E-03
GO:0009607	Response to biotic stimulus	13	6.70E-03
GO:0050832	Defense response to fungus	5	7.10E-03
GO:0050896	Response to stimulus	58	9.70E-03
GO:0006857	Oligopeptide transport	5	1.00E-02
Molecular Function			
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	16	1.00E-04
GO:0016798	Hydrolase activity, acting on glycosyl bonds	18	6.70E-05
GO:0015103	Inorganic anion transmembrane transporter activity	5	1.10E-03
GO:0030599	Pectinesterase activity	5	2.00E-03
GO:0016491	Oxidoreductase activity	41	1.70E-03
GO:0004857	Enzyme inhibitor activity	6	4.30E-03
GO:0043167	Ion binding	59	6.00E-03
GO:0043169	Cation binding	59	5.90E-03
GO:0008509	Anion transmembrane transporter activity	5	9.90E-03

B.4 Significantly enriched GO terms in the down-regulated groups of cold sensitive genotype at NOV conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0010876	Lipid localization	6	9.80E-09
GO:0034641	Cellular nitrogen compound metabolic process	33	2.30E-05
GO:0009877	Nodulation	5	5.70E-05
GO:0051704	Multi-organism process	22	1.80E-04
GO:0044419	Interspecies interaction between organisms	5	3.70E-04
GO:0050896	Response to stimulus	82	3.50E-04
GO:0015833	Peptide transport	8	4.60E-04
GO:0006857	Oligopeptide transport	8	4.10E-04
GO:0044403	Symbiosis, encompassing mutualism through parasitism	5	3.70E-04
GO:0009725	Response to hormone stimulus	23	7.00E-04
GO:0009733	Response to auxin stimulus	14	7.00E-04
GO:0010033	Response to organic substance	28	9.30E-04
GO:0009719	Response to endogenous stimulus	24	1.10E-03
GO:0042221	Response to chemical stimulus	44	1.10E-03
GO:0009791	Post-embryonic development	19	1.30E-03
GO:0043450	Alkene biosynthetic process	6	1.80E-03
GO:0015698	Inorganic anion transport	6	2.60E-03
GO:0009416	Response to light stimulus	16	3.10E-03
GO:0009755	Hormone-mediated signaling pathway	11	4.50E-03
GO:0006869	Lipid transport	6	4.80E-03
GO:0009314	Response to radiation	16	4.30E-03
GO:0070887	Cellular response to chemical stimulus	15	5.00E-03
GO:0032870	Cellular response to hormone stimulus	11	4.80E-03
GO:0051707	Response to other organism	15	4.90E-03
GO:0006555	Methionine metabolic process	6	4.30E-03
GO:0045449	Regulation of transcription	48	3.90E-03
GO:0051171	Regulation of nitrogen compound metabolic process	50	4.30E-03
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	9	5.50E-03
GO:0019219	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	49	5.60E-03
GO:0048856	Anatomical structure development	35	5.70E-03
GO:0043449	Cellular alkene metabolic process	6	6.60E-03
GO:0003006	Reproductive developmental process	20	6.60E-03
GO:0009889	Regulation of biosynthetic process	50	6.50E-03

GO:0031326	Regulation of cellular biosynthetic process	50	6.50E-03
GO:0009734	Auxin mediated signaling pathway	7	6.90E-03
GO:0010556	Regulation of macromolecule biosynthetic process	49	8.70E-03
GO:0006350	Transcription	49	9.30E-03
Molecular Function			
GO:0046524	Sucrose-phosphate synthase activity	5	5.60E-06
GO:0016157	Sucrose synthase activity	5	3.20E-05
GO:0005506	Iron ion binding	25	1.30E-04
GO:0046983	Protein dimerization activity	12	2.40E-04
GO:0016638	Oxidoreductase activity, acting on the CH-NH2 group of donors	6	2.60E-04
GO:0008509	Anion transmembrane transporter activity	7	1.80E-03
GO:0020037	Heme binding	13	1.50E-03
GO:0016491	Oxidoreductase activity	51	1.90E-03
GO:0008236	Serine-type peptidase activity	8	2.30E-03
GO:0017171	Serine hydrolase activity	8	2.30E-03
GO:0004497	Monooxygenase activity	12	4.50E-03
GO:0046906	Tetrapyrrole binding	13	5.90E-03

B.5 Significantly enriched GO terms in the up-regulated groups of cold resistant genotype at FEB conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0034641	Cellular nitrogen compound metabolic process	34	1.20E-08
GO:0005982	Starch metabolic process	18	2.10E-06
GO:0005984	Disaccharide metabolic process	18	2.10E-06
GO:0005985	Sucrose metabolic process	18	1.50E-06
GO:0009311	Oligosaccharide metabolic process	18	3.10E-06
GO:0006073	Cellular glucan metabolic process	19	5.60E-06
GO:0044042	Glucan metabolic process	19	5.80E-06
GO:0016137	Glycoside metabolic process	18	5.20E-06
GO:0044264	Cellular polysaccharide metabolic process	19	1.40E-05
GO:0042221	Response to chemical stimulus	41	1.50E-05
GO:0005976	Polysaccharide metabolic process	21	1.60E-05
GO:0050896	Response to stimulus	68	4.40E-05
GO:0006810	Transport	55	2.60E-04
GO:0051234	Establishment of localization	55	2.80E-04
GO:0051179	Localization	55	4.00E-04
GO:0009607	Response to biotic stimulus	15	8.10E-04
GO:0006520	Cellular amino acid metabolic process	34	1.60E-03
GO:0044106	Cellular amine metabolic process	34	1.60E-03
GO:0009308	Amine metabolic process	36	2.70E-03
GO:0009725	Response to hormone stimulus	17	3.40E-03
GO:0006950	Response to stress	39	3.70E-03
GO:0044262	Cellular carbohydrate metabolic process	22	4.30E-03
GO:0051707	Response to other organism	12	6.30E-03
GO:0006820	Anion transport	6	7.90E-03
GO:0009719	Response to endogenous stimulus	17	8.20E-03
GO:0051704	Multi-organism process	14	9.10E-03
GO:0015833	Peptide transport	5	1.00E-02
GO:0006857	Oligopeptide transport	5	9.50E-03
GO:0000272	Polysaccharide catabolic process	5	9.90E-03
Molecular Function			
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	15	2.60E-04
GO:0004857	Enzyme inhibitor activity	8	1.50E-04
GO:0016798	Hydrolase activity, acting on glycosyl bonds	16	4.80E-04
GO:0016638	Oxidoreductase activity, acting on the CH-NH2 group	5	5.00E-04

	of donors		
GO:0016491	Oxidoreductase activity	40	2.10E-03
GO:0004672	Protein kinase activity	30	2.40E-03
GO:0005215	Transporter activity	31	4.40E-03
GO:0004674	Protein serine/threonine kinase activity	23	3.90E-03
GO:0031072	Heat shock protein binding	6	6.80E-03
GO:0022857	Transmembrane transporter activity	23	7.10E-03
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	31	9.10E-03
GO:0022892	Substrate-specific transporter activity	21	1.00E-02

B.6 Significantly enriched GO terms in the down-regulated groups of cold resistant genotype at FEB conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0009877	Nodulation	8	5.50E-08
GO:0034641	Cellular nitrogen compound metabolic process	42	3.80E-07
GO:0044419	Interspecies interaction between organisms	8	1.00E-06
GO:0044403	Symbiosis, encompassing mutualism through parasitism	8	1.00E-06
GO:0005985	Sucrose metabolic process	23	2.80E-06
GO:0005982	Starch metabolic process	23	4.10E-06
GO:0005984	Disaccharide metabolic process	23	4.20E-06
GO:0009311	Oligosaccharide metabolic process	23	6.40E-06
GO:0016137	Glycoside metabolic process	23	1.20E-05
GO:0006073	Cellular glucan metabolic process	24	2.00E-05
GO:0044042	Glucan metabolic process	24	2.10E-05
GO:0006575	Cellular amino acid derivative metabolic process	29	3.90E-05
GO:0044264	Cellular polysaccharide metabolic process	24	5.50E-05
GO:0005976	Polysaccharide metabolic process	27	5.50E-05
GO:0019438	Aromatic compound biosynthetic process	20	7.10E-05
GO:0019748	Secondary metabolic process	26	2.20E-04
GO:0044262	Cellular carbohydrate metabolic process	36	2.30E-04
GO:0009699	Phenylpropanoid biosynthetic process	13	4.60E-04
GO:0006725	Cellular aromatic compound metabolic process	33	4.80E-04
GO:0005975	Carbohydrate metabolic process	49	4.80E-04
GO:0042398	Cellular amino acid derivative biosynthetic process	19	8.70E-04
GO:0009698	Phenylpropanoid metabolic process	13	9.70E-04
GO:0006118	Electron transport	33	1.00E-03
GO:0006664	Glycolipid metabolic process	6	1.30E-03
GO:0015698	Inorganic anion transport	7	1.30E-03
GO:0051704	Multi-organism process	22	1.70E-03
GO:0006869	Lipid transport	7	2.70E-03
GO:0006519	Cellular amino acid and derivative metabolic process	57	2.70E-03
GO:0019482	Beta-alanine metabolic process	5	3.80E-03
GO:0009813	Flavonoid biosynthetic process	8	3.80E-03
GO:0006720	Isoprenoid metabolic process	11	3.70E-03
GO:0009810	Stilbene metabolic process	5	4.70E-03
GO:0009811	Stilbene biosynthetic process	5	4.70E-03
GO:0006643	Membrane lipid metabolic process	6	5.00E-03

GO:0006857	Oligopeptide transport	7	4.90E-03
GO:0000272	Polysaccharide catabolic process	7	5.20E-03
GO:0009611	Response to wounding	7	5.90E-03
GO:0009812	Flavonoid metabolic process	8	5.50E-03
GO:0010154	Fruit development	14	5.80E-03
GO:0015833	Peptide transport	7	5.40E-03
GO:0006026	Aminoglycan catabolic process	5	5.80E-03
GO:0006820	Anion transport	8	7.00E-03
GO:0019852	L-ascorbic acid metabolic process	5	7.90E-03
GO:0009805	Coumarin biosynthetic process	5	7.40E-03
GO:0009804	Coumarin metabolic process	5	7.40E-03
GO:0030639	Polyketide biosynthetic process	5	8.90E-03
GO:0006979	Response to oxidative stress	14	9.00E-03
GO:0006083	Acetate metabolic process	5	8.90E-03
GO:0019413	Acetate biosynthetic process	5	8.90E-03
GO:0042214	Terpene metabolic process	5	7.90E-03
GO:0030638	Polyketide metabolic process	5	8.90E-03
GO:0030636	Acetate derivative biosynthetic process	5	8.90E-03
GO:0030635	Acetate derivative metabolic process	5	8.90E-03
GO:0042181	Ketone biosynthetic process	5	9.40E-03
GO:0006536	Glutamate metabolic process	5	1.00E-02
Molecular Function			
GO:0016757	Transferase activity, transferring glycosyl groups	34	5.70E-07
GO:0016758	Transferase activity, transferring hexosyl groups	26	1.20E-06
GO:0008194	UDP-glycosyltransferase activity	18	6.80E-06
GO:0046524	Sucrose-phosphate synthase activity	5	1.30E-05
GO:0016157	Sucrose synthase activity	5	7.10E-05
GO:0005506	Iron ion binding	29	6.20E-05
GO:0043169	Cation binding	98	7.30E-05
GO:0043167	Ion binding	98	7.60E-05
GO:0016491	Oxidoreductase activity	64	1.40E-04
GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	13	3.00E-04
GO:0047262	Polygalacturonate 4-alpha-galacturonosyltransferase activity	5	2.80E-04
GO:0020037	Heme binding	16	3.30E-04
GO:0004497	Monooxygenase activity	16	3.70E-04
GO:0003824	Catalytic activity	265	9.10E-04
GO:0008509	Anion transmembrane transporter activity	8	1.20E-03
GO:0046872	Metal ion binding	87	1.70E-03
GO:0046906	Tetrapyrrole binding	16	1.80E-03
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	18	1.90E-03

GO:0016798	Hydrolase activity, acting on glycosyl bonds	20	2.10E-03
GO:0008171	O-methyltransferase activity	5	4.70E-03
GO:0015075	Ion transmembrane transporter activity	23	5.90E-03
GO:0035251	UDP-glucosyltransferase activity	7	6.20E-03
GO:0016706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	6	7.30E-03
GO:0046527	Glucosyltransferase activity	7	1.00E-02

B.7 Significantly enriched GO terms in the up-regulated groups of cold sensitive genotype at FEB conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0034641	Cellular nitrogen compound metabolic process	35	2.60E-07
GO:0006073	Cellular glucan metabolic process	20	2.00E-05
GO:0044042	Glucan metabolic process	20	2.10E-05
GO:0044264	Cellular polysaccharide metabolic process	20	5.00E-05
GO:0005985	Sucrose metabolic process	17	5.50E-05
GO:0042221	Response to chemical stimulus	45	4.60E-05
GO:0009311	Oligosaccharide metabolic process	17	1.00E-04
GO:0009414	Response to water deprivation	10	9.80E-05
GO:0005982	Starch metabolic process	17	7.30E-05
GO:0005984	Disaccharide metabolic process	17	7.50E-05
GO:0009737	Response to abscisic acid stimulus	12	8.90E-05
GO:0009415	Response to water	10	1.30E-04
GO:0016137	Glycoside metabolic process	17	1.60E-04
GO:0009835	Ripening	5	1.90E-04
GO:0005976	Polysaccharide metabolic process	21	1.90E-04
GO:0050896	Response to stimulus	75	2.40E-04
GO:0006810	Transport	60	1.40E-03
GO:0051234	Establishment of localization	60	1.50E-03
GO:0051179	Localization	61	1.30E-03
GO:0006979	Response to oxidative stress	13	2.00E-03
GO:0009607	Response to biotic stimulus	15	4.30E-03
GO:0009628	Response to abiotic stimulus	26	5.40E-03
GO:0010033	Response to organic substance	23	6.20E-03
GO:0006950	Response to stress	44	6.00E-03
GO:0006468	Protein amino acid phosphorylation	29	5.80E-03
GO:0048519	Negative regulation of biological process	12	9.10E-03
GO:0009725	Response to hormone stimulus	18	8.60E-03
GO:0006970	Response to osmotic stress	10	9.20E-03
GO:0009719	Response to endogenous stimulus	19	1.00E-02
Molecular Function			
GO:0004672	Protein kinase activity	35	1.60E-03
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	15	1.60E-03
GO:0016798	Hydrolase activity, acting on glycosyl bonds	17	1.20E-03
GO:0004872	Receptor activity	13	1.50E-03
GO:0004674	Protein serine/threonine kinase activity	28	1.20E-03

GO:0005215	Transporter activity	37	2.00E-03
GO:0031072	Heat shock protein binding	7	4.10E-03
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	37	4.80E-03
GO:0004857	Enzyme inhibitor activity	6	8.90E-03
GO:0022857	Transmembrane transporter activity	26	8.70E-03

B.8 Significantly enriched GO terms in the down-regulated groups of cold sensitive genotype at FEB conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0034641	Cellular nitrogen compound metabolic process	43	4.40E-07
GO:0005982	Starch metabolic process	23	7.80E-06
GO:0005984	Disaccharide metabolic process	23	8.10E-06
GO:0005985	Sucrose metabolic process	23	5.30E-06
GO:0009877	Nodulation	6	1.30E-05
GO:0009311	Oligosaccharide metabolic process	23	1.20E-05
GO:0016137	Glycoside metabolic process	23	2.20E-05
GO:0006073	Cellular glucan metabolic process	24	3.70E-05
GO:0044042	Glucan metabolic process	24	3.90E-05
GO:0051704	Multi-organism process	27	4.40E-05
GO:0044419	Interspecies interaction between organisms	6	1.20E-04
GO:0044264	Cellular polysaccharide metabolic process	24	1.00E-04
GO:0044403	Symbiosis, encompassing mutualism through parasitism	6	1.20E-04
GO:0005976	Polysaccharide metabolic process	27	1.10E-04
GO:0009699	Phenylpropanoid biosynthetic process	14	2.10E-04
GO:0015698	Inorganic anion transport	8	3.50E-04
GO:0009698	Phenylpropanoid metabolic process	14	4.60E-04
GO:0044262	Cellular carbohydrate metabolic process	36	4.90E-04
GO:0051707	Response to other organism	19	1.30E-03
GO:0006857	Oligopeptide transport	8	1.60E-03
GO:0019748	Secondary metabolic process	24	1.80E-03
GO:0015833	Peptide transport	8	1.80E-03
GO:0042398	Cellular amino acid derivative biosynthetic process	18	3.20E-03
GO:0005975	Carbohydrate metabolic process	47	3.10E-03
GO:0006869	Lipid transport	7	3.40E-03
GO:0009607	Response to biotic stimulus	19	4.20E-03
GO:0006541	Glutamine metabolic process	5	5.20E-03
GO:0019438	Aromatic compound biosynthetic process	16	4.80E-03
GO:0043450	Alkene biosynthetic process	6	5.10E-03
GO:0051239	Regulation of multicellular organismal process	11	5.20E-03
GO:0009810	Stilbene metabolic process	5	5.60E-03
GO:0009811	Stilbene biosynthetic process	5	5.60E-03
GO:0048509	Regulation of meristem development	5	6.00E-03
GO:0009791	Post-embryonic development	20	6.10E-03

GO:0000272	Polysaccharide catabolic process	7	6.40E-03
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	10	7.40E-03
GO:0006664	Glycolipid metabolic process	5	7.70E-03
GO:0006519	Cellular amino acid and derivative metabolic process	56	9.10E-03
GO:0019852	L-ascorbic acid metabolic process	5	9.30E-03
GO:0003006	Reproductive developmental process	23	8.80E-03
GO:0006820	Anion transport	8	8.80E-03
GO:0009805	Coumarin biosynthetic process	5	8.80E-03
GO:0009804	Coumarin metabolic process	5	8.80E-03
GO:0030639	Polyketide biosynthetic process	5	1.00E-02
GO:0006083	Acetate metabolic process	5	1.00E-02
GO:0019413	Acetate biosynthetic process	5	1.00E-02
GO:0030638	Polyketide metabolic process	5	1.00E-02
GO:0030636	Acetate derivative biosynthetic process	5	1.00E-02
GO:0030635	Acetate derivative metabolic process	5	1.00E-02
Molecular Function			
GO:0005506	Iron ion binding	31	2.20E-05
GO:0046524	Sucrose-phosphate synthase activity	5	1.50E-05
GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	15	3.60E-05
GO:0016157	Sucrose synthase activity	5	8.60E-05
GO:0016706	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors	9	9.20E-05
GO:0016491	Oxidoreductase activity	65	2.40E-04
GO:0004289	Subtilase activity	5	3.90E-04
GO:0016758	Transferase activity, transferring hexosyl groups	21	3.80E-04
GO:0043167	Ion binding	97	4.90E-04
GO:0043169	Cation binding	97	4.70E-04
GO:0004497	Monooxygenase activity	15	1.50E-03
GO:0016757	Transferase activity, transferring glycosyl groups	26	1.30E-03
GO:0046983	Protein dimerization activity	12	1.50E-03
GO:0008194	UDP-glycosyltransferase activity	14	1.20E-03
GO:0008509	Anion transmembrane transporter activity	8	1.50E-03
GO:0015103	Inorganic anion transmembrane transporter activity	6	1.80E-03
GO:0020037	Heme binding	14	3.60E-03
GO:0046872	Metal ion binding	88	3.60E-03
GO:0016638	Oxidoreductase activity, acting on the CH-NH2 group of donors	5	4.50E-03
GO:0008757	S-adenosylmethionine-dependent methyltransferase activity	8	5.50E-03
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl	17	6.50E-03

	compounds		
GO:0003824	Catalytic activity	267	6.50E-03
GO:0016798	Hydrolase activity, acting on glycosyl bonds	19	7.00E-03
GO:0017171	Serine hydrolase activity	8	8.10E-03
GO:0035251	UDP-glucosyltransferase activity	7	7.60E-03
GO:0008236	Serine-type peptidase activity	8	8.10E-03

B.9 Significantly enriched GO terms in the up-regulated groups of cold resistant genotype at APR conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0005976	Polysaccharide metabolic process	66	1.30E-18
GO:0044264	Cellular polysaccharide metabolic process	56	2.90E-16
GO:0006073	Cellular glucan metabolic process	54	3.00E-16
GO:0044042	Glucan metabolic process	54	3.20E-16
GO:0005975	Carbohydrate metabolic process	95	5.20E-11
GO:0005982	Starch metabolic process	40	7.70E-11
GO:0005984	Disaccharide metabolic process	40	8.30E-11
GO:0005985	Sucrose metabolic process	39	1.30E-10
GO:0016137	Glycoside metabolic process	41	1.50E-10
GO:0034641	Cellular nitrogen compound metabolic process	66	1.50E-10
GO:0009311	Oligosaccharide metabolic process	40	1.70E-10
GO:0044262	Cellular carbohydrate metabolic process	68	4.10E-10
GO:0007018	Microtubule-based movement	21	2.80E-09
GO:0010876	Lipid localization	6	3.30E-07
GO:0007167	Enzyme linked receptor protein signaling pathway	24	4.70E-07
GO:0007017	Microtubule-based process	24	9.70E-07
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	21	1.30E-06
GO:0010383	Cell wall polysaccharide metabolic process	6	1.60E-05
GO:0051258	Protein polymerization	11	1.60E-05
GO:0050826	Response to freezing	6	2.10E-05
GO:0006468	Protein amino acid phosphorylation	63	2.30E-05
GO:0044036	Cell wall macromolecule metabolic process	13	2.80E-05
GO:0007166	Cell surface receptor linked signaling pathway	24	2.90E-05
GO:0045491	Xylan metabolic process	5	3.60E-05
GO:0010410	Hemicellulose metabolic process	5	9.60E-05
GO:0009809	Lignin biosynthetic process	10	1.20E-04
GO:0009808	Lignin metabolic process	10	1.20E-04
GO:0009664	Plant-type cell wall organization	9	2.30E-04
GO:0043687	Post-translational protein modification	81	3.30E-04
GO:0009765	Photosynthesis, light harvesting	7	4.50E-04
GO:0006118	Electron transport	47	6.20E-04
GO:0009225	Nucleotide-sugar metabolic process	8	1.00E-03
GO:0042545	Cell wall modification	8	1.00E-03
GO:0019321	Pentose metabolic process	6	1.50E-03

GO:0006464	Protein modification process	85	1.50E-03
GO:0000272	Polysaccharide catabolic process	10	1.70E-03
GO:0006687	Glycosphingolipid metabolic process	6	1.90E-03
GO:0006022	Aminoglycan metabolic process	9	1.90E-03
GO:0006026	Aminoglycan catabolic process	7	2.10E-03
GO:0006011	UDP-glucose metabolic process	5	2.20E-03
GO:0000280	Nuclear division	7	2.30E-03
GO:0007067	Mitosis	7	2.30E-03
GO:0000279	M phase	11	2.50E-03
GO:0000087	M phase of mitotic cell cycle	7	2.50E-03
GO:0016998	Cell wall macromolecule catabolic process	7	3.30E-03
GO:0043412	Macromolecule modification	86	3.70E-03
GO:0042726	Riboflavin and derivative metabolic process	7	3.80E-03
GO:0006771	Riboflavin metabolic process	7	3.80E-03
GO:0009863	Salicylic acid mediated signaling pathway	5	4.00E-03
GO:0022403	Cell cycle phase	11	4.10E-03
GO:0009409	Response to cold	13	4.10E-03
GO:0009832	Plant-type cell wall biogenesis	7	4.40E-03
GO:0018298	Protein-chromophore linkage	6	4.60E-03
GO:0009069	Serine family amino acid metabolic process	40	5.30E-03
GO:0051707	Response to other organism	23	5.70E-03
GO:0016310	Phosphorylation	65	6.40E-03
GO:0009810	Stilbene metabolic process	6	6.90E-03
GO:0009811	Stilbene biosynthetic process	6	6.90E-03
GO:0034622	Cellular macromolecular complex assembly	15	7.60E-03
GO:0009739	Response to gibberellin stimulus	7	8.20E-03
GO:0010033	Response to organic substance	40	8.60E-03
GO:0043623	Cellular protein complex assembly	11	8.60E-03
GO:0006796	Phosphate metabolic process	72	9.10E-03
GO:0043450	Alkene biosynthetic process	7	9.30E-03
GO:0006793	Phosphorus metabolic process	72	9.60E-03
GO:0006664	Glycolipid metabolic process	6	1.00E-02
Molecular Function			
GO:0005507	Copper ion binding	30	6.10E-14
GO:0016682	Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	16	6.90E-13
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	44	6.70E-12
GO:0016798	Hydrolase activity, acting on glycosyl bonds	48	9.00E-12
GO:0008471	Laccase activity	13	1.90E-11
GO:0016762	Xyloglucan:xyloglucosyl transferase activity	14	2.40E-11
GO:0016679	Oxidoreductase activity, acting on diphenols and related substances as donors	17	1.10E-10

GO:0016758	Transferase activity, transferring hexosyl groups	34	1.50E-06
GO:0005516	Calmodulin binding	18	2.10E-05
GO:0016757	Transferase activity, transferring glycosyl groups	40	4.10E-05
GO:0016837	Carbon-oxygen lyase activity, acting on polysaccharides	7	1.10E-04
GO:0030570	Pectate lyase activity	7	1.10E-04
GO:0004674	Protein serine/threonine kinase activity	51	4.10E-04
GO:0004672	Protein kinase activity	65	5.50E-04
GO:0008017	Microtubule binding	6	1.20E-03
GO:0030599	Pectinesterase activity	8	1.50E-03
GO:0015631	Tubulin binding	6	1.70E-03
GO:0047262	Polygalacturonate 4-alpha-galacturonosyltransferase activity	5	1.90E-03
GO:0016760	Cellulose synthase (UDP-forming) activity	5	2.20E-03
GO:0004194	Pepsin A activity	7	2.50E-03
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	69	2.70E-03
GO:0016759	Cellulose synthase activity	5	2.80E-03
GO:0016787	Hydrolase activity	132	4.10E-03
GO:0016168	Chlorophyll binding	6	5.40E-03
GO:0008171	O-methyltransferase activity	6	6.90E-03
GO:0005509	Calcium ion binding	19	7.90E-03
GO:0004091	Carboxylesterase activity	16	8.90E-03
GO:0043169	Cation binding	126	9.60E-03
GO:0043167	Ion binding	126	9.90E-03

B.10 Significantly enriched GO terms in the down-regulated groups of cold resistant genotype at APR conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0005983	Starch catabolic process	5	1.50E-06
GO:0000272	Polysaccharide catabolic process	9	2.50E-06
GO:0009251	Glucan catabolic process	5	5.50E-06
GO:0044247	Cellular polysaccharide catabolic process	5	5.50E-06
GO:0043467	Regulation of generation of precursor metabolites and energy	5	3.10E-05
GO:0009699	Phenylpropanoid biosynthetic process	10	1.20E-04
GO:0019438	Aromatic compound biosynthetic process	13	1.90E-04
GO:0009698	Phenylpropanoid metabolic process	10	2.30E-04
GO:0019748	Secondary metabolic process	17	2.70E-04
GO:0006118	Electron transport	22	4.10E-04
GO:0009813	Flavonoid biosynthetic process	7	4.20E-04
GO:0005976	Polysaccharide metabolic process	16	5.40E-04
GO:0009812	Flavonoid metabolic process	7	6.10E-04
GO:0005985	Sucrose metabolic process	11	2.30E-03
GO:0006022	Aminoglycan metabolic process	5	2.70E-03
GO:0005982	Starch metabolic process	11	2.70E-03
GO:0005984	Disaccharide metabolic process	11	2.80E-03
GO:0009311	Oligosaccharide metabolic process	11	3.40E-03
GO:0016052	Carbohydrate catabolic process	11	4.00E-03
GO:0016137	Glycoside metabolic process	11	4.50E-03
GO:0044036	Cell wall macromolecule metabolic process	5	4.90E-03
GO:0005975	Carbohydrate metabolic process	27	5.20E-03
GO:0042967	Acyl-carrier-protein biosynthetic process	8	8.00E-03
GO:0006073	Cellular glucan metabolic process	11	9.40E-03
GO:0044042	Glucan metabolic process	11	9.50E-03
Molecular Function			
GO:0016758	Transferase activity, transferring hexosyl groups	18	1.50E-06
GO:0016757	Transferase activity, transferring glycosyl groups	20	2.80E-05
GO:0008194	UDP-glycosyltransferase activity	12	2.60E-05
GO:0043167	Ion binding	60	4.40E-05
GO:0043169	Cation binding	59	7.90E-05
GO:0008378	Galactosyltransferase activity	5	1.10E-04
GO:0035251	UDP-glucosyltransferase activity	7	1.80E-04
GO:0020037	Heme binding	11	3.50E-04

GO:0046527	Glucosyltransferase activity	7	3.20E-04
GO:0016491	Oxidoreductase activity	37	7.70E-04
GO:0046872	Metal ion binding	53	7.90E-04
GO:0004497	Monooxygenase activity	10	1.40E-03
GO:0046906	Tetrapyrrole binding	11	1.30E-03
GO:0005506	Iron ion binding	16	1.70E-03
GO:0008415	Acyltransferase activity	9	3.20E-03
GO:0016747	Transferase activity, transferring acyl groups other than amino-acyl groups	9	8.00E-03

B.11 Significantly enriched GO terms in the up-regulated groups of cold sensitive genotype at APR conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0005976	Polysaccharide metabolic process	54	3.20E-15
GO:0006073	Cellular glucan metabolic process	44	3.10E-13
GO:0044264	Cellular polysaccharide metabolic process	46	1.90E-13
GO:0044042	Glucan metabolic process	44	3.30E-13
GO:0005975	Carbohydrate metabolic process	82	2.40E-10
GO:0005982	Starch metabolic process	34	1.20E-09
GO:0044262	Cellular carbohydrate metabolic process	58	3.70E-09
GO:0009311	Oligosaccharide metabolic process	33	8.10E-09
GO:0005985	Sucrose metabolic process	32	8.20E-09
GO:0005984	Disaccharide metabolic process	32	1.50E-08
GO:0016137	Glycoside metabolic process	33	2.00E-08
GO:0007018	Microtubule-based movement	18	2.40E-08
GO:0007017	Microtubule-based process	21	2.20E-06
GO:0000038	Very-long-chain fatty acid metabolic process	6	5.90E-06
GO:0009809	Lignin biosynthetic process	10	2.90E-05
GO:0009808	Lignin metabolic process	10	2.90E-05
GO:0051258	Protein polymerization	9	1.00E-04
GO:0006022	Aminoglycan metabolic process	10	1.40E-04
GO:0006026	Aminoglycan catabolic process	8	1.50E-04
GO:0034641	Cellular nitrogen compound metabolic process	41	3.00E-04
GO:0009225	Nucleotide-sugar metabolic process	8	3.30E-04
GO:0009699	Phenylpropanoid biosynthetic process	15	5.90E-04
GO:0006869	Lipid transport	9	7.80E-04
GO:0009698	Phenylpropanoid metabolic process	15	1.30E-03
GO:0009664	Plant-type cell wall organization	7	1.60E-03
GO:0019438	Aromatic compound biosynthetic process	20	1.70E-03
GO:0042196	Chlorinated hydrocarbon metabolic process	7	1.80E-03
GO:0042197	Halogenated hydrocarbon metabolic process	7	1.80E-03
GO:0019497	Hexachlorocyclohexane metabolic process	6	2.00E-03
GO:0006030	Chitin metabolic process	5	2.40E-03
GO:0006032	Chitin catabolic process	5	2.40E-03
GO:0007167	Enzyme linked receptor protein signaling pathway	14	2.90E-03
GO:0042398	Cellular amino acid derivative biosynthetic process	21	3.20E-03
GO:0010015	Root morphogenesis	9	3.20E-03
GO:0006118	Electron transport	38	3.40E-03

GO:0006012	Galactose metabolic process	10	3.40E-03
GO:0009719	Response to endogenous stimulus	31	3.50E-03
GO:0007169	Transmembrane receptor protein tyrosine kinase signaling pathway	12	4.70E-03
GO:0042221	Response to chemical stimulus	59	4.60E-03
GO:0009624	Response to nematode	5	4.80E-03
GO:0044036	Cell wall macromolecule metabolic process	8	5.50E-03
GO:0006771	Riboflavin metabolic process	6	6.30E-03
GO:0042726	Riboflavin and derivative metabolic process	6	6.30E-03
GO:0000272	Polysaccharide catabolic process	8	6.10E-03
GO:0010033	Response to organic substance	35	7.20E-03
GO:0042545	Cell wall modification	6	6.70E-03
GO:0050896	Response to stimulus	109	7.10E-03
GO:0070887	Cellular response to chemical stimulus	20	6.50E-03
GO:0009832	Plant-type cell wall biogenesis	6	7.20E-03
GO:0010224	Response to UV-B	5	6.80E-03
GO:0015837	Amine transport	8	8.10E-03
GO:0009725	Response to hormone stimulus	27	1.00E-02
Molecular Function			
GO:0005507	Copper ion binding	23	2.00E-10
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	37	2.40E-10
GO:0016798	Hydrolase activity, acting on glycosyl bonds	40	4.70E-10
GO:0008471	Laccase activity	10	5.90E-09
GO:0016762	Xyloglucan:xyloglucosyl transferase activity	10	4.80E-08
GO:0016682	Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	10	1.20E-07
GO:0016679	Oxidoreductase activity, acting on diphenols and related substances as donors	11	1.70E-06
GO:0008171	O-methyltransferase activity	9	1.80E-05
GO:0016758	Transferase activity, transferring hexosyl groups	26	9.40E-05
GO:0016757	Transferase activity, transferring glycosyl groups	31	9.30E-04
GO:0043167	Ion binding	115	9.40E-04
GO:0043169	Cation binding	115	9.00E-04
GO:0003993	Acid phosphatase activity	6	2.00E-03
GO:0046914	Transition metal ion binding	81	3.00E-03
GO:0004568	Chitinase activity	5	4.80E-03
GO:0003777	Microtubule motor activity	5	4.80E-03
GO:0016787	Hydrolase activity	112	5.30E-03
GO:0030599	Pectinesterase activity	6	9.20E-03
GO:0046872	Metal ion binding	103	1.00E-02

B.12 Significantly enriched GO terms in the down-regulated groups of cold sensitive genotype at APR conditions

GO Accession Number	Description	Number of Genes	P-value
Biological Process			
GO:0005983	Starch catabolic process	6	5.30E-08
GO:0043467	Regulation of generation of precursor metabolites and energy	7	1.10E-07
GO:0009251	Glucan catabolic process	6	2.60E-07
GO:0044247	Cellular polysaccharide catabolic process	6	2.60E-07
GO:0000272	Polysaccharide catabolic process	10	3.00E-07
GO:0006664	Glycolipid metabolic process	5	4.70E-04
GO:0016052	Carbohydrate catabolic process	13	4.60E-04
GO:0006118	Electron transport	22	4.60E-04
GO:0005976	Polysaccharide metabolic process	16	5.90E-04
GO:0044272	Sulfur compound biosynthetic process	6	1.80E-03
GO:0006643	Membrane lipid metabolic process	5	1.60E-03
GO:0005975	Carbohydrate metabolic process	29	1.60E-03
GO:0006022	Aminoglycan metabolic process	5	2.80E-03
GO:0019684	Photosynthesis, light reaction	6	2.80E-03
GO:0044036	Cell wall macromolecule metabolic process	5	5.10E-03
GO:0042967	Acyl-carrier-protein biosynthetic process	8	8.40E-03
GO:0005982	Starch metabolic process	10	8.40E-03
GO:0044275	Cellular carbohydrate catabolic process	9	7.60E-03
GO:0008610	Lipid biosynthetic process	13	7.40E-03
GO:0006073	Cellular glucan metabolic process	11	9.90E-03
Molecular Function			
GO:0016758	Transferase activity, transferring hexosyl groups	18	1.60E-06
GO:0004497	Monooxygenase activity	14	5.30E-06
GO:0046906	Tetrapyrrole binding	14	2.90E-05
GO:0020037	Heme binding	13	2.20E-05
GO:0005506	Iron ion binding	20	3.10E-05
GO:0043167	Ion binding	60	5.70E-05
GO:0043169	Cation binding	59	1.00E-04
GO:0016757	Transferase activity, transferring glycosyl groups	19	9.40E-05
GO:0008378	Galactosyltransferase activity	5	1.10E-04
GO:0046872	Metal ion binding	52	1.60E-03
GO:0008194	UDP-glycosyltransferase activity	9	2.00E-03
GO:0016491	Oxidoreductase activity	34	5.40E-03

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PUBLICATIONS

Zeybek E., Önde S. and Kaya Z. (2012). Improved *in vitro* micropropagation method with adventitious corms and roots for endangered saffron. Cent. Eur. J. Biol., 7(1), 138-145.

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