Active Oxygen Involvement in Developmental Processes in *Populus*

With Emphasis on HipI-Superoxide Dismutase

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Cover: a xylem vessel from AS-SOD9 plant; see Paper II
(Photo: Vaibhav Srivastava)

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Abstract

In plants, oxidative stress is result of disruption of the cellular redox metabolism and is caused by a variety of stress conditions (abiotic and/or biotic). This leads to the induction of several mechanisms that protect against disruption of the redox balance, as well as mechanisms to assist in recovery from toxicity/damage caused by increased cellular levels of reactive oxygen species (ROS). The superoxide dismutase enzymes (SODs) are key component of the reactive oxygen species gene network and represent the first line of defense against ROS, by converting superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and water (H_2O). Therefore, SODs play an important role in protection against oxidative stress in all aerobic organisms.

In this thesis, I describe the characterization of an SOD isoform from *Populus*, hipI-SOD, which has a high iso-electric point. The global response to oxidative stress is also discussed.

Different forms of hipI-SOD transcripts were found in vascular tissue, one of which was produced by alternative splicing. HipI-SOD proteins were found to be mainly localized extracellularly, in the primary and secondary cell walls of vascular tissues. These results together with analysis of transgenic *Populus* trees with suppressed expression of hipI-SOD strongly indicate roles for hipI-SOD in regulating ROS levels in vascular tissue.

ROS are important regulators of plant stress responses. Nevertheless, oxidative stress often affects plants growth and development. In order to understand the basis of oxidative stress tolerance, the diversity of stress responses needs to be investigated. To achieve this we first developed an O2PLS-based multivariate methodology for the integration of multiple datasets originating from three different platforms (transcriptomics, proteomics and metabolomics). Subsequently this data integration method was utilized for a comprehensive study of the overall responses to oxidative stress in *Populus*. The findings may facilitate the development of stress-tolerant plants with improved survival rates and yields under stressed conditions.

Keywords: alternative splicing, *Populus,* superoxide dismutase, hipI-SOD, vascular tissue, cell wall, reactive oxygen species, oxidative stress, O2PLS.

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To my loving parents

"Success is never ending and failure is not the end"

Contents

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I   <u>Srivastava V</u>, Srivastava MK, Chibani K, Nilsson R, Nicolas R, Melzer M. and Wingsle G. (2009) Alternative splicing studies of reactive oxygen species gene network in *Populus* reveal two isoforms of high-isoelectric-point Superoxide Dismutase. *Plant Physiology* 149, 1848-1859

II <u>Srivastava V</u>[†], Schinkel H[†], Witzell J, Hertzberg M, Torp M, Srivastava MK, Karpinska B, Melzer M. and Wingsle G. (2007) Downregulation of high-isoelectric-point extracellular superoxide dismutase mediates alterations in the metabolism of reactive oxygen species and developmental disturbances in hybrid aspen. *The Plant Journal* 49, 135-148

III Bylesjö M[†], Nilsson R[†], <u>Srivastava V</u>, Grönlund A, Johansson AI, Jansson S, Karlsson J, Moritz M, Wingsle G. and Trygg J. (2009) Integrated analysis of transcript, protein and metabolite data to study lignin biosynthesis in Hybrid Aspen. *Journal of Proteome Research* 8, 199-210

IV <u>Srivastava V,</u> Hvidsten TR, Ryden P, Nilsson R, Freyhult E, Bygdell J, Quarnström J, Moritz T, Karlsson J, Trygg J. and Wingsle G. A Systems Biology approach to studying global responses to oxidative stress in *Populus. Manuscript*

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[†] Equal Contribution

Abbreviations

$\cdot$OH	Hydroxyl radical
$^1O_2$	Singlet Oxygen
4CL	Hydroxycinnmate-CoA ligase
ABA	Abscisic acid
AOX	Alternative Oxidase
APX	Ascorbate Peroxidase
AS	Alternative Splicing
C3H	4-coumarate 3-hydroxylase
C4H	Cinnamate-4-hydroxylase
CAD	Cinnamylalcohol dehydrogenase
CAT	Catalase
CCoAOMT	Caffeoyl-CoA O-methyltransferase
CCR1	Cinnamoyl-CoA Reductase1
cDNA	Complementary DNA
COMT	Caffeic acid 3-O-methyltransferase
E. coli	*Escherichia coli*
ESI	ElectroSpray Ionization
EST	Expressed Sequence Tag
GC-MS	Gas Chromatography Mass Spectrometry
GO	Gene Ontology
H_2O_2	Hydrogen Peroxide
hipI-SOD	High-isoelectric-point superoxide dismutase
HPLC	High-performance liquid chromatography
HR	Hypersensitive Response
IMAC	Immobilized Metal Affinity Chromatography
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
miRNAs	MicroRNAs
MS	Mass Spectrometry
MVA	MultiVariate Analysis
O_2	Oxygen
O_2^-	Superoxide Radical
O2PLS	Bidirectional OPLS
O_3	Ozone
OPLS	Orthogonal Projections to Latent Structures
PAL	Phenylalanine Ammonia Lyase
PCA	Principal Component Analysis

PCD	Programmed Cell Death
pre-mRNA	Pre-messenger RNA
ROIs	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
SODs	Superoxide Dismutases
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
WT	Wild Type

1 Introduction

In our solar system, the Earth is the only planet known to contain molecular oxygen (O_2) in its atmosphere; this supports aerobic life. However, about 4.5 billion years ago, when the Earth was formed, its atmosphere was very different; it was reducing and essentially free of oxygen. Life is thought to have begun in the depths of the ocean as anaerobic bacteria deriving their energy by fermentation in the O_2-depleted environment. O_2 was probably produced through the photolytic dissociation of water by solar ultraviolet (UV) and other types of high energy radiation. Later the evolution of O_2-producing cyanobacteria increased the concentrations of free O_2 in the Earth's atmosphere. Following O_2 accumulation, evolution of more complex organisms was possible (Berkner and Marshall, 1965). Thus, O_2 is an essential element for the survival of aerobic life; it is present at a concentration of ~21% in the atmosphere.

1.1 Reactive Oxygen Species

Reactive oxygen species (ROS) or reactive oxygen intermediates (ROIs) are partially reduced or activated forms of atmospheric oxygen (O_2). Ever since O_2 entered our atmosphere as a result of photosynthesis, ROS have been the unavoidable companions of aerobic metabolism (Mittler, 2002). Unlike O_2, ROS are highly reactive and toxic and can cause the oxidative destruction of cells (Bhattachrjee, 2005; Scandalios, 2005). The ROS capable of causing oxidative damage include superoxide radicals (O_2^-), perhydroxy radicals (HO_2^-), hydrogen peroxide (H_2O_2), hydroxy radicals (^-OH), alkoxy radicals ($RO\cdot$), peroxy radicals ($ROO\cdot$), organic hydroperoxide (ROOH), singlet oxygen ($^1O_2$) and excited carbonyls ($RO\cdot$) (Bhattachrjee, 2005; Quan et al., 2008). During the reduction of O_2 to H_2O, ROS such as O_2^-, H_2O_2 and ^-OH can be formed (Fig. 1B).

1.1.1 The ROS Gene Network in Plants

It is well documented that ROS are toxic molecules but that they also control many different processes in plants (Karpinski et al., 1999; Neill et al., 2002; Foreman et al., 2003; Kwak et al., 2003; Overmyer et al., 2003; Laloi et al., 2004). Therefore it is very important to maintain ROS in a stable state, to avoid toxicity whilst allowing cells to utilize ROS for the

control and regulation of biological processes (Foreman et al., 2003; Overmyer et al., 2003; Laloi et al., 2004). In plants, the level of ROS is strictly controlled by a large gene network encoding ROS-producing and ROS-scavenging proteins (Table I). In *Arabidopsis* the ROS gene network is composed of at least 152 genes (Mittler et al., 2004). The reactive oxygen gene network adjusts ROS homeostasis in different cellular compartments for signaling purposes as well as to provide protection against oxidative damage. Various genes encoding ROS-producing and ROS-scavenging proteins are briefly described in the following subsections.

1.1.1.1 Generation of ROS

Oxygen is a bio-radical; its two outermost valence electrons occupy separate orbits, with parallel spins (Fig. 1A). In order to oxidize another species O_2 needs to react with a partner whose electron spin is anti-parallel, and thus fits into its free electron orbital. However, pairs of electrons characteristically have opposite spins, so that O_2 must abstract electrons from other species, this makes it relatively unreactive. In others words, other species with spin paired electrons cannot transfer more than one electron at a time to O_2 (Imlay, 2003). Nevertheless, the reactivity of O_2 can be significantly increased by energy transfer or electron transfer reactions. ROS are typically the products of the excitation of O_2 to form singlet oxygen ($^1O_2$) or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical ($\cdot OH$) (Fig. 1B). O_2^- and H_2O_2 are weaker oxidizing agents than $\cdot OH$. Under normal conditions, the half life of H_2O_2 is probably 1ms; the half lives of other forms of ROS ($^1O_2$, $\cdot OH$, O_2^-) are much shorter, about 2-4 µs (Bhattachrjee, 2005).

In plants ROS are continually produced as by-products of many metabolic pathways that are localized in different cellular compartments like chloroplast, mitochondria and peroxisomes (Foyer and Harbinson, 1994). Under normal growth condition, ROS production in cells is low (Alscher et al., 1997), although many factors (abiotic and biotic stresses) that disturb the cellular (redox) homeostasis of cells cause enhanced production of ROS, leading to oxidative stress in plants (Bolwell, 1999; Bolwell et al., 2002; Mittler, 2002; Apel and Hirt, 2004; Scandalios, 2005). These stress-related factors include drought, salt, chilling, heat shock, heavy metals, UV-exposure, high light levels, air pollutants such as ozone (O_3) and SO_2, mechanical stress, nutrient deprivation and pathogen attack.

A rapid increase in ROS concentration is called an "oxidative burst" (Apel and Hirt, 2004). The steady state level of ROS in different compartments is determined by the interplay between multiple ROS-producing pathways, and ROS-scavenging mechanisms (Mittler, 2002; Mittler et al., 2004; Scandalios, 2005).
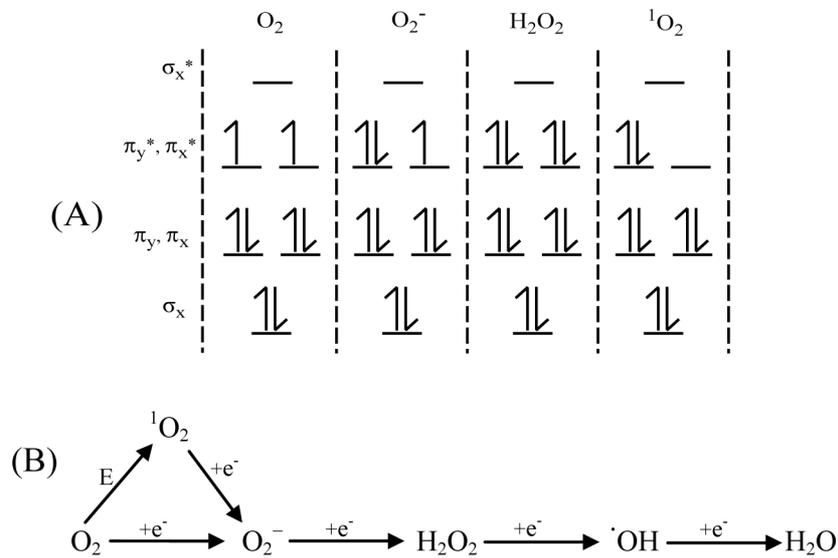
Figure 1. (A) Molecular orbital diagrams of molecular oxygen (O_2), the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). (Based on Imlay, 2003)
(B) Pathways in the univalent reduction of oxygen (O_2), to water (H_2O), leading to the generation of various intermediate ROS. E=energy; e^-=electron.

ROS produced from O_3 degradation (a notorious air pollutant) are associated with necrotic damage and programmed cell death (PCD) in plants (Kangasjärvi et al., 2005). Plant hormones such as ethylene have been shown to promote endogenous ROS formation under different stress conditions (Overmyer et al., 2003). Reactions associated with metabolic pathways such as photosynthesis, photorespiration and respiration are major sources of ROS production within plant cells (Ort and Baker, 2002).

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During photosynthesis O_2^- is continuously produced by reduced electron transport components associated with photosystem I (PSI) and $^1O_2$ is produced by photosystem II (PSII) (Apel and Hirt, 2004). Photoinhibition by strong light and UV-radiation leads, respectively, to excessive production of $^1O_2$ and O_2^- in spinach leaves (Hideg et al., 2002). About 1-5% of mitochondrial O_2 consumption leads to H_2O_2 production (Quan et al., 2008). In peroxisomes ROS are produced by glycolate oxidases and fatty acid β-oxidation (Mittler, 2002).

Other sources of ROS production in plants include NADPH-oxidases, oxalate oxidases, xanthine oxidases, amine oxidases, and cell wall bound peroxidases (Mittler et al., 2004). NADPH-oxidase catalyzes the production of O_2^- by single-electron reduction of O_2 with NADPH as the electron donor. In plants, NADPH-oxidase homologs known as respiratory burst oxidase homologs (Rboh), have been shown to be a source of ROS in response to pathogen attack (Alan and Fluhr, 1997) or wounding (Torres et al., 2002), environmental stresses (e.g. drought or osmotic stress) and during plant development (Lamb and Dixon, 1997; Potikha et al., 1999; Gapper and Dolan 2006). In addition to NADPH-oxidase, it has been suggested that germin-like oxalate oxidases, pH-dependent cell wall peroxidases and amine oxidases generate H_2O_2 in the apoplast. These are tightly regulated and are involved in the production of ROS during plant defence responses, wounding, PCD and development (Foreman et al, 2003; Bindschedler et al., 2006; Yoda et al., 2006; Angelini et al., 2008). Many peroxidases, localized in the apoplastic space, are ionically or covalently bound to cell wall polymers. In the presence of phenolic substrates and H_2O_2 they are involved in lignin and phenolic polymer biosynthesis (Apel and Hirt, 2004). In addition to these activities, *in vitro* studies have shown that horseradish peroxidase can reduce H_2O_2 to $^.OH$, in a similar manner as in the Haber-Weiss or Fenton reaction (Chen and Schopfer, 1999). During pathogenesis or wounding, ROS production is primarily apoplastic, whereas during salt stress ROS is produced from internalized membranes (endosomes) by NADPH-oxidases in a phosphatidylinositol 3-kinase dependent manner (Leshem et al., 2006, 2007). ROPs (Rho-related GTPases from plants) trigger H_2O_2 production followed by oxidative burst, probably by activating NADPH-oxidase (Agrawal et al., 2003).

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1.1.1.2 Detoxification of ROS

ROS are extremely reactive and cytotoxic in all organisms. ROS can interact with proteins, lipids and nucleic acids and cause severe molecular damage (Scandalios, 2005). Consequently, to minimize the deleterious effects of ROS, the evolution of all aerobic organisms has been dependent upon the development of an efficient ROS-scavenging mechanism based on non-enzymatic and enzymatic antioxidants.

Non-enzymatic antioxidant defense includes compounds with basic antioxidant properties, such as glutathione (GSH), ascorbate (Vitamin C), phenolic compounds (α-tocopherol, flavonoids, phenolic acid), nitrogen compounds (alkaloids, chlorophyll derivatives, polyamines) and carotenoids (β-carotene, zeaxanthin), that are capable of quenching ROS (Karpinski, 1994). Antioxidants help maintain cellular defenses against free radicals by many ways, such as by changing their redox state, being targeted for destruction, regulating oxidative processes involved in signal transduction, and affecting gene expression and pathways of cell proliferation, differentiation and death (Foyer and Noctor, 2005; Scandalios, 2005). The protection of cells against $^1O_2$ is believed to be mediated by carotenoids. Ascorbate and GSH, which are found at high concentrations, are crucial for plant defense against oxidative stress (Conklin and Barth, 2004; Noctor, 2006). Maintaining a high reduced:oxidized ratio of ascorbate and GSH is important for ROS scavenging within cells. This ratio is maintained by glutathione reductase (GR), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR), using the reducing power of NADPH (Conklin and Barth, 2004). The accumulation of phenolics has been described as a defensive or stress response in plants (Dixon and Paiva, 1995). Alpha-tocopherol plays an important role in protecting membranes in plants, which are highly susceptible to oxidative stress (Munné-Bosch, 2005). The overall balance between the different antioxidants has to be tightly controlled within the cell (Foyer and Noctor, 2005).

Proteins that have the capacity to bind to metals such as Fe and Cu (e.g. cytochrome, porphyrin, ferritin etc.) also come under this category of defense, preventing metal-catalyzed reactions. One good example of a metal-catalyzed reaction that produces a highly toxic ˙OH is the Haber-Weiss or Fenton reaction (Mittler, 2002). Since there are no known scavengers of ˙OH, its damaging effect can only be avoided by preventing the reactions that generate it.

Table I. ROS producing and scavenging gene network in plants[1]

	Enzyme and Reaction	Localization
1.	Alternative Oxidase (AOX) $2e^- + 2H^+ + O_2 \rightarrow H_2O$	mit, chl
2.	Amine oxidase $RCH_2NH_2 + O_2 + H_2O_2 \rightarrow RCHO + NH_3 + H_2O_2$	apo
3.	Ascorbate Peroxidase (APX) $2\ Asc + H_2O_2 \rightarrow 2\ MDA + 2H_2O$	chl, cyt, per, mit, apo
4.	Blue copper protein $Cu + P \rightarrow P\text{-}Cu$	
5.	Catalase (CAT) $2H_2O_2 \rightarrow 2H_2O + O_2$	per
6.	Dehydroascorbate Reductase (DHAR) $DHA + 2\ GSH \rightarrow Asc + GSSG$	chl, mit
7.	Ferritin $Fe + P \rightarrow P\text{-}Fe$	chl
8.	Glutaredoxin (GLR) $DHA + 2\ GSH \rightarrow Asc + GSSG$	cyt
9.	Glutathione Peroxidase (GPX) $H_2O_2 + 2\ GSH \rightarrow 2H_2O + GSSG$	cyt
10.	Glutathione Reductase (GR) $GSSG + NAD(P)H \rightarrow 2\ GSH + NAD(P)^-$	cyt, chl, mit
11.	Glycolate oxidase a-hydroxy acid $+ O_2 \rightarrow$ a-keto acid $+ H_2O_2$	per
12.	Monodehydroascorbate Reductase (MDAR) $MDA + NAD(P)H + H^+ \rightarrow Asc + NAD(P)^-$	chl, mit
13.	NADPH oxidase $NADPH + e^- + O_2 \rightarrow NADP^- + O_2^- + H^+$	mem
14.	NADPH oxidase-like $NADPH + e^- + O_2 \rightarrow NADP^- + O_2^- + H^+$	mem
15.	Oxalate oxidase $oxalate + O_2 + 2H^+ \rightarrow 2CO_2 + H_2O_2$	apo
16.	Peroxidases $Donor + H_2O_2 \rightarrow oxidized\ donor + H_2O$	CW, cyt, vac
17.	Peroxiredoxin (PrxR) $2P\text{-}SH + H_2O_2 \rightarrow P\text{-}S\text{-}S\text{-}P + 2H_2O$	chl, mit
18.	Superoxide Dismutase (SOD) $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$	chl, cyt, per, mit, apo
19.	Thioredoxins (Trx) $P\text{-}S\text{-}S\text{-}P + 2H^+ \rightarrow 2P\text{-}SH$	mit, chl
20.	Xanthine oxidase (XO) $xanthine + O_2 + H_2O_2 \rightarrow urate + H_2O_2$	per

Abbreviations: chl, chloroplast; cyt, cytosol; vac, vacuole; CW, cell wall; mem, membrane; mit, mitochondria; apo, apoplast; per, peroxisomes.
[1]Based on Mittler 2002 and Mittler et al., 2004

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Enzymatic antioxidant defenses in plants comprise mainly superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and peroxiredoxin (PrxR). SODs catalyze the dismutation of O_2^- to H_2O_2 whereas CAT, APX, GPX and PrxR reduce H_2O_2 to $2H_2O$. The balance between SOD and different H_2O_2-scavenging enzymes in cells is crucial for determining the steady state levels of O_2^- and H_2O_2. It has been shown that altering the balance among these enzymes induces a compensatory mechanism (Mittler, 2002). APX and GPX use ascorbate and GSH respectively as the reducing agent in detoxification of H_2O_2 to H_2O (Apel and Hirt, 2004). In plants the major ROS-scavenging pathways in which all these enzymes are involved include the water-water cycle in chloroplasts, and the ascorbate-glutathione cycle in chloroplasts, cytosol, mitochondria, apoplasts, and peroxisomes (Mittler, 2002). SODs, which constitute the first line of defense, are found in almost all plant cellular compartments (Fink and Scandalios, 2002). In contrast, CAT is mainly present in peroxisomes but it is crucial for ROS detoxification during oxidative stress. Unlike other H_2O_2 degrading enzymes, CAT degrades H_2O_2 without consuming any of the cellular reducing equivalents (Scandalios, 2005). In response to both biotic and abiotic stress, high levels of ROS have been found in transgenic tobacco plants that have reduced CAT levels (Mittler, 2002).

A group of enzymes called alternative oxidases (AOXs) has been shown to decrease ROS production through alternative channeling of electrons in the electron transport chains of chloroplasts and mitochondria. They use these electrons to decrease the overall level of O_2 by reducing it to water (Mittler, 2002). Plants with lower levels of mitochondrial AOXs are more sensitive to oxidative stress (Maxwell et al., 1999). Furthermore, chloroplast AOX is induced by high light levels in normal plants and also in transgenic plants lacking APX and /or CAT (Mittler, 2002). Redundancy has also been shown to exist among the ROS-scavenging mechanisms. For instance, plants with suppressed APX production induce SOD, CAT and GR, whereas plants with suppressed CAT production induce APX, GPX and mitochondrial AOX (Mittler, 2002). Recently, it has been reported that peroxiredoxins function as redox sensors, linking redox signaling and the ROS network in cells (Dietz, 2008); they have also been implicated in detoxifying peroxynitrite ($ONOO^-$), a potent oxidizing and nitrating species formed by a reaction between nitric oxide and O_2^- (Hong et al., 2008; Wilson et al., 2008).

1.1.2 ROS Signaling in Plants

ROS are found in almost all intracellular organelles or compartments as a result of normal metabolic activity. Normally, in cells, ROS exist in balance with antioxidant molecules and enzymes. Oxidative stress occurs when this critical balance is disturbed due to depletion of antioxidants or excess accumulation of ROS (Mittler, 2002; Foyer and Noctor, 2005; Scandalios, 2005). Increased levels of ROS within cells, either as a result of normal metabolic activity or of different types of stress, can interact with proteins, lipids and nucleic acids to cause severe molecular damage. Oxidative damage to lipids occurs due to ROS reacting with fatty acids in several ways in the membrane lipid bilayer, leading to membrane leakage and cell death. Oxidative damage to proteins can occur in many ways, including: fragmentation of the peptide chain; site-specific amino acid modifications (specific amino acids differ in their susceptibility to ROS attack); increased susceptibility to proteolysis; oxidation of Fe-S centers, which destroys enzymatic function; oxidation of specific amino acids, which "marks" proteins for degradation by specific proteases; oxidation of specific amino acids (e.g. Try), which leads to cross-linking; and aggregation of cross-linked reaction products. Oxidative damage to DNA occurs via DNA deletions, mutations, translocations, base degradation, single-strand breakage and cross-linking of DNA to proteins. Incomplete repair of such damage would lead to its accumulation over time resulting in age-related deterioration. Recently it was suggested that ROS toxicity plays an important role in longevity (Scandalios, 2005). ROS play a critical role in natural senescence, during which lipid peroxidation is an inherent feature (Bhattachrjee, 2005).

Recently, it has become apparent that ROS are not always toxic metabolic byproducts. A new role for ROS has been identified: the control and regulation of biological processes, such as growth, cell cycles, programmed cell death, hormone signaling, biotic and abiotic stress responses and development (Gapper and Dolan, 2006). H_2O_2 is accepted as being a secondary messenger for signals generated by means of ROS because of its relative longevity and ability to cross membranes (Quan et al., 2008). It has been speculated that H_2O_2 diffuses through aquaporins (Bienert et al., 2007). Therefore, ROS produced at a specific cellular site can affect other cellular compartments. For instance, light stress in *Arabidopsis*, which results in enhanced production of ROS in the chloroplast, induces the cytosolic scavenging mechanism (Karpinski et al., 1997). Superoxide radicals are membrane impermeable due to their negative charge in ambient pH conditions (e.g. cytosolic pH, which is

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approximately 7.0). However, under low pH conditions, superoxide can be protonated and, in this form, has been shown to cross yeast membranes (Wallace et al., 2004). In plants, the normal physiological extracellular pH is around 5, at which a small percentage of superoxide would be in the membrane-permeable hydroperoxyl ($HO_2^.$) form. Thus, external pH status could moderate the movement across the membrane of O_2^- produced by NADPH-oxidase (Sagi and Fluhr, 2006).

ROS produced by NADPH-oxidases have been shown to play a role in plant development, such as root elongation, through the activation of Ca^{+2} channels (Foreman et al., 2003), abscisic acid signaling in guard cells (Kwak et al., 2003), leaf shape and apical dominance (Sagi et al., 2001) and in plant defense responses (Torres et al., 2002). ROS have also been implicated in the loosening of cell walls (to allow cell expansion) in growing tissue (Rodriguez et al., 2002; Liszkay et al., 2004) and making cell walls rigid (to inhibit cell expansion) as growth ceases and cells differentiate (Hohl et al., 1995; Pothikha et al., 1999; Ros-Barcelo et al, 2002). Many studies have shown that H_2O_2 is important for lignification and secondary wall formation in plants (Potikha et al., 1999; Karlsson et al., 2005; Ros-Barcelo, 2005). H_2O_2 together with ethylene has been shown to be involved in the formation of hypoxia-induced lysigenous aerenchyma in *Arabidopsis* (Muhlenbock et al., 2007).

ROS play a central role in plant pathogen defense (Bolwell and Wojtaszek, 1997; Torres et al., 2002; Bindschedler et al., 2006). Rapid generation of O_2^- and H_2O_2 is a characteristic early feature of hypersensitive response (HR) following pathogen avirulence signals (Bolwell and Wojtaszek, 1997). In addition, in order to act as a direct antimicrobial agent, H_2O_2 also functions as a substrate for oxidative cross linking in the cell wall, as a threshold trigger for hypersensitive cell death, and as a diffusible signal for the induction of cellular defense genes in surrounding cells (Quan et al., 2008). Salicylic acid and nitric oxide play important roles in ROS accumulation during pathogen defense (Quan et al., 2008). Singlet oxygen-induced cell death depends on the blue light/UVA-specific photoreceptor cryptochrome and differs from PCD triggered by H_2O_2/O_2^- (Danon et al., 2006).

Ozone-induced oxidative burst results in a cell death process similar to the HR during plant pathogen interaction (Kangasjärvi et al., 2005). The plant hormones salicylic acid, jasmonic acid, ethylene and abscisic acid are involved in determining the duration and extent of O_3-induced cell death and its propagation (Overmyer et al., 2003). During abiotic stress, the role of ROS seems to be the opposite of that during plant-pathogen interactions

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and is associated with both signaling and oxidative damage (Breusegem et al., 2008). ROS have also been shown to induce autophagy in order to degrade oxidized proteins in *Arabidopsis* (Xiong et al., 2007). During abiotic stress, ROS-scavenging enzymes are induced in order to decrease the concentration of toxic intracellular ROS (Apel and Hirt, 2004). ROS-induced activation of various genes, including transcription factors, either directly or indirectly appears to be central for mediating cellular responses to multiple stresses (Apel and Hirt, 2004, Mittler et al., 2004). A generalized model of the ROS signal transduction pathway is shown in Figure 2 below.
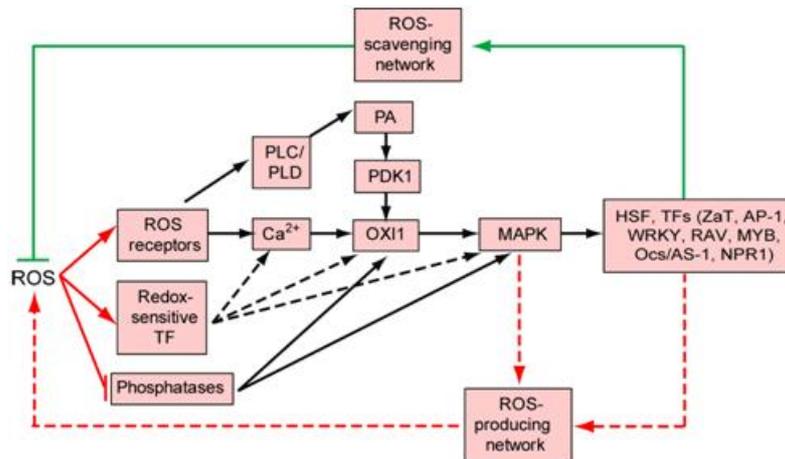
Figure 2. Generalized model of the reactive oxygen species (ROS) signal transduction pathway. ROS can be detected by at least three mechanisms (ROS receptors, redox sensitive transcription factors and phosphatases). Detection of ROS by receptors results in the generation of Ca^{2+} signals and the activation of a phospholipase C/D (PLC/PLD) activity that generates phosphatidic acid (PA). PA and Ca^{2+} are thought to activate the protein kinase OXI1. Activation of OXI1 results in the activation of a mitogen-activated protein kinase (MAPK) cascade and the induction or activation of different transcription factors that regulate the ROS-scavenging and ROS-producing pathways. The activation or inhibition of redox-sensitive transcription factors by ROS might also affect the expression of OXI1 or other kinases and/or the induction of ROS-specific transcription factors. Inhibition of phosphatases by ROS might result in the activation of kinases such as OXI1 or MAPK. A localized or general defense response (solid green line) can be activated to suppress ROS, whereas a localized amplification response (red dashed line) can be activated to enhance ROS signals. Abbreviations: HSF, heat shock factor; PDK, phosphoinositide-dependent kinase; TFs, transcription factors. (Based on Mittler et al., 2004).

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1.2 Alternative Splicing (AS) in Plants

Pre-messenger RNA (pre-mRNA) splicing is an important regulatory step in the expression of many eukaryotic genes. The AS of pre-mRNA results in the generation of multiple transcripts from a single gene, thereby increasing the coding capacity and proteome diversity of eukaryotic genomes (Ast, 2004; Stamm et al., 2005). In addition to affecting several aspects of RNA metabolism, AS affects proteins in many ways, including production of protein isoforms with the associated loss or gain of function, altered cellular localization, protein stability, enzyme activity and posttranscriptional modifications (Reddy, 2007). The number of genes displaying AS (using various bioinformatic, transcriptomic and other functional genomic tools) has increased tremendously in recent years, from a few hundred to many thousands (Reddy, 2007). It has recently been established that AS is a further frequent, important regulatory mechanism of gene expression in *Caenorhabditis elegans*, human, mouse and *Drosophila.* Analyses of the developmental stage and/or tissue-specific expression patterns of AS variants have become important components of biological research (Lareau et al., 2004; Pajares et al., 2007). In humans, it is estimated that about 60% of genes undergo AS (Modrek and Lee, 2002), exon skipping (ExonS) being the most common form and intron retention (IntronR) the least common (Kim et al., 2007; Fig. 3). Most of the alternatively spliced genes in humans are involved in signalling and regulation (Valdivia, 2007).

Alternative splicing in plants is a relatively unexplored area. However it is interesting because of the clear differences in plant splicing compared with that in fungi and animals; in addition there are splicing differences between monocots and dicots and between different species of dicots (Wang and Brendel, 2006; Reddy, 2007). Recent studies suggest that AS in plants is an important posttranscriptional regulatory mechanism in modulating gene expression and consequently plant form and function (Brown and Simpson, 1998; Reddy, 2007). Relatively few plant genes have been reported to undergo AS (Wang and Brendel, 2006). In an extensive study, Ner Gaon et al. (2007) analyzed 11 plant species using ESTs and genomic sequences, and found indications that AS occurs in all tested species. However, the proportions of alternatively spliced genes found in these plants varied substantially. In both *Arabidopsis* and rice, AS was found to affect ca. 21% of the genes, and IntronR to be the most prevalent form (Wang and Brendel, 2006). Functional studies conducted with a few splice variants indicate important functions of AS in plants. One of the isoforms of Rubisco activase, produced by AS, is redox-regulated (Zhang

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et al., 2002). Alternative splicing of FCA (a nuclear ABA receptor) pre-mRNA controls the developmental switch from the vegetative to the reproductive phase (Quesada et al., 2003). A few reports indicate that AS of pre-mRNAs of resistance genes plays an important role in plant defense responses (Dinesh-Kumar et al., 2000). In tomatoes, subcellular localization of protein phosphatase isoforms is determined by AS (de la Fuente van Bentem et al., 2003).

The appearance of new splice variants has been shown as a result of different stresses in plants (Reddy, 2007). Furthermore, these splice variants either increased or decreased as a result of abiotic stress. Exposure of maize seedlings to cadmium increased the level of an intron-containing transcript (Marrs et al., 1997) and the AS profile of *Arabidopsis* genes was affected by cold and other stresses (Iida et al., 2004). It has been suggested that various stresses alter serine/arginine rich (SR) protein expression which, in turn, alters the splicing of other pre-mRNAs. Positive and negative feedback auto-regulation or regulation of SR pre-mRNAs by other SR proteins may be involved in stress-regulated AS (Reddy, 2004 and 2007). This enables plants rapidly to alter their transcriptome posttranscriptionally in response to changing environmental conditions. Together these studies indicate that pre-mRNA splicing plays important role in stress responses.
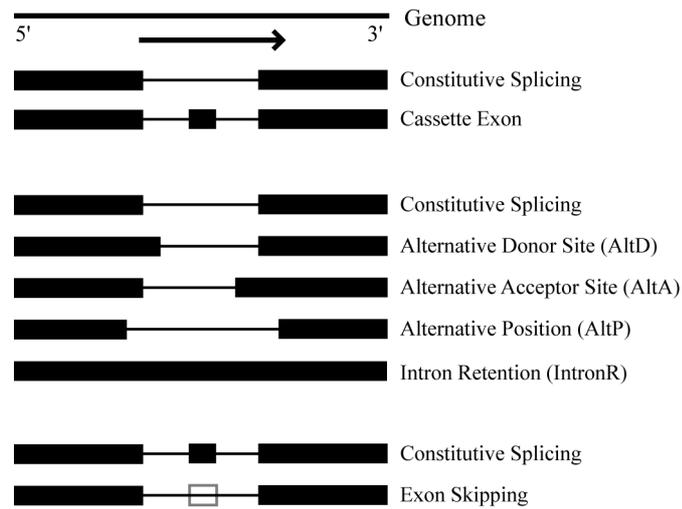
Figure 3. Visualization of different types of alternative splicings. The top black line represents the genome sequence. Filled boxes denote exons. Thin lines connecting the boxes indicate introns. Arrow indicates the direction of transcription. The open box represents a skipped exon. (Based on Wang and Brendel, 2006).

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1.3 Superoxide Dismutases

Superoxide dismutases (SODs, EC 1.15.1.1) are a family of metalloenzymes which catalyzes the dismutation reaction of toxic superoxide radicals to molecular oxygen and hydrogen peroxide.

$$O_2^- \ + \ O_2^- \ + \ 2H^+ \ \xrightarrow{\ \ SOD\ \ } \ O_2 \ + \ H_2O_2$$
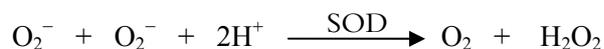
This reaction forms a crucial part of the cellular antioxidant defense mechanism by preventing the oxidation of various biological molecules, either by the radicals themselves or by their derivatives (Fridovich 1986; Bowler et al., 1992 and 1994). While all compartments of the cell are possible sites for O_2^- formation, the chloroplast, mitochondria, and peroxisomes are thought to be the most important sources of ROS (Bolwell, 1999; Bolwell et al., 2002; Mittler, 2002; Apel and Hirt, 2004; Scandalios, 2005). It has been shown that phospholipid membranes are impermeable to charged O_2^- and therefore it is crucial that SODs are present for the removal of O_2^- in the compartments where it is formed (Takahashi and Asada 1983; Alscher et al., 2002). SOD was first isolated from bovine blood (Mann and Keilin, 1938) whose biological function was believed to be copper storage. The true catalytic function of SODs (i.e. dismutation of O_2^- to H_2O_2 and O_2) was discovered by McCord and Fridovich (1969). The concentration of SODs in most of the cell is ~10^{-5} M (Fink and Scandalios, 2002). SODs are among the fastest enzymes known with a V_{max} of 2.4 x 10^9 $M^{-1}s^{-1}$. The efficiency of the reaction is partly due to electrostatic guidance of the substrate into the catalytic pocket of the enzyme (Getzoff et al., 1983).

SODs have been isolated and characterized from a wide variety of organisms (Marklund 1982; Kanematsu and Asada, 1989; Battistoni and Rotilio 1995; Karpinska et al., 2001; Schinkel et al., 2001; Fink and Scandalios, 2002; Kasai et al., 2006; Kim et al., 2008). On the basis of metal co-factor requirements, SOD enzymes can be classified into four groups. One class consists of SOD with Cu (II) plus Zn (II) at the active site (Cu/Zn-SOD), another with the Mn (III) at the active site (Mn-SOD), a third with Fe (III) at the active site (Fe-SOD) and a fourth with Ni (II/III) at the active site (Ni-SOD) (Fink and Scandalios, 2002). Fe-SODs are generally found in prokaryotes, in algae and in some higher plant chloroplasts. Mn-SODs are found in prokaryotes, in mitochondria and in peroxisomes. Cu/Zn-SODs are generally found in eukaryotes (Bowler et

al., 1994; Karpinska et al., 2001; Kasai et al., 2006; Kim et al., 2008) (Fig. 4). Ni-SODs are found in the *Streptomyces* genus (Fink and Scandalios, 2002). All these SOD enzymes appear to be encoded in the nucleus and can be distinguished from each other by inhibition studies. Cyanide and H_2O_2 inhibit Cu/Zn-SODs while Fe-SODs are inhibited by H_2O_2, but not cyanide, and Mn-SODs are insensitive to both chemicals (Schinkel, 2001).

Multiple forms of SOD have been found in all plants examined to date. Unlike most other organisms, plants have multiple forms of each class encoded by more than one gene (Fink and Scandalios, 2002).
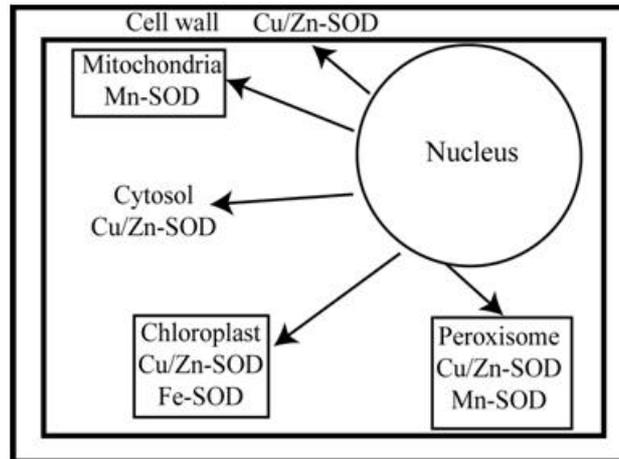
Figure 4. Location of SODs within the plant cell

1.3.1 Cu/Zn-SODs, Role and Regulation in Plants

Cu/Zn-SOD is the most abundant class of SOD that exists in plants. The electrical properties of Cu/Zn-SOD differ greatly from those of Fe and Mn-SODs. Cu/Zn-SODs have no sequence similarity to Mn and Fe-SODs and probably evolved separately in eukaryotes (Fink and Scandalios, 2002).

A Cu/Zn-SOD is often a homodimer with a molecular weight around 32kD, but other types of SODs do exist. Monomeric forms of Cu/Zn-SOD have been reported in plants (Kanematsu and Asada, 1989; Schinkel et al., 2001) and in *Escherischia coli* (Battistoni and Rotilio, 1995). Mammals have a glycosylated homotetrameric extracellular Cu/Zn-SOD with a molecular weight of 135kD (Marklund 1982).

Each subunit contains one Cu (II) and one Zn (II) atom. During the catalytic reaction of Cu/Zn-SODs, the Cu^{2+} is cyclically reduced and oxidized during successive encounters with the superoxide substrate at the active site. In the first step, an electron from one O_2^- radical is donated to the catalytic center leading to the formation of O_2 and one Cu^{1+} which, in turn, donates one electron to a second O_2^- radical to produce, together with two protons, hydrogen peroxide (Brodo et al., 1994). Zn^{2+} stabilizes the structure of the active site (Strange et al., 2007). The metal binding site in the enzyme is highly conserved (Kitagawa et al., 1991; Brodo et al., 1994; Hornberg et al., 2007).

Cu/Zn-SODs are found almost in all compartments of plant cell. They are mainly present in the cytosol (Kim et al., 2008), chloroplasts (Kliebenstein et al., 1998; Kim et al., 2008) peroxisomes (Bueno et al., 1995), apoplasts (Strellar and Wingsle 1994, Karpinska et al., 2004; Karlsson et al., 2005; Kasai et al., 2006; Kim et al., 2008) and nucleus (Ogawa et al., 1996; Kim et al., 2008). In *Arabidopsis* three Cu/Zn-SOD genes, CSD1, CSD2, and CSD3 have been identified (Kliebenstein et al., 1998). CSD1 and CSD2 activities are detected in roots, leaves, stems and siliques and their proteins are localized in the cytosol and chloroplasts, respectively (Kliebenstein et al., 1998).

The cytosolic Cu/Zn-SOD is considered to be a general stress response enzyme (Bowler et al., 1992). Transgenic plants with high SOD activity have enhanced tolerance of oxidative stress (Perl et al., 1993; Sen-Gupta et al., 1993) Overexpression of a cytosolic SOD from pea in transgenic tobacco plants increased ozone tolerance (Pitcher and Zilinskas, 1996). In a similar way, overexpression of a chloroplastic Cu/Zn-SOD from pea in
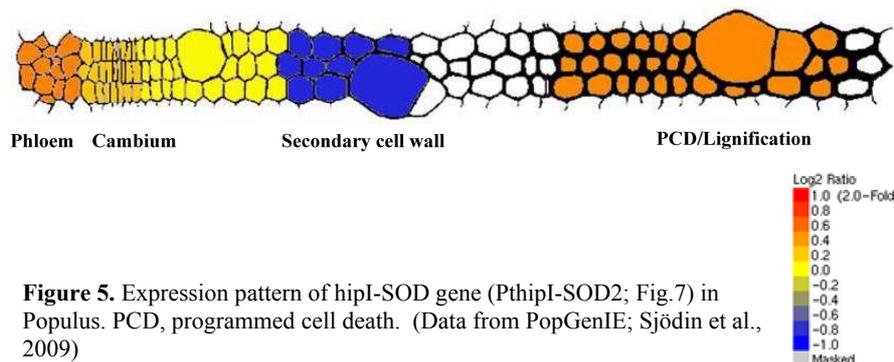
transgenic tobacco plants resulted in increased tolerance of high light levels and low temperatures (Sen Gupta et al., 1993). These studies clearly indicate the importance of Cu/Zn-SODs in plant defense mechanisms. The putative function of Cu/Zn-SOD in the nucleus is protection of DNA against oxidative stress (Corpas et al., 2006). Rizhsky et al. (2003) suggested that, in *Arabidopsis*, chloroplastic Cu/Zn-SOD protects plants from photooxidative stress. It has been suggested that extracellular Cu/Zn-SODs play an important role in detecting exogenous signal molecules, in enhancing cell wall-based defense and in acting as a downstream control of signal transmission pathways that leads to diverse defense responses, primary cell wall elongation and expansion (Kasai et al., 2006). In normal conditions it may also participate in cell wall growth and other developmental process, such as covalent cross-linking between polysaccharides and proteins in cell walls, lignification etc. (Ogawa et al., 1997; Karlsson et al., 2005; Kim et al., 2008).

A large number of investigations have been undertaken to examine the regulation of Cu/Zn-SODs. Cu/Zn-SODs also seem to be regulated by the redox equilibrium in the cell (Herouart et al., 1993; Wingsle and Karpinski, 1996). Members of the SOD family are differentially regulated; CSD1 and CSD2, in particular, are upregulated in response to stress that generates ROS, including O_3, UV-B and high light levels (Kliebenstein et al., 1998). Karpinski et al. (1992) showed a significant increase of both chloroplastic and cytosolic Cu/Zn-SOD mRNA level in pine exposed to air pollutants such as SO_2 and NO_2. Also microRNAs (miRNAs), short double-stranded RNAs that suppress gene expression in plants and animals, have been shown to posttranscriptionally regulate Cu/Zn-SODs (CSD1 and CSD2) expression in *Arabidopsis* (Sunkar et al., 2006). It has been shown that CSD1 and CSD2 are continuously transcribed *in vivo* and induction of their transcripts by oxidative stress depends on the suppression of miRNA (miR398) rather than increased transcription (Sunkar et al., 2006). In addition, miRNAs can also act as translational repressors of Cu/Zn-SODs in the absence of a target site i.e. SODs transcripts (Dugas and Bartel, 2007). Therefore it can be concluded that, in *Arabidopsis*, under both normal and stress conditions, the levels of Cu/Zn-SODs are strictly regulated by miRNAs.

1.3.2 HipI-Superoxide Dismutase

High-isoelectric-point superoxide dismutase (hipI-SOD), a Cu/Zn-SOD, was first found in Scots pine (Karpinska et al., 2001). Later its existence was shown in hybrid aspen (Schinkel et al., 2001) and *Zinnia elegans* (Karlsson et al., 2005). This enzyme has substantially higher pI value than other known SODs (> 9.5), as well as monomeric masses of about 16 kD. In pine, hipI-SOD has been detected by immunolocalization in the plasma membrane of phloem sieve cells, in the Golgi apparatus of albuminous cells and in secondary walls and intercellular spaces in the xylem cells (Karpinska et al., 2001). In hybrid aspen, hipI-SOD activity has been found in apical tissues as well as in the phloem and xylem (Schinkel et al., 2001). Expression patterns of hipI-SOD from various microarray analyses have shown that it is highly expressed in the phloem and secondary xylem (Fig. 5). The implications of the expression of hipI-SOD and the presence of H_2O_2 in the development of secondary cell walls have been discussed with respect to *Zinnia* (Karlsson et al., 2005).

There are indications that an isoform of hipI-SOD that is present in pine may be active as a monomer, unlike other SODs, which are active in their dimeric or tetrameric forms (Schinkel et al., 1998). This provides increased evidence that various isoforms of hipI-SOD could play different roles in metabolic processes.

Phloem   Cambium   **Secondary cell wall**   **PCD/Lignification**

Figure 5. Expression pattern of hipI-SOD gene (PthipI-SOD2; Fig.7) in Populus. PCD, programmed cell death. (Data from PopGenIE; Sjödin et al., 2009)
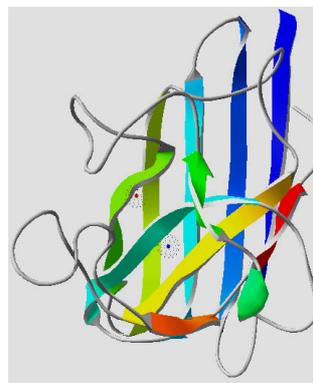
1.3.3 Molecular Structure of Cu/Zn-SOD

The molecular structure of Cu/Zn-SODs from spinach (Kitagawa et al., 1991), human (Parge et al., 1992), yeast (Djinovic et al., 1992a) and bovine (Djinovic et al., 1992b) has been determined by means of X-ray crystallography. Subsequent studies have explored its structure by analyzing several WT and mutant SOD enzymes (Rosenzweig and Halloran 2000; Tiwari et al., 2003; Lindberg et al., 2004; and 2005; Hornberg et al., 2007).

These proteins have a very similar structure, with eight anti-parallel β-strands forming a Greek key motif and seven loops. The main-chain hydrogen bonds primarily link the β-strands. This type of structure renders the protein more stable (Fink and Scandalios, 2002). The single disulphide bridge (Cys-57 and 146) and the salt bridge (Arg-79 and Asp-101) stabilize the loop region of the structure. Because of the low α- helix content the enzyme is less sensitive to heat, guanidine and organic solvent treatments than other enzymes.

The active site of the enzyme consists of one Cu^{+2} and one Zn^{+2} metal ion bridged by the imidazole ring of the His-63, a unique feature differentiating all the enzymes of this class. The Cu^{+2} is coordinated by three additional histidine ligands (His-46, -48, -120) and a water molecule, while Zn^{+2} is liganded by two additional histidines (His-71, -80) and an asparatic acid (Asp-83). Thus His-63 is the common ligand of Cu^{+2} and Zn^{+2} and the distance between the two metals is 6.1 Å. The orientations of the side chains and main chains of the metal-liganding residues are stabilized by a complex network of hydrogen bonds (Kitagawa et al., 1991). It has been suggested that Arg-143 participates in directing O_2^- to the active site. Several residues that are important for the activity are highly conserved (Bordo et al., 1994). The numbers given here refer to

Figure 6. Molecular Structure of Spinach Cu/Zn-SOD. Cu^{+2} and Zn^{+2} metal ions are shown in blue and red dots respectively.

Spinach Cu/Zn-SOD. Since Cu/Zn-SODs are a family of proteins with a high degree of sequence conservation (Bordo et al., 1994), the model of spinach Cu/Zn-SOD (Fig. 6) should be a very useful template for determining the structure of other plant Cu/Zn-SODs.

2 Objectives

The main objective of this PhD project was to investigate the role(s) of hipI-SOD in *Populus*. There are indications that hipI-SOD plays important roles in lignification, and possibly other processes, in various plant genera, including *Pinus* and *Zinnia*.

While characterizing hipI-SOD transgenic *Populus* plants it was found that besides affecting lignification, elevated levels of ROS also severely affected the plants growth. Therefore, the emphasis of the project was shifted to analyses of overall alterations in general metabolism in these plants.

In these contexts, the following were undertaken:

- Functional studies and cellular localization of hipI-SOD in *Populus*.

- Biochemical characterization of hipI-SOD.

- Developing a method for integrative analysis of transcript, protein and metabolite data.

- A study of the effect of oxidative stress on plant development in *Populus* by comprehensive analysis of gene expression using the data integration method.

3 Methodological overview

3.1 *Populus* as Model System

A model organism is a species that is extensively studied to develop our understanding of particular biological phenomena, with the expectation that discoveries made by studying the model organism will provide insights into the workings of other organisms. Model systems in plant biology include a range of species from "well-established" to "emerging" models, depending on the degree to which they have been developed. The choice of a particular model organism is usually dictated by a number of factors; of particular importance is the availability of tools and resources such as genomic and EST sequences, mutant collections, seed stocks, microarrays etc for a given organism. The choice of a particular model system can also be based on the need to study important plant processes that are absent or poorly developed in other model systems.

The model system used in the studies underlying this thesis was mainly hybrid aspen (*Populus tremula* x *Populus tremuloides).* Aspen (*Populus tremula*) was also used to a lesser extent.

Populus is an established model system for woody plants (Jansson and Douglas, 2007) and it provides an opportunity to study processes for which herbaceous models are less well suited (e.g. wood formation). It has a small genome (about 4x the size of *Arabidopsis*), fast growth, and it is easy to transform genetically and propagate. The availability of sequenced ESTs from different cDNA libraries (Sterky et al., 2004), a fully sequenced genome (Tuskan et al., 2006) and the availability of microarrays make it attractive for plant biologists to use as a model plant.

Populus is a close relative of *Arabidopsis*, so gene functions are often conserved (Jansson and Douglas, 2007). Other model plants currently used include tobacco, maize and wheat, each of which has specific advantages. Using more than one model system can also be very useful for assessing the generality of an investigated mechanism.

Most of the methods or techniques used in the studies described in this thesis are well known and are described in the respective papers. However some of them will also be briefly outlined in following sections.

3.2 Recombinant protein expression and purification

Extraction and purification of active proteins directly from plant or animal tissue, to produce large, homogeneous volumes is a tedious process, especially when the protein occurs at very low levels. Downstream processes after purification, such as structural characterization, require large quantities of pure protein (Geng and Wang, 2007).

Escherichia coli (*E. coli*) is one of the most commonly used host cells for recombinant protein production because of its ability to grow rapidly and at high densities on inexpensive substrates, its well characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. There is no guarantee however that the recombinant gene product will accumulate in *E. coli* at a higher level, at its full length or in a biologically active form. High-level production of functional proteins in *E. coli* usually leads to the formation of inactive protein aggregates called inclusion bodies (Gu et al., 2001). Protein folding or protein renaturation is the preferred way to produce active proteins expressed as inclusion bodies in *E. coli.* (Geng and Wang, 2007). However the overall yield of target protein is largely dependent on the efficiency of the protein refolding process. In recent years, liquid chromatography (LC) has been used to refold proteins and achieve higher yields (Wang et al., 2007). Advantage of the LC method is that it not only prevents the unfolded protein molecules from aggregation with each other, but it also simultaneously purifies the proteins during the chromatography process; therefore is known as protein folding liquid chromatography (PFLC, Geng and Wang, 2007).

To investigate function of hipI-SOD proteins (Paper I) and its three dimensional structure (ongoing work); it was expressed in *E. coli*. The majority of the protein produced was expressed as inclusion bodies. Since the hipI-SOD protein was expressed together with a His-tag to its C-terminal, immobilized metal affinity chromatography (IMAC) was used to refold and purify the protein. IMAC is based on the affinity interaction between the ligand and the histidine tag, usually at the end of target protein. In the presence of high denaturant concentration, the histidine tail of the target protein can still bind to IMAC, therefore it can accomplish refolding and purification simultaneously. IMAC has been widely used to refold proteins to achieve higher yield and purity (Zhu et al., 2005).

3.3 Methods for gene expression analysis

Analysis of the amount of transcripts is often used to determine the expression pattern of a particular gene in a certain tissue (e.g. xylem), in a specific condition (e.g. normal/stressed) or at a particular time (e.g. time series experiments). As a result, conclusions can be drawn about the involvement of that gene in regulating a specific process, its protein abundance etc. Microarray analysis has become a popular technique for identifying differentially expressed genes. Once identified, the varying expression levels of specific mRNAs must be confirmed. A number of widely used procedures exist for evaluating the expression pattern of a particular mRNA across RNA samples.

3.3.1 RT-PCR and Q-PCR

Reverse Transcription, coupled with the Polymerase Chain Reaction (RT-PCR), has revolutionized the study of gene expression. It is now possible to detect the RNA transcript of any gene, regardless of the amount of starting material or the relative abundance of the specific mRNA. In RT-PCR, an RNA template is copied into a complementary DNA transcript (a cDNA) using a reverse transcriptase. The cDNA sequence of interest is then amplified exponentially using PCR. Detection of the PCR product is typically performed by agarose gel electrophoresis and ethidium bromide staining or by the use of radiolabeled nucleotides or primers in the PCR. In the studies reported in Paper I, semi quantitative RT-PCR was performed to detect different forms of hipI-SOD in vascular tissues (Paper I, Fig. 4).

A more sensitive approach for detecting gene expression is quantitative RT-PCR (Q-PCR), which differs from classical PCR by the measurement of the amplified PCR product during each cycle throughout the PCR reaction, in terms of increased incorporation of a fluorescent dye (in this study SYBRgreen) into the PCR products (Wong and Medrano, 2005). Thus, real-time PCR allows the amplification to be followed in real-time during the exponential phase of the run, and thus allows the amount of starting material to be determined precisely. In contrast to end-point PCR techniques, the result is not suffering from saturation of the PCR reaction. The importance of Q-PCR has been reviewed by several authors (e.g. Klein, 2002; Gachon et al., 2004). Q-PCR was used to check the relative expression of a few of the phenylpropanoid biosynthesis genes in hipI-SOD antisense plants, compared to the WT (Paper II). In all cases the reference gene used was 18S, because of its high and stable expression (Norberg et al., 2005).

3.3.2 Microarray

Microarray technology is an important comprehensive tool for studying gene expression in plants. It allows global expression analysis of genes in a single experiment. It was first described by Schena and co workers (1995) and has been reviewed several times (e.g. Yang and Speed 2002; Kennedy and Wilson, 2004). Quantification of a large number of mRNA transcripts using microarray technology provides detailed insights into the cellular processes involved in the regulation of gene expression.

Briefly, DNA probes for a large number of genes are immobilized on a solid surface (typically glass) at pre-defined positions. RNA from the biological sample is reverse transcribed, labeled with fluorescent dyes and hybridized to the DNA probes on the array. The fluorescent signals from the labeled transcripts are detected using a laser scanner and the resulting images are converted into numerical data describing the relative expression level of each microarray element.

All microarray experiments described in this thesis (Papers II-IV) were performed on spotted cDNA microarrays using targets (two RNA samples) labeled with two fluorophores (cyanine dye Cy3 and Cy5). The first microarray, as used in Paper II, contained 2995 EST clones (Hertzberg et al., 2001) obtained from the wood-forming zone. Papers III and IV made use of *Populus* POP2.3 array, which consists of 25,000 cDNAs representing about 16,000 gene models from 19 different libraries (Sterky et al., 2004; http://poppel.fysbot.umu.se/index.html). A number of control spots were present on each array, these included human and archaebacterial cDNA clones as well as synthetic controls from a Lucidea Scorecard kit (GE Healthcare).

Microarrays are valuable screening tools for hypothesis generation and for later gene-based experimental approaches. Conclusive information about the expression of any particular gene(s), obtained by microarray analysis, must be confirmed using more accurate methods such as Q-PCR.

3.4 Mass Spectrometry

Proteomics, the systematic study of proteins and protein networks, requires qualitative and quantitative analysis of proteins and peptides. Mass spectrometry (MS) is currently a key analytical technique in proteomics and modern mass spectrometers generate large amounts of high-quality data that, in turn, allow protein identification, annotation of secondary modifications, and determination of the absolute or relative abundance of individual proteins (Jensen, 2006; Matthiesen and Jensen, 2008). Currently most large scale protein identification work in proteomics is based on mass spectrometry (Kocher and Superti-Furga, 2007).

Determination of the molecular mass of hipI-SOD proteins (Paper I) was conducted by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of bio-molecules (biopolymers such as proteins, peptides and sugars) and large organic molecules. The ionization is triggered by a laser beam (normally a nitrogen laser). A matrix is used to protect the bio-molecules from being destroyed by directing the laser beam and facilitating vaporization and ionization (Gevaert and Vandekerckhove, 2000).

For protein identification and quantification, proteins are usually digested by trypsin (an enzyme that specifically and efficiently cleaves the amide bond C-terminal to arginine and lysine residues unless a proline is the next residue) to generate peptides; these are then analyzed using mass spectrometry-based strategies, including peptide mass mapping by MS (to determine accurately the molecular mass of peptides) and peptide sequencing (amino acid sequencing) by tandem mass spectrometry (MS/MS).

Prior to mass spectrometric analysis, peptide separation by LC is advantageous when analyzing complex peptide samples. LC equipment is readily interfaced with mass spectrometers, so called LC-MS and LC-MS/MS systems depending on the application. One of the critical steps is to convert molecules (peptides) from liquid phase to gas phase ions prior to mass spectrometry. A process frequent employed for this purpose is ElectroSpray Ionization (ESI). A number of overviews of mass spectrometry-based proteomics have been published in recent years (Gevaert and Vandekerckhove, 2000; Aebersold and Mann, 2003; Matthiesen and Jensen, 2008).

Determination of the relative abundance level of proteins in an organism or tissues exposed to different physiological or environmental conditions is essential when studying particular processes and cellular

responses to stress (Matthiesen and Jensen, 2008). Biological systems are always studied by comparing different states, for example, a control state vs. a perturbed state. Quantitative methods for proteomic analysis are required in these situations and are achieved by peptide intensity profiling (Wang et al., 2008) or by stable isotope labeling (Yan and Chen, 2005). Since there is some degree of instrument-introduced variation in the intensity of the signals, it is important to replicate measurements.

With the exception of molecular mass determination of hipI-SOD, other mass spectrometric analyses for protein identification and quantification in Papers I, III and IV were performed on reverse-phase LC-ESI-MS/MS using a nano flow capillary liquid chromatography (HPLC/UPLC) system coupled to an electrospray ionization quadrupole-time of flight (ESI-Q-TOF) mass spectrometer. In the research described in this thesis, we compared the proteomic profile of various tissues (e.g. xylem and cambium) from WT and antisense plants (Papers III and IV) by intensity profiling, which is based on comparisons between the measured intensity signals of peptides in different samples. Triplicate LC-MS analysis was performed on the tryptic digestion obtained from each of the states described in Papers III and IV. Peptide retention times, peptide masses, and intensities were extracted from the LC-MS raw data by the MarkerLynx program. More information can be found in the respective papers.

3.5   Multivariate Analysis

With the increasing availability of high throughput systems (e.g. microarray, GC-MS, LC-MS etc) for parallel monitoring of multiple variables, massive amounts of data can be collected from a single experiment. Extracting useful information from such a system is not a trivial task, and requires powerful computational methods to identify common trends and to help detect any underlying biological patterns.

One valuable technique from the field of chemometrics is multivariate analysis (MVA), which refers to any statistical technique used to analyze data that arises from few or many variables. Multivariate methods can be used to extract information from large or small tables of data. Among the numerous data-driven methods available in chemometrics, the latent variable based modeling approach has been widely and successfully used to characterize the systematic properties of a system (Bylesjö, 2008). The latent variable based modeling method is well suited to handling high-
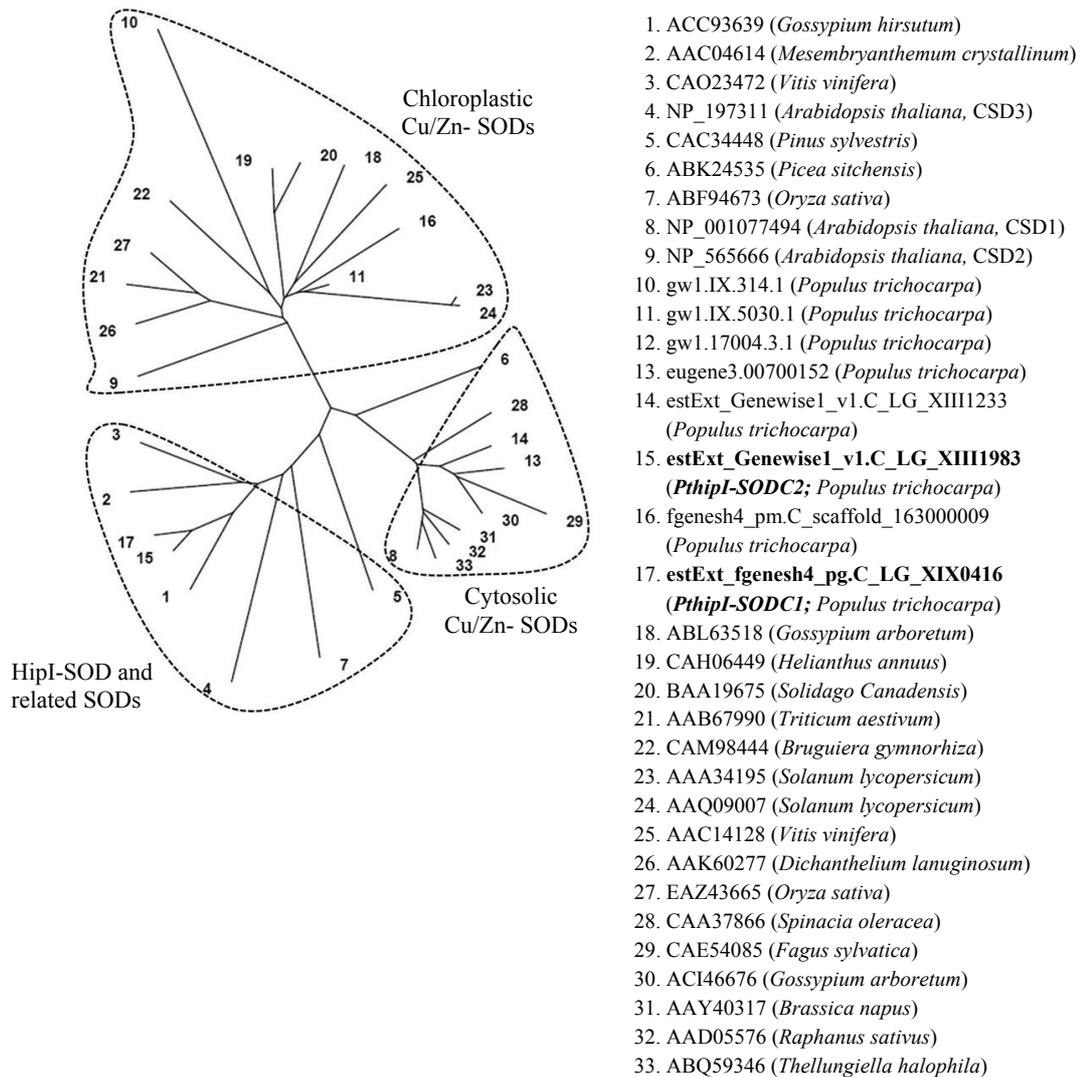
dimensional, noisy data with strong multicollinearity (e.g. biological or chemical data) (Bylesjö et al., 2007).

Principal component analysis (PCA) and O2PLS, both of which are latent variable based multivariate modeling methods, were used in the studies discussed in this thesis (Papers III and IV). PCA produces an overview of the information in the data by providing a measure and graphical representation of the relationships between the observations and also correlations between variables (Wold et al., 1987; Jolliffe, 2002). O2PLS is a bidirectional multivariate regression method (Trygg and Wold, 2003). The unique property of O2PLS, compared to other two block modeling approaches, is its capacity to identify joint variation between two datasets, while acknowledging also systematic variation that is unique to each dataset.

4 Results and Discussion

4.1 Different forms of hipI-SOD in *Populus* (Paper I)

Previously, hipI-SOD enzymes have been studied in pine (Karpinska et al., 2001) and *Zinnia* (Karlsson et al., 2005). In *Populus* hipI-SOD was first cloned by screening a cDNA library with a probe based on Scots pine (Schinkel et al., 2001). The availability of both *Populus* EST (Sterky et al., 2004) and genome (Tuskan et al., 2006) databases facilitated the identification of hipI-SOD ESTs and gene models, respectively. It also enabled global analysis of AS in this plant species. SODs are key components of the ROS gene network in plants. Since the genes involved in the ROS gene network in plants adjust ROS homeostasis in the cell as well as protecting against oxidative damage, it is very likely that AS may play an important role in regulating these genes posttranscriptionaly, depending on cellular status. To investigate AS events in *Populus*, we first extracted and compared all nucleotide sequences (ESTs, cDNA and gene models) for each gene in *Populus* that has been shown to be part of the ROS gene and signaling network in *Arabidopsis* (Mittler et al., 2004). The study revealed that all known types of AS events occur in the chosen set of genes in *Populus* (Paper I, Table I). Intron retention was the most common form of AS in the ROS-related dataset, accounting for 54% of the AS events detected in the *Populus* ROS gene network; this value is similar to the percentages reported for *Arabidopsis* and rice (Wang and Brendel, 2006). About half of the closest homologs in *Arabidopsis* to the genes showed AS in *Populus* in our study were not reported to be alternatively spliced, thus indicating that they may fulfill important roles in the regulation of tree-specific genes. Analysis of sequences around exon-intron boundaries for ROS-related genes showed high degrees of similarity to sequences reported for *Arabidopsis* and rice (Paper I, Fig. 1) but not to human and mouse genes. This clearly indicates the difference between splice site preferences in plants and animals (Wiebauer et al., 1988; Brendel et al., 1998; Reddy, 2007).

1. ACC93639 (*Gossypium hirsutum*)
2. AAC04614 (*Mesembryanthemum crystallinum*)
3. CAO23472 (*Vitis vinifera*)
4. NP_197311 (*Arabidopsis thaliana*, CSD3)
5. CAC34448 (*Pinus sylvestris*)
6. ABK24535 (*Picea sitchensis*)
7. ABF94673 (*Oryza sativa*)
8. NP_001077494 (*Arabidopsis thaliana*, CSD1)
9. NP_565666 (*Arabidopsis thaliana*, CSD2)
10. gw1.IX.314.1 (*Populus trichocarpa*)
11. gw1.IX.5030.1 (*Populus trichocarpa*)
12. gw1.17004.3.1 (*Populus trichocarpa*)
13. eugene3.00700152 (*Populus trichocarpa*)
14. estExt_Genewise1_v1.C_LG_XIII1233
    (*Populus trichocarpa*)
15. **estExt_Genewise1_v1.C_LG_XIII1983**
    (***PthipI-SODC2; Populus trichocarpa***)
16. fgenesh4_pm.C_scaffold_163000009
    (*Populus trichocarpa*)
17. **estExt_fgenesh4_pg.C_LG_XIX0416**
    (***PthipI-SODC1; Populus trichocarpa***)
18. ABL63518 (*Gossypium arboretum*)
19. CAH06449 (*Helianthus annuus*)
20. BAA19675 (*Solidago Canadensis*)
21. AAB67990 (*Triticum aestivum*)
22. CAM98444 (*Bruguiera gymnorhiza*)
23. AAA34195 (*Solanum lycopersicum*)
24. AAQ09007 (*Solanum lycopersicum*)
25. AAC14128 (*Vitis vinifera*)
26. AAK60277 (*Dichanthelium lanuginosum*)
27. EAZ43665 (*Oryza sativa*)
28. CAA37866 (*Spinacia oleracea*)
29. CAE54085 (*Fagus sylvatica*)
30. ACI46676 (*Gossypium arboretum*)
31. AAY40317 (*Brassica napus*)
32. AAD05576 (*Raphanus sativus*)
33. ABQ59346 (*Thellungiella halophila*)

Figure 7. Phylogenetic relationship among Cu/Zn-SODs in plants. The phylogenetic tree was constructed using the ClustalX (2.0.10; Larkin et al., 2007) and TreeView (1.6.6; Page, 1996) programs. The Genbank accession number of the protein sequences shown as numbers in the phylogentic tree is given to the left except for numbers 10-17 which represent gene model IDs for all Cu/Zn-SODs in *Populus*. Organism name is shown in brackets next to the Genbank accession numbers. Numbers 15 and 17 are the two genes (shown in bold) for hipI-SOD in *Populus*.

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In *Populus*, four ESTs (corresponding to two contigs POPLAR.5824.C1 and POPLAR.5824.C2) and two gene models (*PthipI-SODC1* and *PthipI-SODC2;* Fig. 7) of hipI-SOD were identified from *Populus* EST (PopulusDB; http://poppel.fysbot.umu.se/) and genome (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) databases, respectively. Both of these hipI-SOD genes consist of seven exons separated by six introns (Paper I, Supplemental Fig. S1). The contig POPLAR.5824.C2, represented by two ESTs, both termed as *hipI-SODC2*, corresponds to the *PthipI-SODC2* gene (Paper I, Fig. 2). The contig POPLAR.5824.C1, also represented by two *hipI-SOD* ESTs, termed as *hipI-SODC1s* and *hipI-SODC1b*, corresponds to *PthipI-SODC1*. Alignment of these sequences with *PthipI-SODC1* showed that *hipI-SODC1b* is 69 bp longer than *hipI-SODC1s*, and thus encodes an 18.7 kD protein of 181 amino acids (Paper I, Fig. 2); this is 23 amino acids longer than the sequence encoded by *hipI-SODC1s* (158 amino acids, 16 kD) Nevertheless, comparative analysis of the ESTs and *PthipI-SODC1* sequences revealed that the C-terminal part of all the predicted polypeptides is similar. It was found that exon 6 in *hipI-SODC1b* is larger than the corresponding exon in *PthipI-SODC1*, and that the extra 23 amino acids are encoded by the starting portion of intron 6, thereby making it 69 bp longer. Splicing at this new splice site ligates the seventh exon to the extended exon 6, thereby increasing the size of its transcript (Paper I, Fig. 3).

Furthermore, we checked the expression patterns of all three transcripts (*hipI-SODC1s*, *hipI-SODC1b* and *hipI-SODC2*) in vascular tissue. The splice variant *hipI-SODC1b* was differentially expressed throughout the vascular tissue, being clearly expressed in cambial tissue and the xylem, but not in the phloem (Paper I, Fig.4). Alternative splicing related differences in tissue-specific patterns of transcripts have been detected in several other plants (Tamaoki et al., 1995; de la Fuente van Bentem et al., 2003 and Marsh et al., 2003). Immunolocalisation confirmed the presence of hipI-SOD proteins in vascular tissue and they were mainly found extracellularly (Paper I, Fig. 5). The antibody used here was generated against the N-terminal part of the hipI-SOD (Schinkel et al., 2001) and thus, theoretically, should not be able to distinguish between the different hipI-SOD forms. Nevertheless, the presence of hipI-SOD proteins matched the transcriptional expression of *hipI-SOD* in the different tissues. Despite its extracellular localization, no typical secretory signal peptide was found in hipI-SOD. However, while most proteins that are exported out of a cell have signal peptides, many plant Cu/Zn-SODs that have no signal sequence at the N-terminus have been shown to be localized extracellularly (Ogawa

et al. 1996; Karpinska et al. 2001; Kasai et al., 2006; Kim et al., 2008). There are reports of extracellular proteins that contain internal hydrophobic signal sequences that are not cleaved off (Ye et al., 1988; Revest et al., 2000). There may be one (Ye et al., 1988) or two (von Heijne et al., 1991) hydrophobic domains in the N-terminal part of the proteins. HipI-SOD has such two domains (Schinkel, 2001).

In order to separate the different isoforms, we further purified hipI-SOD proteins from phloem and xylem samples. An interesting observation after western blot analysis was the detection of one band in phloem tissue and two bands in xylem. The size of these bands was also different (close to 18kD) than the expected size of hipI-SOD monomers (16 kD) (Paper I, Fig. 6). The discrepancy between the theoretical and experimental molecular masses may be due to the presence of two isoforms, differing slightly in molecular mass, or to the presence of post translational modification (e.g. glycosylation). All the bands identified as hipI-SODs by western blot analysis were used for tryptic digestion followed by mass spectrometry. Several peptides belonging to different hipI-SOD isoforms were identified (Paper I, Fig. 2) but we were unable to identify any specific peptide belonging to the extended exon 6 of *hipI-SODC1b*. Since all three hipI-SODs transcripts were found throughout the vascular tissue (Paper I, Fig. 4), but their respective proteins did not exhibit a similar pattern, it may be concluded that hipI-SOD proteins are restricted to a particular tissue and their level is independent of the corresponding m-RNA level. The biochemical functions of the spliced gene products were assessed by expressing recombinant hipI-SOD proteins and *in vitro* SOD activity assays. The results indicated that expressed hipI-SODC1s is functionally active as a dimer while its monomeric form is inactive. Despite forming a dimer, hipI-SODC1b exhibited no activity, like its monomeric form (Paper I, Fig. 7). Irrespective of their exact size, which was confirmed by MALDI-MS (Paper I, Fig. 8), expressed hipI-SOD proteins were found to migrate differently on Tris-glycine SDS-PAGE, in the same way as the native hipI-SOD proteins.

The existence of a splice variant, i.e. *hipI-SODC1b*, found to be differentially expressed in vascular tissue but forming a functionally inactive and unstable protein, indicated that it may be involved in regulating hipI-SOD gene expression at the posttranscriptional level (e.g. negative or positive feedback regulation, or a storage form) instead of actively participating in producing active proteins itself.

4.2 HipI-SOD as a regulator of ROS within lignifying cells (Paper II)

Expression and localization studies of a hipI-SOD in other plant species (Karpinska et al., 2001; Karlsson et al., 2005), and gene expression data from *Populus* transcript profiles (Schrader et al., 2004), suggest that the enzyme could be involved in the development and lignification of secondary cell walls. To elucidate the function of hipI-SOD, we generated transgenic hybrid aspen plants expressing the hipI-SOD gene in antisense orientation and confirmed that two lines (AS-SOD9 and AS-SOD24) exhibited phenotypic differences from the WT (Paper II, Fig. 3 and 4). As expected, the antisense plants contained reduced quantities of hipI-SOD proteins in their bark and xylem tissues (Paper II, Fig. 5). Compared with the WT, the growth rate of antisense plants was reduced, stems were thinner and leaves smaller and had developmental abnormalities (Paper II, Fig. 3 and 4). Plants of the AS-SOD9 line seem to have lost apical dominance, having a sloping stem habit and developing numerous lateral shoots (Paper II, Fig. 3). Furthermore, it was observed that vascular tissue of the antisense plants became lignified earlier. Increased accumulation of H_2O_2 and O_2^- was found in the vascular tissue of antisense plants (Paper II, Fig. 6). Elevated O_2^- was also localized in the living cells of the cambium in the transgenic lines. Further histological analysis showed that, in the transgenic plants, the cells in the cambial region of the stem were compressed and looked 'disorganized' and cell division and expansion were impeded towards both the phloem and xylem sides, possibly accounting for the stunted phenotype. This may be a direct effect of increased oxidative burst caused by the down-regulation of hipI-SOD in these tissues, thus inhibiting cell division, as reported earlier (Reichheld et al., 1999). Xylem fibers and vessels were shorter and thinner in antisense plants than in the WT (Paper II, Fig. 9). Since, in antisense plants, the lignification process is initiated prematurely, at a time when the vessels and fibers are still expanding, their cell walls may become more rigid, thereby limiting cell expansion. It could also be one of the reasons for the dwarfed phenotype of the transgenic plants. Since lignification started earlier in antisense plants we were interested to see whether the overall lignin content was affected. However, only a slight difference in lignin content was found between transgenic plants and the WT, indicating that the down-regulation of hipI-SOD in antisense plants affected the initiation of lignification but not the overall lignin content (Paper II, Fig. 7a).

On the basis of the above observations, we assumed that antisense plants might be under stress from the start of their growth. We examined

the phenomenon further by checking the differentially expressed gene in the apical part of the primary shoot using microarray analysis. Many genes that are involved in signaling (such as GTPase, IRE homolog, serine/threonine protein kinase, RAS related protein Rab 11C and calmodulins), stress responses (phospholipase A2 and a patatin-like protein) and lignification (basic peroxidases, COMT, CAD and 4CL) were found to be upregulated in antisense plants (Paper II, Table I). The upregulated proteins phospholipase A2 and a patatin-like protein have been shown to be rapidly induced during the HR in tobacco (Dhondt et al., 2000). The microarray results were further confirmed by real time PCR for a number of genes involved in the lignification process (Paper II, Fig. 10).

Reduced expression of hipI-SOD in the antisense lines leads to an increased localized accumulation of H_2O_2 and O_2^-. In the absence of SOD, O_2^- has been shown to be dismutated at a non-enzymatic rate (Torres et al., 2002), which may cause enhanced production of H_2O_2 compared with the situation when it is dismutated in a SOD-dependent manner (Liochev and Fridovich, 1994). The increased accumulation of ROS seems to induce both defense responses, as occurs during pathogen infections and ozone treatment of plants (Kangasjärvi et al., 2005), and signal transduction pathways important for developmental processes. In addition, the increased H_2O_2 in the transgenic plants may also participate directly in the peroxidase-mediated oxidation of monolignols to monolignol radicals during lignin polymerization (Ogawa et al., 1997). A further indication that the transgenic plants are stressed is the elevated level of phenolics in their stems. Accumulation of phenolic acids in transgenic poplar stems might cause the activation of an antioxidative system to diminish the oxidative stress (Sakihama et al., 2002), or may be involved in increased lignification (Robertsen and Svalheim, 1990). Accumulation of phenolics has also been described as a defensive or stress response in plants (Dixon and Paiva, 1995). The localization of hipI-SOD in phloem fibers and xylem vessels also suggests a role for the enzyme as a regulator of ROS levels in these cells.

4.3 O2PLS, a method for integrative analysis of transcript, protein and metabolite data (Paper III)

Plant metabolism involves a complex set of processes (Hirai et al., 2004). The focus of functional genomics studies in the post-genomics era has been on comprehensive characterization of samples using global profiling techniques involving parallel monitoring of transcripts (transcriptomics), proteins (proteomics) and biochemical process (metabolomics) (Bylesjö, 2008). A precise picture of a whole-cell process can be described by systems biology approach by integrating all the 'omics' sciences; however, massive amounts of data are generated from all the associated techniques (e.g. microarrays, LC-MS, GC-MS etc.). Data integration involving these huge amounts of data is one of the key challenges in studies of post-genomic functional genomics. Numerous strategies are described in the literature for integration of data from parallel sources (Bylesjö et al., 2007).

In the present study, we described a strategy for planning and integrated analysis of data derived from transcript, protein and metabolite profiling technologies (Paper III, Fig 2). The O2PLS method was used as a tool for data integration (Paper III, Fig. 1). Bylesjö et al. (2007) demonstrated the utility of the O2PLS method for integrating transcript and metabolite data. O2PLS is a bidirectional multivariate regression method that allows separate modeling of the covariance between two data sets as well as systematic variation specific to each data set (Bylesjö et al., 2007).

The study involved profiling steady state systems of three genotypes of hybrid aspen. The first genotype was the WT plant, which was included as a reference. The second and third genotypes were denoted G5 and G3 and contained four and one anti-sense constructs of the gene *PttMYB21a*, respectively (MYB transcription factor; Karpinska et al., 2004). Plants from both of the antisense lines displayed distinct phenotypes, with slower growth compared to the WT (Karpinska et al., 2004). Xylem samples from all three genotypes were collected from three internode positions on all of the plants (denoted internodes A-C), corresponding to a developmental gradient (Paper III, Fig. 2). This comprehensive sampling made it possible to study both the internode and genotype effects separately as well as any potential synergism between these factors. The O2PLS method was originally developed for integrated modeling of pairs of data sets. Here, however, three sets of transcript, protein and metabolite data were characterized in parallel. Multiple O2PLS models were utilized for this purpose in order to identify joint covariance from the transcript data through the protein data to the metabolite data. The steps utilized for identification of joint and platform-specific variation are summarized in

Fig. 3 and Table I (Paper III). The joint variation (Paper III, Fig. 7) describes two distinct effects that are common to the data derived from all of the profiling techniques. The first joint effect is that of an internode gradient, reflecting the common growth patterns of the samples, regardless of their genotypes (Paper III, Fig. 4). The second joint effect is a separation of the genotypes, regardless of the internode (Paper III, Fig. 5). These effects, hereafter referred to as the *internode effect* and the *genotype effect*, respectively, essentially reflect the variations included in the experimental setup (Paper III, Fig. 2). This confirmed the hypothesis that the integrative approach can capture the essential characteristics of the samples, since no information regarding the experimental setup was incorporated in the modeling.

The integrated analysis both confirmed known links between transcripts, proteins and metabolites and revealed a number of potential novel associations. Transcripts found to be strongly associated with the internode effect are related to photosynthesis and protein translation. Their expression tended to be strongest in the primary growth region (internode A), and declines basipetally. Most of the identified proteins were related to protein translation, elongation and glucose metabolism. The increased metabolism in the highest internodes requires essential amino acids, which were elevated at the metabolite level in these internodes. The perturbed gene, *PttMYB21a*, is known primarily to affect lignin biosynthesis and plant growth (Karpinska et al., 2004). It should be noted that the normal growth gradient and the separation of the mutant genotypes appear to be independent of one another. This suggests that both the G5 and G3 mutants share the essential developmental processes of a normal plant but with a few exceptions that cause their growth to be retarded. Several transcripts coding for factors that are essential for cell growth, including tubulin, actin-depolymerizing factor and protein translation elongation factors, showed decreased transcription levels in the G5 mutant. The behavior of the protein translation elongation factors was of particular interest since they were affected both by the internode and the genotype effect. Numerous factors involved in lignin biosynthesis were also heavily affected in the mutants; notably, in G5 mutants, reductions in transcript levels of several enzymes involved in lignin biosynthesis, e.g. CCoAOMT, COMT, CCR1, CAD and chorismate synthase. The changes at the transcript level can, in turn, be linked to changes at the metabolite level, for instance changes in levels of quinic acid, which is also related to lignin biosynthesis. The effects on tubulin, COMT and CCoAOMT could also be seen at the protein level, but in the opposite direction. While transcript levels were decreased in the G5

mutant, the protein levels were elevated. This negative correlation did not apply to all identified transcripts and proteins, and hence was in part specific to the G5-related changes in transcript and protein abundance. This can be partly explained by the complex post-transcriptional and/or post-translational regulatory mechanisms that are present in many higher-level organisms. These results indicated that *PttMYB21a* regulates lignin biosynthesis pathway in *Populus*, however, further investigations and follow-up experiments are required to conclude firmly.

Monitoring at multiple levels made it possible to separate systematic variation that was platform-specific from variation that could be linked across the different platforms (Paper III, Fig. 7). The platform-specific systematic variation signified events that have no correspondence across the omics data sets; nevertheless, they were interesting and important to study from a biological perspective. It has already been shown that the joint variation captures all known effects related to the sample properties (Paper III, Fig. 2), hence no other trends were expected to be seen in the specific sources of variation. Investigation of the systematic omics-specific sources of variation revealed that this was indeed the case (data not shown). These effects were, instead, largely linked to housekeeping-like events, which were not traceable to corresponding molecular events by the other omics technologies, and were either the result of the effects being completely unrelated or, possibly, they reflect the technologies' lack of full comprehensiveness.

Results from O2PLS models allowed easy access to model components that were useful for interpretation of the results. Because of this model transparency, all of the identified (internode and genotype) effects can be directly related to the variables of interest (transcript, proteins and metabolites) in order to put the results in a biological context.

4.4 Study of global responses to oxidative stress by systems biology approach (Paper IV)

Suppression of hipI-SOD expression in transgenic hybrid aspen trees resulted in overall impaired growth (Paper II). It was found that the altered phenotype was mainly due to oxidative stress (secondary effect) caused by increased accumulation of ROS in these trees. Based on this observation, these trees provided a good model system to study the global effect of oxidative stress on plant development. We focused our study on the cambial region of the stem, which showed severe developmental defects as well as high level of O_2^-, (Paper II, Fig 6 and 8).

Oxidative stress leads to the induction of several protective mechanisms for the recovery of redox balance and also to recover from toxicity/damage caused by ROS (Mittler et al., 2004). This whole process is controlled and coordinated by a complex regulatory network; however a clear picture is still not known (Mittler, 2002). Therefore parallel monitoring and integration of transcripts, proteins and metabolites is important in understanding of underlying mechanism of whole-cellular process under stress. Systems biology is an emerging area in plant research which integrates multidimensional biological information into networks and models (Yuan et al., 2008). As described earlier a multivariate method, O2PLS has been successfully utilized for data integration in plant biology (Paper III; Bylesjö et al., 2007).

In this study, transcriptome, proteome and metabolome analysis was conducted in parallel in the tissue extracted from cambial region (internodes 5-18) of transgenic hipI-SOD plants. To find out correlation among transcripts, proteins and metabolites during oxidative stress, a data integration multivariate method, O2PLS was used (Paper III). O2PLS models identified joint covariance (connection) among three data sets (transcriptomics, proteomics and metabolomics). The joint covariance captures effect that is common for all profiling techniques. This effect, referred as genotype effect (Paper IV), is distinct separation of transgenic plants compared to WT (Paper IV, Fig. 2).

Expression of several phenylpropanoid metabolism related genes (e.g. PAL, C3H, CCoAOMT, laccases) was up-regulated in transgenic plants (Paper IV, Fig. 2). However, at protein level they were not identified, probably because of their low level of expression as compared to other proteins. Nevertheless, related metabolites such as aromatic amino acids (phenylalanine, tryptophan) were found in higher amount in transgenic plants. Aromatic amino acids serve as precursors for indole glucosinolates, phytoalexins, alkaloids, lignins, flavonoids, isoflavonoids, and

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hydroxycinnamic acids (Dixon, 2001). Phenypropanoids compounds have been shown to be induced by various stress conditions (Dixon and Paiva, 1995). Their accumulation was described as defensive or stress response in plants (Dixon and Paiva, 1995). Our results indicated that oxidative stress had a considerable impact on primary metabolism. Many genes (fructokinase, Glucose-3 phosphate dehydrogenase), proteins (fructokinase, Glucose-3 phosphate dehydrogenase) and metabolites (Glucose-6-phosphate, fructose-6-phosphate, 3-phosphoglycerate) related to glycolysis were up-regulated in transgenic plants. It is tempting to speculate that the low ATP level under oxidative stress triggers up-regulation of genes needed for energy production.

Despite having high level of ascorbate peroxidase transcript and protein, ascorbate levels was found low in transgenic plants. It may indicate more prolonged and severe oxidative stress. Moreover, there was a pronounced accumulation of threonate, a breakdown product of ascorbate. Ascorbate is one of the principal antioxidant molecules in the cell (Horemans et al., 2000) and the production of ascorbate breakdown products indicates a failure to recycle all of the oxidized ascorbate via the ascorbate-glutathione cycle (Baxter et al., 2007). High level of expression of many genes and proteins related to ROS detoxification and maintaining redox balance within the cell were also found such as thioredoxin, glutaredoxin, peroxidase etc. (Mittler et al., 2002). Both at gene and protein level, HSPs (heat shock proteins), which play a crucial role in protecting plants against stress by re-establishing normal protein conformation and homeostasis, were found up-regulated (Celedon et al., 2007).

Based on the results obtained from the combined profiling approach (systems biology) for the study of overall effect of oxidative stress on transgenic hipI-SOD hybrid aspen plants it seems that plants try to alleviate the oxidative stress by up-regulation of defence related genes, protein and metabolites. A second clear response to the oxidative stress was upregulation of the lignification related gene expression and accumulation of aromatic amino acids that are precursors of lignin biosynthesis, which is in accordance with the early lignification seen in these transgenic, trees (Paper II). The diversity of the abiotic stress tolerance mechanism in plants was seen in this study. It has been shown that abiotic stress can also induce cell death in plants (Dat et al., 2003). The cause of premature transition into maturation in transgenic hipI-SOD plants could possibly be due to the severity and prolongation of the stress.

5 Conclusion and Future Perspectives

HipI-SOD seems to comprise a new subgroup of Cu/Zn-SODs that differs both in structure and localization from other isoforms. Different forms of hipI-SOD have been identified throughout vascular tissue in *Populus*. The existence of a splice variant, i.e. *hipI-SODC1b*, gave indications for its involvement in regulation of hipI-SOD gene expression posttranscriptionally. This type of regulatory mechanisms has to be explored in future experiments. Because of the extracellular localization of hipI-SOD in the absence of signal peptides, more controlled experiments need to be conducted (e.g. expression of the hipI-SOD gene with an epitope tag) to reveal its currently unknown pathway of migration outside the cell (this is on-going work). In addition to the cell wall, GhCSD3 was also found to be localized in the cytosol and nucleus (Kim et al., 2008). This protein is the closest homolog of hipI-SOD in cotton. It should be investigated whether hipI-SOD proteins in *Populus* have some roles at these locations as well.

Compared to their actual molecular masses, the migration of native as well as recombinant hipI-SOD proteins was found to be higher on SDS-PAGE. This strongly suggests that the discrepancy between theoretical and experimental molecular masses seen on SDS-gel is mainly because of the three dimensional structure of hipI-SOD proteins, which somehow interferes with their migration, and not because of any post translational modification as previously thought. The reason for the difference between the calculated (~7) and experimental (>9.5) iso-electric points of hipI-SOD is still unclear. However charge distribution on the hipI-SOD protein was found to be different compared to the cytosolic and chloroplastic forms of Cu/Zn-SOD (Schinkel, 2001). Determination of its crystal structure is expected to resolve this discrepancy (this is on-going work).

The function of hipI-SOD has not yet been satisfactorily determined. Transgenic plants, which were produced to elucidate the functions of hipI-SOD, showed reduced growth and viability and increased accumulation of ROS. Nevertheless, extracellular hipI-SOD localization and the effects on ROS metabolism and plant development caused by its down-regulation indicate that it is involved in ROS-regulated processes within vascular tissue such as lignification. High accumulation of O_2^- was also found in the cambial region of tissues such as leaves and roots, of the hipI-SOD transgenic plants (unpublished data). The types of cells related to this

region do not show lignification. These observations suggest additional roles (e.g. defense related action) for hipI-SOD, not related to lignification. Whether both hipI-SOD isoforms have different role in different parts of the plant should be investigated in future experiments. These studies could also enlighten about the process of subfunctionalization and/or neofunctionalization of both hipI-SOD genes, one of which originated after gene duplication in *Populus*. An interesting experiment would be to observe the growth of these plants in presence of ROS quenchers. It would also be interesting to examine the behavior of plants over-expressing hipI-SOD.

The results obtained from our combined profiling (systems biology) analysis of global responses to oxidative stress indicate the occurrence of two clear responses (defense and maturation) to this stress in the cambial region of the transgenic hipI-SOD hybrid aspen plants. The results can be interpreted so that the increased accumulation of ROS induces, in the initial phase responses that can alleviate mild oxidative stress including the up-regulation of defense-related genes, proteins and metabolites. However, cambial development is severely affected in the plants, presumably due to the severity and prolongation of the stress. Consequently, in later stages, after the defense responses have failed to cope fully with the oxidative stress, a second phase occurs, in which maturation and lignification signals are induced. In the same way, abiotic stress conditions has been shown to induce primarily a range of stress responsive genes (Cheong et al., 2002) supporting overlap between the ROS accumulation in hipI-SOD transgenic plants and abiotic stress. Therefore, our results suggest that the premature maturation event (e.g. cell death) occur also in response to prolonged abiotic stress. These maturation responses seem to be rather slower compared to what has been observed during HR (Bolwell and Wojtaszek, 1997), ozone exposure or wounding (Cheong et al., 2002). It would be appealing to conduct similar studies to examine other tissues found to have enhanced levels of O_2^-. This system-level understanding of ROS-metabolism could also help in the development of stress tolerant plants, improving survival and yield under stressed conditions.

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