Hydrogen peroxide in inducible plant stress responses

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Academic dissertation
To be presented for public criticism, with permission of the Faculty of Science, University of Helsinki, in the auditorium 1041 of the Biocenter, Viikinkaari 5 on December 7th, 2001, at 12 o'clock noon

Helsinki 2001

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ISSN 1239-9469
ISBN 952-91-4117-3 (nid.)
ISBN952-10-0216-6 (PDF)

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Abbreviations

ABA	abscisic acid
AOX	alternative oxidase
APX	ascorbate peroxidase
ARE	antioxidant responsive element
CAT	catalase
Cat1AS	CAT1 antisense
cDNA	complementary DNA
CHS	chalcone synthase
CHX	cycloheximide
DAB	3′3- diaminobezidine
DAO	diamine oxidase
DDRT-PCR	differential display reverse transcriptase- PCR
DPI	diphenylene iodonium
ECM	extracellular matrix
EC-SOD	extracellular superoxide dismutase
ERELEE4	ethylene responsive element, *Lycopersicon esculentum* E4
ERF	ethylene responsive element binding factor
EST	expressed sequence tag
G	glucose
GO	glucose oxidase
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione-S-transferase
HL	high light
HPLC	high performance liquid chromatography
HR	hypersensitive response
HRGP	hydroxyproline-rich glycoprotein
JA	jasmonic acid
MJ	methyl jasmonate
MPT1	mitochondrial phosphate translocator1
MRE	metal responsive element
mRNA	messenger RNA
MTF-1	metal response element binding transcription factor 1
NahG	bacterial gene encoding salicylate hydroxylase
PAL	phenylalanine ammonia-lyase
PAO	polyamine oxidase
PCD	programmed cell death
PCR	polymerase chain reaction
PiC	phosphate carrier
POX	peroxidase
PR10	pathogenesis related protein 10
PR- protein	pathogenesis related protein
Q	quinacrine
RbcS	Rubisco small subunit
rbohA	respiratory burst oxidase homolog A
rcd1	radical induced cell death 1
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SOD	superoxide dismutase
TEM	transmission electron microscopy
TMV	tobacco mosaic virus
UV	ultraviolet
VOC	volatile organic compound
WRKY protein	DNA- binding protein containing WRKYGQC -amino acid sequence in the N- terminus

Original publications

This thesis is based on the following publications, which will be referred to in the text with their Roman numerals. Additional unpublished data will also be presented in the text.

I **Tuomainen J, Pellinen R, Roy S, Kiiskinen M, Eloranta T, Karjalainen R and Kangasjärvi J.** 1996. Ozone affects birch (*Betula pendula* Roth) phenylpropanoid, polyamine and active oxygen detoxifying pathways at biochemical and gene-expression level. J.Plant Physiol. 148, 179-188.

II **Pellinen R, Palva T and Kangasjärvi J.** 1999. Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. Plant J. 20(3), 349-356. (Short communication).

III **Pellinen R, Korhonen M, Kiiskinen M, Utriainen M, Overmyer K, Lapinjoki S, Palva ET and Kangasjärvi J.** H_2O_2 activates cell death and defense gene-expression in birch (*Betula pendula*) (Manuscript).

IV **Dat JF, Pellinen R, Beeckman T, Kangasjärvi J, Inzé D and Van Breusegem F.** H_2O_2 primes an active cell death process in tobacco (Manuscript).

V **Wulff A, Anttonen S, Pellinen R, Savonen E-M, Sutinen M-L, Heller W, Sandermann Jr H and Kangasjärvi J.** 1999. Birch (*Betula pendula* Roth) responses to high UV-B radiation. Boreal Env. Res. 4, 77-88.

Summary

Plant inducible defence responses during stress were studied in the commercially important forest tree species Silver birch (*Betula pendula* Roth). A model system, catalase1 antisense tobacco (*Nicotiana tabacum*)(Cat1AS, deficient in catalase activity), was used to reveal further mechanisms underlying oxidative stress -induced cell death process. Numerous environmental factors affect the productivity of plants during their life span. Some of these are stress inducing and they may be biotic (such as pathogens) or abiotic (such as air pollutants or irradiation). Many biotic and abiotic stresses have in common the ability to induce the accumulation of apoplastic and intracellular reactive oxygen species (ROS) in plant tissues. These ROS have numerous tasks in inducible plant defence responses. Both in Silver birch and tobacco a ROS species, H_2O_2, is being actively produced in response to stress. In Silver birch the apoplastic H_2O_2 is produced by the plasma membrane NADPH oxidase together with cell wall peroxidases. Similarly in Cat1AS tobacco, abiotic stress induces NADPH oxidase dependent H_2O_2 accumulation. In both species stress-induced H_2O_2 accumulation precedes cell death, correlating spatially with lesion formation. H_2O_2 alone is sufficient to induce cell death, which appears to be an active process, and has features resembling programmed cell death (PCD). In both plant systems various morphological features of PCD occur as a response to oxidative stress. In addition to the ability of H_2O_2 to induce cell death, it can induce the expression of numerous stress- related genes. These stress -inducible genes share some common regulatory features in their promoter sequences. Stress causing agents that do not involve H_2O_2 production induce a distinct set of stress-inducible genes, differing from that induced by oxidative stresses. H_2O_2 is therefore a crucial component in signalling oxidative stress related responses and PCD in Silver birch and tobacco.

1 Introduction

1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) include H_2O_2, $\cdot O_2^-$, hydroxyl radicals ($\cdot$OH) and singlet oxygen. They are all formed during normal cellular metabolism, but under stress conditions their formation is accelerated (Noctor and Foyer, 1998).

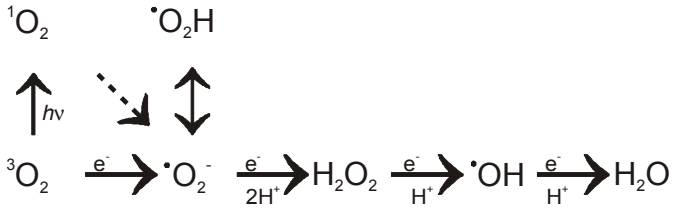

Figure 1. Formation of $\cdot O_2^-$, H_2O_2 and $\cdot$OH.

Generation of $\cdot O_2^-$, that exists in equilibrium with its protonated form hydroperoxyl radical ($\cdot O_2H$), requires energy (Figure 1). At physiological pH, $\cdot O_2^-$ is relatively non-toxic against cellular macromolecules and in aqueous solutions it disproportionates to H_2O_2 and O_2 either spontaneously or by the action of superoxide dismutase (SOD) (Wojtaszek, 1997). During $\cdot O_2^-$ disproportionation H_2O_2 is always formed. H_2O_2 is a relatively stable form of ROS and it is electrically neutral. It has an ability to pass through membranes, and therefore it reaches cellular components distant from its site of synthesis. It may be destroyed by catalases (CAT) or peroxidases. H_2O_2 can also be produced by peroxidases as shown in Figure 2 (Wojtaszek, 1997).

$\cdot RH + O_2 \rightarrow \cdot O_2^- + RH$
Peroxidase + $\cdot O_2^- \rightarrow$ Compound III
Compound III + $RH_2 \rightarrow$ Peroxidase (Fe^{3+}) + $\cdot RH$
$2 \cdot O_2^- + 2\ H \leftrightarrow H_2O_2 + O_2$
$RH_2 + \cdot O_2^- \rightarrow \cdot RH + H_2O$

Figure 2. H_2O_2 generation by peroxidases. RH and $\cdot$RH represent organic hydrocarbons and their respective radicals.

$\cdot$OH, produced in the Haber-Weiss reaction (Figure 3), is the most harmful of ROS forms in the plant tissue (Wojtaszek, 1997).

$H_2O_2 + \cdot O_2^- \rightarrow \cdot OH + OH^- + O_2$

Figure 3. Haber-Weiss reaction.

Under normal cellular conditions Haber-Weiss reaction proceeds very slowly and only very low amounts of $\cdot$OH are formed. It is however, formed in significant

amounts in the Fenton reaction (Figure 4) with transition metals such as Cu^{+} and Fe^{2+} and a subsequent reaction with $\cdot O_2^-$ (Wojtaszek, 1997).

$$H_2O_2 + Fe^{2+} (Cu^{+}) \rightarrow Fe^{3+} (Cu^{2+}) + \cdot OH + OH^-$$
$$\cdot O_2 + Fe^{3+} (Cu^{2+}) \rightarrow Fe^{2+} (Cu^{+}) + O_2$$

Figure 4. The Fenton reaction.

Since transition metals act as catalysts in the formation of ·OH, their subcellular localization also determines the location of ·OH synthesis. ·OH can initiate radical chain reactions and it is believed to be the main ROS responsible for alterations in cellular macromolecules and organellar damage (Wojtaszek, 1997). Hydroxyl radicals and singlet oxygen are extremely toxic to living cells and therefore must be efficiently removed. H_2O_2 and $\cdot O_2^-$, however, are formed continuously in the cells where they are involved in many biological processes. H_2O_2 and $\cdot O_2^-$ toxicity results mainly from their ability to initiate reactions leading to the formation of more harmful ROS species (Noctor and Foyer, 1998).

ROS can be produced also enzymatically within the plant tissues. At the moment the two most intensively studied enzyme systems involved in apoplastic ROS production conferring to the oxidative burst are the NADPH oxidase complex and the pH dependent cell wall peroxidases. A germin/oxalate oxidase system is also able to produce H_2O_2 in response to pathogen challenge (Wojtaszek, 1997) and both diamine and polyamine oxidases (DAO and PAO, respectively) produce H_2O_2 as a response to external stimuli (Smith, T.A., 1985).

Desikan et al. (1996) isolated protein components from *Arabidopsis* extracts that share immunological properties with the mammalian NADPH oxidase complex. Also, Keller et al. (1998) isolated an *Arabidopsis rbohA* (*respiratory burst oxidase homolog A*) gene, that has pronounced similarity to one of the subunits of the neutrophil respiratory burst NADPH oxidase. In addition, plant cell lines were found to express proteins resembling those of the mammalian NADPH oxidase complex in response to elicitation (Dwyer et al., 1996). Plant NADPH oxidase homolog *rbohA* also contains Ca^{2+} binding domains similar to the ones in their mammalian counterparts. Unlike mammalians, no cytosolic components of the enzyme complex were recognised (Keller et al., 1998).

According to data obtained from plant oxidative burst studies and mammalian NADPH oxidase complex, the oxidative burst reaction begins with the recognition of the elicitor molecule by a corresponding receptor molecule that lies on the plasma membrane. Receptors isolated in plants so far are still putative and only partially characterized (Wojtaszek, 1997). Components of the signalling pathway downstream of the receptor include at least GTP binding proteins, ion channels, protein kinases (Rajasekhar et al., 1999), phosphatases, phospholipases A and C, and cyclic AMP. Finally, NADPH oxidase is activated, $\cdot O_2^-$ produced and dismutated to H_2O_2 (Wojtaszek, 1997). Dismutation of $\cdot O_2^-$ to H_2O_2 in the apoplast is thought to occur via ex-

tracellular SOD (EC-SOD). Involvement of the EC-SOD is mainly based on inhibitor studies (Bestwick et al., 1997; Jabs et al., 1997), but EC-SOD activity has been determined (Desikan et al., 1996) and the gene has actually been isolated in Scots pine (Streller and Wingsle, 1994).

In the model where oxidative burst is created by pH dependent cell wall peroxidases, signalling components are less important while pre-existing components present in the extracellular matrix (ECM) are of utmost importance. In this model an elicitor is recognized by a receptor molecule, which leads to the activation of ion channels (Wojtaszek, 1997). Ion fluxes in turn cause transient alcalinization of the ECM, leading to the activation of pH dependent cell wall peroxidases, and finally H_2O_2 is formed as shown in Figure 2.

1.2 Plants and the environment

Plants can survive even in the most extreme environmental conditions, but also in areas where growing conditions are relatively good, environmental factors can affect photosynthesis and hence plant productivity is rarely optimal. Environmental changes force plants to adapt to surrounding conditions on a daily, or even an hourly basis (Etherington, 1988). Industrialisation has made the environment even more complex for plant survival by adding numerous air pollutants into the atmosphere (Oleksyn and Innes, 2000). Some of the most tragic air pollution problems among forest ecosystems have taken place in the boreal and temperate climate zones (Luttermann and Freedman, 2000). The most harmful air pollutants affecting forests are ozone (O_3), sulphur dioxide (SO_2), hydrogen sulphide (H_2S), nitrous oxides (NO_x), ammonia (NH_3) and fluorides (especially HF). These pollutants may cause visible symptoms as well as numerous physiological level alterations (Luttermann and Freedman, 2000).

In Finland 66% of the land is covered by forest. The most important forest tree is Scots pine (*Pinus sylvestris*), which takes up 45% of the area, while Norway spruce (*Picea abies*) has the second place. The deciduous trees Silver and Downy birch (*Betula pendula* and *Betula pubescens*), many willow (*Salix* sp.) and oak (*Quercus robur*) are less important. However, the importance of birch is increasing in Finland and in Sweden where it is used for the reforestation of farmland and to increase the productivity of coniferous forests (Selldén et al., 1997). The importance of deciduous trees also increases as the climate change is predicted to favour their growth rather than the growth of coniferous species (Kellomäki et al., 1996).

1.2.1 Environmental stress

Defining stress in plants is almost impossible, since no fixed stress points can be set. Factors that can cause stress to plants can be classified to seven main classes, represented in Table 1 (Elstner and Osswald, 1994). In a broad sense, plant stress can be defined as "any unfavourable condition or substance that affects or blocks a plant's

metabolism, growth, or development" (Lichtenthaler, 1996). In this case however, it is important to differentiate between low stress responses, that can partially be overcome by acclimation and repair, from strong or chronic stress effects, which may cause irreversible damage and cell death (Lichtenthaler, 1996).

Table 1. Stress causing agents in plants

Light	High intensity
	Low intensity
Radiation	UV (ultraviolet)
	γ
	α
	β
	X- ray
Temperature	High temperature
	Low temperature
	-Freezing
	-Chilling
Hydration	Drought
	Flooding
Chemical factors	Salts
	Heavy metals
	pH
	Air pollutants
	-O_3
	-SO_2
	-H_2S
	-NO_x
	-NH_3
	-HF
Mechanical factors	Wind
	Lightning
	Fire
	Snow
	Cutting, biting
	Pressing
Biological influence	Flowering
	Fruit ripening
	Insects
	Infections
	Allelopathy factors
	Competition

In many of the stresses mentioned in Table 1, ROS are involved as central signalling components (Noctor and Foyer, 1998). Of these ROS related stresses, O_3, light, pathogen infections and mechanical stress will be described in more detail in the following chapters. UV-B irradiation will be discussed also since ROS are involved in UV-B induced stress responses in some cases (Green and Fluhr, 1995).

1.2.1.1 Ozone (O_3)

Stratospheric O_3 filters the deleterious UV-B irradiation before it reaches earth (Attridge, 1990b). In the troposphere, however, O_3 is a secondary air pollutant, formed through the photo-oxidation of volatile organic compounds (VOCs) in the presence of NO_x. NO and hydrocarbons are produced as by -products in combustion processes in traffic and industry (Luttermann and Freedman, 2000). Although the overall reactions in the formation of photochemical "smog" are well known, many features in the formation of O_3 still remain unclear (Rao et al., 2000). O_3 is considered the most harmful of all photochemical air pollutants because of the small difference between ambient and toxic concentrations (Laurila, 1995).

Phytotoxic O_3 concentrations occur in Fennoscandia regularly. 100 nL L^{-1} is considered as a harmful level for vegetation (The Finnish Meteorological Institute, http://interim.fmi.fi/o3tietoa.html), but the proposed long term and short term critical levels for O_3 are preferably expressed as cumulative exposure over the threshold concentration of 40 nL L^{-1}. This exposure index is referred to as the AOT40 (accumulated exposure over a threshold of 40 nL L^{-1}). The AOT40 is calculated as the sum of the differences between the hourly O_3 concentrations in nL L^{-1} and 40 nL L^{-1} for each hour when the concentration exceeds 40 nL L^{-1} (Kärenlampi and Skärby, 1996). The highest O_3 concentrations in Finland in the year 2000 varied between 50-75 nL L^{-1}. The maximum value measured so far has been 95 nL L^{-1} in 1996 in Evo (The Finnish Meteorological Institute, http://interim.fmi.fi/o3tietoa.html). O_3 risk is highest in the southern coastal part of the country in comparison to the inland sites due to higher background O_3 concentrations (Pääkkönen et al., 1997). O_3 concentrations vary during the day (Sanz and Millán, 2000), and depend on the season, the highest concentrations occurring in the summer especially when the weather is hot and dry (de Leeuw and van Zantvoort, 1997; Luttermann and Freedman, 2000).

Forest vegetation suffers from O_3 depending on the concentration and time of exposure. Acute injury occurs when plants are subjected to high concentrations (200-300 nL L^{-1}) for short periods of time (2-4 h). Sensitive plant species show acute symptoms already when exposed to O_3 concentrations as low as 80 nL L^{-1} (Luttermann and Freedman, 2000), and e.g. in birch, O_3 sensitivity varies with age (Pääkkönen et al., 1995b). Transport of O_3 from urban sources to forested areas can lead to extended periods of exposure to moderate O_3 levels (Luttermann and Freedman, 2000). O_3 is considered likely to be the primary cause of forest decline in Europe (Schmieden and Wild, 1995), although it has more recently appeared that the link between O_3 and forest damage in Europe cannot be unequivocally drawn (Skärby et al., 1998; Matyssek and Innes, 1999). It appears that O_3 affects forests together with CO_2 and nitrogen depositions and that the major target of O_3 on mature tree fitness is resource allocation rather than growth (Matyssek and Innes, 1999).

Concern of O_3 toxicity partly arises from the fact that it can be transported over long distances from heavily polluted areas to forested areas (Luttermann and Freedman, 2000). O_3 injury was first described in 1940's in California, USA, and in the fifties they were referred to as "smog markings". In Europe, first O_3 caused damage to

vegetation was recorded in the 1970's (Davison and Barnes, 1998). In the case of O_3, high acute peak concentrations generally lead to cell death and therefore to visible symptoms (Heath and Taylor, 1997).
At the ultrastructural level, morphological changes in Silver birch, which is an O_3 sensitive tree, are first detected in the mesophyll cells around stomatal cavities (i.e. where O_3 enters the leaf) as exudates on the cell walls. It continues with the disintegration of cytoplasm and cell collapse. In the end the epidermal cells also collapse (Günthardt-Goerg et al., 1993). In detailed transmission electron microscopy (TEM) studies it has been shown that the number of irregularly shaped and more electron dense chloroplasts increases (Pääkkönen et al., 1995a; Selldén et al., 1997), thylakoids become dilated and distorted and the granulation in the stroma increases (Pääkkönen et al., 1995a, 1997), mitochondria become disintegrated, the amount of cytoplasmic lipids increases (Pääkkönen et al., 1996) and the amount of chloroplastic starch decreases (Pääkkönen et al., 1995b). Also alterations in the morphology of Golgi bodies, ER, and nuclear envelopes in O_3 treated spinach have been detected (Miyake et al., 1984).

Mechanisms leading to O_3 symptoms are only partly known. O_3 can damage cuticles, but hardly any O_3 penetrates the leaves through it. Instead, O_3 enters leaves through stomata and passes into the intercellular space (Guderian, 1985). Therefore, any factor affecting the stomata (e.g. drought), will subsequently affect the intracellular O_3 concentration (Sanz and Millán, 2000). The most critical events take place immediately as O_3 enters the aqueous apoplastic space, where it degrades rapidly forming ROS such as H_2O_2, hydroxyl radicals ($\cdot OH$), and superoxide ($\cdot O_2^-$). Due to this high reactivity of O_3 in aqueous environment containing lipids (such as the apoplast), and its short calculated half-life within plant tissue ($70 * 10^{-9}$ s), it is very unlikely that any O_3 as such would penetrate cells. In addition to these degradation products, plant cells begin to produce ROS rapidly in response to O_3 treatment (Heath and Taylor, 1997; Schraudner et al., 1998; Rao and Davis, 1999; Overmyer et al., 2000).

1.2.1.2 Light

41% of solar radiation is visible light, 50% infra-red light and the remaining 9% consists of x-rays and gamma rays as well as UV irradiation. The light received by a plant leaf varies with latitude, season, time of day, aspect, leaf inclination and cloud cover. Of the light that actually reaches the plant, a portion is reflected, absorbed or transmitted (Attridge, 1990b). Light absorption occurs by various light-absorbing pigments including photosynthetic pigments (chlorophylls and carotenoids), as well as the phytochrome (Attridge, 1990a) and UV-A (320-400 nm) and -B (280-320 nm) photoreceptors (Batschauer, 1999).

Light is indispensable for plant growth, photosynthesis and development. In addition to growth, many physiological processes are light dependent. These include germination, inhibition of hypocotyl growth, chloroplast differentiation, plant greening and expression of numerous genes (Beligni and Lamattina, 2000). No matter how

crucial and vital light is for plant development and productivity, it can also be a strong stress causing factor when received in excess. Excess white light leads to photooxidative stress (Karpinski et al., 1999), which damages the photosynthetic apparatus. During photooxidative stress H_2O_2 is formed in the chloroplasts (Foyer, 1997). Excess light is able to cause necrosis, as well as morphological changes, including the deformation of chloroplasts with pockets of cytoplasm, thylakoid swelling, and increases in the number of plastoglobuli (Cushman et al., 1995).

Stratospheric O_3 depletion has lead to a remarkable increase in the amount of UV-B irradiation on earth affecting both animals and plants (Rousseaux et al., 1999). No visible injury is detected in broadleaf trees under naturally occurring UV-B doses (Dillenburg et al., 1995; Sullivan et al., 1996; Zeuthen et al., 1997). However, in *Arabidopsis thaliana* leaf yellowing occurs as a response to UV-B irradiation (Lois, 1994). UV-B irradiation affects the leaf anatomical features differently in conifers and broadleaf species. Conifers seem to be more tolerant to the irradiation and in broadleaf species tissue thickening in the palisade parenchyma layer is the most prominent anatomical response to UV-B irradiation (Nagel et al., 1998).

In animals UV-B can induce carcinogenesis (Davies, 1995), and in both animals and plants DNA damage by the formation of covalent pyrimidine dimers (Rousseaux et al., 1999). The primary defence mechanism in plants against UV-B irradiation is the production of pigments, flavonoids, which absorb harmful UV wavelengths (Logemann et al., 2000), and therefore protect the plant DNA from damage (Kootstra, 1994). ROS production in plant cells also takes place in UV-B treated plants (Green and Fluhr, 1995).

1.2.1.3 Plant -pathogen interactions

Plants are attacked by a wide array of microorganisms during their life span, including fungi, bacteria, viruses and nematodes. Plants have the ability to protect themselves from microorganisms by both pre-existing and inducible defence responses. Pre-existing defence mechanisms include structural barriers and stored antimicrobial compounds (Hutcheson, 1998). Primary induced responses take place in the cells in direct contact with the pathogen, and lead to cell death. Secondary responses are induced in the cells surrounding the infection site and the third class of inducible responses consists of the systemic acquired resistance (SAR) that is induced in the whole plant (Hutcheson, 1998). Microbial infection leads to a disease when the microorganism both overcomes the pre-existing defences and avoids to induce the active defence responses (Hutcheson, 1998). In plant- pathogen interaction, the winner is either the pathogen, if it can proliferate fast enough in the plant tissue, or the plant, if it can respond fast enough with correct defence responses (Dong, 1998).

Pathogen infiltration has been shown to cause formation of necrotic lesions in plant leaves within 30 hours of injection (Levine et al., 1996; Alvarez et al., 1998). These necrotic lesions bear great resemblance to that seen in O_3- injury. In soybean (*Glycine max*) suspension cultures infected with pathogen, profound morphological

changes occur. These alterations include plasma membrane blebbing, cell shrinkage, and condensation of the cytoplasm and nucleus (Levine et al., 1996).

One of the defence responses taking place during plant-pathogen interactions is cell death that can be seen as restricted necrotic lesions at the infection site, distinct from the surrounding healthy tissue, known as the hypersensitive response (HR). Albeit some host tissue is damaged in the process, this localised cell death effectively restricts the spread of pathogens within the tissue (Tenhaken et al., 1995). Salicylic acid (SA) accumulates during HR, and the highest concentrations are found just around lesions (Enyedi et al., 1992). HR is considered nowadays as a form of programmed cell death (PCD), known as apoptosis in mammalian tissues (Alvarez et al., 1998).

An integral part of HR is the oxidative burst producing ROS. The oxidative burst can be defined as a rapid production of high levels of ROS in response to external stimuli (Wojtaszek, 1997). The transient ROS accumulation during oxidative burst is very rapid; in suspension cultured plant cells it begins as quickly as 1-2 min after the addition of an elicitor and in plant segments or intact plants ROS can be detected 2-12 hours after the elicitation (Wojtaszek, 1997). This rapid ROS accumulation is proposed to be non-specific (Draper, 1997). Upon recognition of the invading pathogen, a second burst of ROS accumulation takes place, and this sustained accumulation leads to the formation of HR (Draper, 1997). In addition to the local pathogen induced ROS accumulation, minor oxidative bursts can appear in distant locations from the original infection site. These minor oxidative bursts also lead to HR, only the lesion size is greatly diminished (Alvarez et al., 1998).

In mammals macrophages kill invading bacteria by phagocytosis. During this process oxygen is consumed and the reaction is called the respiratory burst. The $\cdot O_2^-$ accumulating during the respiratory burst is produced by the NADPH oxidase enzyme complex, and it is a major feature in antibacterial activity of phagocytes (Wojtaszek, 1997). The major difference between plant and animal systems in the oxidative burst is that in phagocytosis only the pathogen is killed, whereas in HR the plant tissue surrounding infection site is also destroyed (Bolwell et al., 1995).

Mechanical stress on plants may also lead to ROS production within tissues (Orozco-Cardenas and Ryan, 1999). This mechanical stress can be caused by insect feeding or by some other destructive event on the plant tissue (Maleck and Dietrich, 1999). Wounding causes bruising in potato (*Solanum tuberosum*) tubers (Partington et al., 1999), and in barley leaves it leads to necrotic lesions surrounded by chlorotic halos at the wound sites (Ledford and Richardson, 1994).

ROS from the oxidative burst have many important functions in the process of the HR and in other defence responses. They also drive the cross linking of cell wall polymers, trigger localized cell death and act as signalling components leading to other cellular responses such as the induction of transcription of stress related genes (Levine et al., 1994). In addition to pathogens, O_3 (Sharma and Davis, 1997), high light (HL) (Foyer, 1997), UV-B (Green and Fluhr, 1995) and wounding (Orozco-Cardenas and Ryan, 1999) are able to cause ROS production in plant tissues. Thus the

oxidative burst can be stimulated by many agents and it may lead to coordinated defence responses that are strikingly similar for various stresses (Sandermann, 1998).

1.3 Defence responses

Plants respond to stresses by exclusion, tolerance, compensation and/or repair (Heath and Taylor, 1997). Concentration and duration of, exposure to the stress factor as well as environmental conditions and developmental and metabolic state of the plants affect the response (Guzy and Heath, 1993).

1.3.1 Antioxidant enzymes

Antioxidant enzymes either catalyse reactions where an antioxidant molecule(s) is able to quench ROS without being transformed into a destructive radical itself or to process ROS directly. Each of the antioxidant enzymes comprise of several isoforms (Noctor and Foyer, 1998). Although they are often induced in similar stress situations, their responses may be differential (Adám et al., 1995). The total foliar activities of these enzymes may be misleading, since the compartments' activities are the ones that count in the local scavenging reactions. Since ROS are also considered important signalling molecules in plant inducible defence responses, the role for antioxidant enzymes may not be to control ROS level only but also to modulate gene expression through the generation of appropriate signal molecules and the destruction of unnecessary signal molecules (Noctor and Foyer, 1998).

1.3.1.1 Superoxide dismutase (SOD)

SOD dismutates $\cdot O_2^-$ to H_2O_2 (Noctor and Foyer, 1998), therefore converting one harmful oxidant to a less harmful one. Three classes of SOD activities are recognized and they differ by their metal cofactors, which are Cu, Mn or Fe and Zn. Subcellular localization of the isoenzymes is known, MnSOD being mitochondrial, FeSOD plastidic and CuZnSOD plastidic and cytosolic (Kliebenstein et al., 1998). EC-SOD in Scots pine is CuZnSOD and it has four isoforms (Streller and Wingsle, 1994).

Total or isoform specific enzyme activities and changes in mRNA levels for different SODs in different stress responses vary remarkably as shown in Table 2. This is rather natural considering the numerous SOD isoforms and distinct compartmentalisation (Kliebenstein et al., 1998). In any case, transgenic plants over- expressing SOD have been shown to be more stress tolerant against oxidative stress (Pitcher and Zilinskas, 1996; Noctor and Foyer, 1998; McKersie et al., 2000), although in some cases no changes in damage formation are detected (Allen, 1995). Both chloroplastic and cytoplasmic targeting of the transgene result in increased stress tolerance (Pitcher and Zilinskas, 1996; McKersie et al., 2000).

Table 2. Changes in the activities or mRNA levels of different SOD isoforms upon various stress treatments. Cyt = cytosolic and Chl= chloroplastic, - = no change, ↓ = down -regulation, ↑ = up -regulation.

Stress factor	MnSOD	FeSOD	Cyt CuZnSOD	Chl CuZnSOD
O_3	- (Willekens et al., 1994a; Kliebenstein et al., 1998)	↓ (Willekens et al., 1994a; Conklin and Last, 1995)	↑ (Kliebenstein et al., 1998; Overmyer et al., 2000)	↓ (Kliebenstein et al., 1998; Overmyer et al., 2000)
Pathogen	↓ (Adám et al., 1995)		↑ (Adám et al., 1995)	
Light	- (Kliebenstein et al., 1998)	↑ (Kliebenstein et al., 1998)	↑ (Kliebenstein et al., 1998)	↑ (Kliebenstein et al., 1998)
UV-B irradiation	↑ (Kliebenstein et al., 1998)	- (Kliebenstein et al., 1998)	↑ (Kliebenstein et al., 1998)	↑ (Kliebenstein et al., 1998)

1.3.1.2 Peroxidase

Peroxidases scavenge H_2O_2 by reducing it to H_2O in the presence of a reductant. They are found throughout the cell and they have higher affinity to H_2O_2 than CAT. In plant cells, the most important reducing substrate for peroxidase is ascorbate (Noctor and Foyer, 1998).

Peroxidases (either ascorbate peroxidase [APX], glutathione peroxidase [GPX] or total peroxidase) are up -regulated either at mRNA, protein or enzyme activity level under numerous stresses as shown in Table 3.

Table 3. Stress- induced changes in the mRNA , protein or enzyme activity level in peroxidases under various stress treatments. TMV= tobacco mosaic virus, - = no change, ↓ = down -regulation, ↑ = up -regulation.

Stress factor	APX	GPX	Total peroxidase
O_3	↑ (Conklin and Last, 1995) -(Willekens et al., 1994a; Overmyer et al., 2000), ↓ (Tanaka et al., 1985)	↑ (Willekens et al., 1994a; Schraudner et al., 1998) ↓ (Overmyer et al., 2000)	↑ (Tingey et al., 1975)
Pathogen	↑ (Schenk et al., 2000)		↑ (Adám et al., 1995; Bestwick et al., 1998), ↓ isoforms 2-3 (Adám et al., 1995)
TMV	↑ (Ward et al., 1991; Mittler et al., 1998)		
Light	↑ (Karpinski et al., 1997; Willekens et al., 1997), ↑ APX2, - APX3 (Karpinski et al., 1999)	↑ (Willekens et al., 1997)	
UV-B irradiation	- (Willekens et al., 1994a)	↑ (Willekens et al., 1994a)	
Wounding			↑ (Angelini et al., 1990)

Transgenic approaches show a clear role for cytosolic *Apx* in the protection of plant tissues against pathogens (Mittler et al., 1999), O_3 (Örvar and Ellis, 1997) and peroxisomal oxidative stress (Wang, J. et al., 1999). Chloroplastic oxidative stress, however, appears not to be avoided by over -expression of *Apx* (Tör et al., 1994; Wang, J. et al., 1999).

1.3.1.3 Glutathione reductase (GR)

Glutathione reductase (GR) catalyses a bi-directional reaction in which glutathione disulphide (GSSG) is reduced to glutathione (GSH). GR activity in green tissue is mainly localized in the chloroplasts, but there is also a cytoplasmic, and in pea (*Pisum sativum*) even a mitochondrial isoform (Greissen et al., 1996).

Both pathogen and high light (HL) treatments induce GR activity or mRNA level (Karpinski et al., 1997; Vanacker et al., 1998). GR activity increases in response to O_3 in spinach (Tanaka et al., 1985) and wheat (Rao et al., 1995), but is not affected in Scots pine needles (Wingsle et al., 1992). *Gr* mRNA levels generally decrease in a response to O_3 (Conklin and Last, 1995; Rao and Davis, 1999; Overmyer et al., 2000). However, in different tobacco (*Nicotiana tabacum*) cultivars GR activity correlates positively with the O_3 tolerance of the plants (Tanaka et al., 1990).

Gr over -expression leads to increased resistance to oxidative stress (Aono et al., 1991; Foyer et al., 1995), but to gain more O_3 tolerant plants over -expression is required both in chloroplasts and mitochondria (Broadbent et al., 1995).

1.3.1.4 Catalase (CAT)

Catalases (CAT) convert H_2O_2 to water and molecular oxygen (Noctor and Foyer, 1998). *Cat* genes exist in three isoforms (*Cat1-3*), which have different functions and locations in plant tissues. *Cat1* is peroxisomal and involved in scavenging photorespiratory H_2O_2, *Cat2* is preferentially expressed in the vascular tissue, while *Cat3* has a role in glyoxysomal processes (Willekens et al., 1994b). H_2O_2 itself can cause a decrease in the transcript levels of these isoforms in low concentrations, but their expression is induced at high concentrations (Polidoros and Scandalios, 1999).

CAT enzyme activity decreased in O_3 treated spinach (Tanaka et al., 1985), but increased in soybean more than 10 -fold (Tingey et al., 1975). However, when two different *Cat* family members were studied at the transcript level, it was clear that mRNA levels of *Arabidopsis Cat1* and *Cat3* were increased at least two fold in both WT and *rcd1* (*radical induced cell death1*, a mutant exhibiting O_3 and $\cdot O_2^-$ inducible lesion formation) (Overmyer et al., 2000). Three tobacco *Cat* genes responded very differently to O_3 exposure: *Cat1* was first down -regulated, rising little at the end of the experiment. *Cat2* was not affected, whereas *Cat3* transcript level clearly increased (Willekens et al., 1994a). Maize (*Zea mays*) *Cat1, 2* and *3* genes were induced also by H_2O_2 treatment (Polidoros and Scandalios, 1999). Increase in CAT activity upon pathogen invasion appeared to be cultivar dependent (Vanacker et al., 1998, 2000) and it also varied depending on plant species (Adám et al., 1995; Schenk et al., 2000).

Role of CAT in oxidative stress has been extensively studied with catalase antisense tobacco (Cat1AS) plants, which retain only 10% of wild type catalase activity. This deficiency leads to accumulation of H_2O_2 in the tissue under excess light causing photo-oxidative stress, and subsequent white necrotic lesions (Chamnongpol et al., 1996, 1998; Willekens et al., 1997). These plants undergo severe cellular damage under HL conditions, and it has therefore been postulated that CAT is a cellular sink for H_2O_2 produced during photo-oxidative stress (Willekens et al., 1997). In HL treated *Arabidopsis*, *Cat1* transcript level has been shown to increase 2.5 fold (Karpinski et al., 1997). UV-B irradiation of tobacco leaves caused inducion of *Cat2* and *3*, while suppressing *Cat1* (Willekens et al., 1994a). Similarly to the Cat1AS plants, which were hypersensitive to pathogen treatment (Mittler et al., 1999), over -expression of tobacco *Cat2* in potato lead to enhanced disease resistance in these transgenic plants (Yu et al., 1999).

1.3.1.5 Glutathione-S-transferase (GST)

Glutathione-*S*-transferases (GST) are a family of enzymes that catalyse the conjugation of glutathione via the sulfydryl group to a variety of electophilic centers of

hydrophobic compounds. This reaction makes the compound in question more hydrophilic and enables its transport to vacuoles or the apoplast. Phenolic oxidants and anthocyanins are among GST substrates. GSTs are also responsible for the detoxification of highly reactive lipid peroxidation products, generated from oxidative stress damaged membranes (Polidoros and Scandalios, 1999). O_3 (Sharma and Davis, 1994; Overmyer et al., 2000), pathogens (Dudler et al., 1991; Schenk et al., 2000), wounding (Reymond et al., 2000), H_2O_2 (Levine et al., 1994; Chen et al., 1996; Polidoros and Scandalios, 1999), and $\cdot O_2^-$ (Jabs et al., 1996) are able to increase the *Gst* transcript level.

1.3.2 Phenylpropanoid synthesis

Accumulation of phenylpropanoid compounds is detected in numerous plant species as a response to different stresses. Phenylpropanoid biosynthetic pathway leads to the synthesis of a vast array of biologically active secondary metabolites (Hahlbrock and Scheel, 1989), such as SA, phytoalexins (Smith, C.J., 1996), stilbenes, UV absorbing flavonoids and isoflavonoids and structural molecules such as lignin as well as anthocyanin pigments (Hahlbrock and Scheel, 1989) (Figure 5).

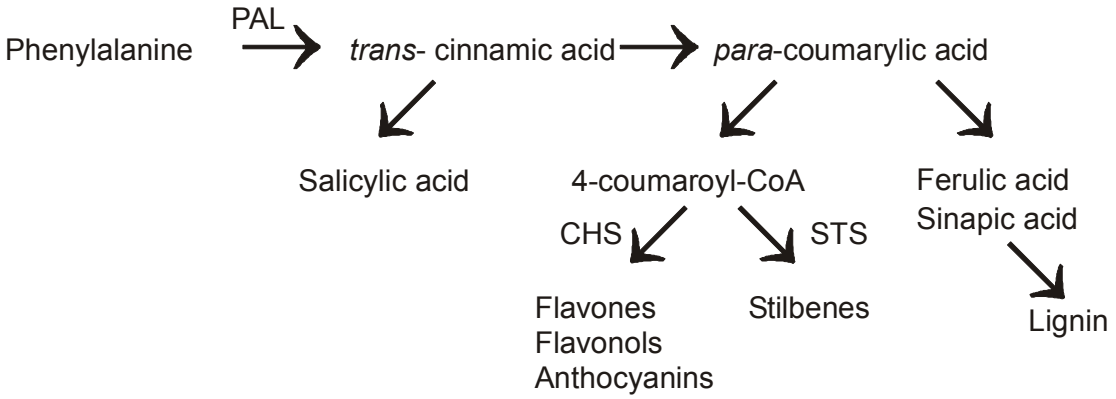

Figure 5. Phenylpropanoid biosynthesis.

Phenylpropanoid biosynthesis starts from the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL). PAL controls the flux of carbon into the pathway, and therefore efficiency of the whole pathway (Hahlbrock and Scheel, 1989). From 4-coumaryoyl-CoA the pathway branches to the flavonoid and anthocyanin biosynthetic pathway. This pathway is in turn controlled by chalcone synthase (CHS) (Dooner et al., 1991). Flavonoids are synthesised as a response to various environmental stimuli. Most importantly they protect plant tissues from harmful UV-B irradiation by absorbing light in the UV region, and therefore prevent the UV-B induced DNA damage (Kootstra, 1994).

Cell walls are strengthened at the pathogen penetration sites by incorporation and oxidative cross-linking of proteins and various phenolic subunits (Grant and Mansfield, 1999). Fortifying the plant cell wall gives various advantages to the plant.

It prevents leakage of cytoplasmic contents and creates an excellent barrier. Lignin precursor molecules and free radicals formed in the cross-linking reactions may as such disrupt pathogen membranes or inactivate bacterial enzymes and toxins (Hammond-Kosack and Jones, 1996). A localized oxidative burst is often detected during cell wall fortification, but the accumulation of ROS is much below the level seen during the HR (Grant and Mansfield, 1999). Rapid oxidative cross-linking of basic hydroxyproline-rich glycoproteins (HRGPs) with pathogenesis related-proteins (PR-proteins) may be one of the earliest defence responses linked to the oxidative burst (Bradley et al., 1992).

1.3.3 Polyamines

The plant polyamines consist of diamine putrescine, and polyamines spermine and spermidine (Langebartels et al., 1991). Polyamines have crucial roles in numerous aspects of plant life, including cell division, macromolecule synthesis, senescence, and stress responses (Angelini et al., 1990). For example, in stress situations polyamines may have a role in preventing O_3 injury by chelating metal ions that catalyse peroxidation reactions leading to lipid peroxidation and changes in plasma membrane permeability, both known consequences of O_3 exposure (Kangasjärvi et al., 1994). The ability of polyamines to prevent O_3 injury is likely to be due to membrane stabilization and scavenging of oxygen radicals (Evans and Malmberg, 1989; Rowland-Bamford et al., 1989). Radical scavenging ability of polyamines is due to their phenolic hydroxy groups (Bors et al., 1989). Pathogen infection can also affect plant polyamine levels and these alterations are able to modulate the pathogen defence response (Yamakawa et al., 1998).

1.3.4 Lipids

In plant cells, lipids act as major components of the biological membranes, energy reserves, precursors for waxes, cutin and suberin, and signal transduction chain components (Miguel et al., 2000). For example, the lipid derived signal molecule jasmonic acid (JA) has been implicated of being responsible for wounding induced gene expression in plants (Martín, M. et al., 1999). Also, inositol lipid turnover may transduce the elicitor induction of *Pal* via protein kinases (Kamada and Muto, 1994a).

Lipid peroxidation is detected in plants undergoing HR, and it can be triggered by adding ROS generating systems into plants (Rogers et al., 1988). O_3 causes drastic changes in the lipid compositions which may result from these peroxidation events (Sakaki et al., 1994).

1.3.5 Signalling in stress responses

Signalling takes place in plant development as well as in sensing environmental stimuli. Signalling events include recognition of the stimuli and subsequent intracel-

lular events. ROS may also act as signalling molecules (Noctor and Foyer, 1998). The most studied signalling molecules in plant stress responses include SA, JA, ethylene, and abscisic acid (ABA). SA and ethylene and their role in mediating oxidative stress responses will be discussed in more detail in the following chapters.

1.3.5.1 Salicylic acid (SA)

SA is derived from the phenylpropanoid biosynthetic pathway (Figure 5) (Ward et al., 1991). It plays a central role in defence against pathogen invasion, especially when resistance is achieved via HR. In the early phase of pathogen invasion, $\cdot O_2^-$ and H_2O_2 are produced locally, and at the same time local induction of defence related genes takes place (Draper, 1997). Some of these genes encode enzymes of the phenylpropanoid biosynthetic pathway, like *Pal*, which catalyses production of SA precursors (Mauch-Mani and Slusarenko, 1996; Draper, 1997; Smith-Becker et al., 1998). Also the enzyme responsible for SA synthesis from benzoic acid has been shown to be induced by H_2O_2 (León et al., 1995). Draper (1997) has proposed a model where initial pathogen infection would lead to rapid and short lived production of H_2O_2. This H_2O_2 would activate SA synthesis, leading to a potentiating phase where SA would in turn induce secondary prolonged H_2O_2 production at the same time with actual pathogen recognition. Like in the first phase, H_2O_2 would again induce SA synthesis, that would now lead to HR, cell death and resistance as well as to defence gene induction (also induced by H_2O_2 from the second phase) and SAR (Draper, 1997). SA enhances ROS accumulation and cell death in numerous plant species (Kauss and Jeblick, 1995; León et al., 1995; Shirasu et al., 1997; Kawano et al., 1998; Hückelhoven et al., 1999; Asai et al., 2000) and it seems that SA potentiates the HR signalling pathway (Alvarez, 2000).

O_3 induced HR- like symptoms and antioxidant defence responses were greatly diminished in *NahG* plants (*Arabidopsis* plants expressing bacterial salicylate hydroxylase, therefore deficient in SA) in comparison to WT, suggesting that SA could potentiate responses conferring to plant's O_3- sensitivity (Rao and Davis, 1999). Potentiation was also important in hybrid poplar where O_3- sensitive clone seemed to be insensitive to SA and therefore unable to produce a full defence response (Koch et al., 2000).

SA also induces binding activity of a stress-inducible transcription factor that binds to a region found in more than 30 different stress responsive genes (Goldsbrough et al., 1993). WRKY DNA-binding proteins (recognize elicitor responsive elements in parsley [*Petroselinum crispum*] *PR1* promoter, and contain WRKYGQC - amino acid consensus sequence in their N- termini) may also regulate gene expression during pathogen and SA induced defence responses (Yang et al., 1999), with numerous other proteins binding to SA- responsive elements (Hennig et al., 1993; Guevara-García et al., 1998; Zhou et al., 2000).

1.3.5.2 Ethylene

Ethylene is a gaseous plant hormone and its concentration increases shortly after exposure to various stresses. In O_3 stress the amount of this stress ethylene correlates with the visible injury formation (Langebartels et al., 1991, 2000; Telewski, 1992; Wellburn and Wellburn, 1996). It has been suggested that a plant's capability to produce stress ethylene would be the key determinant of its O_3 sensitivity (Kangasjärvi et al., 1994). In an O_3 sensitive *Arabidopsis* mutant *rcd1*, O_3 induced $^{\cdot}O_2^-$ production is ethylene dependent. Ethylene therefore promotes the ROS triggered lesion formation in O_3 treated *Arabidopsis* (Overmyer et al., 2000). Ethylene may also modulate elicitor induced cell death (Asai et al., 2000).

Ethylene can regulate gene expression through various ethylene responsive elements. One of these, the GCC -box may mediate either negative or positive stress responses (Fujimoto et al., 2000). Another ethylene responsive *cis*-element, ERELEE4 (ethylene responsive element in *Lycopersicon esculentum* ethylene responsive gene E4), has been shown to regulate senescence related gene expression (Itzhaki et al., 1994).

1.4 Programmed cell death (PCD)

HR cell death in plants has been shown to be genetically programmed resembling the apoptosis in mammals (Sasabe et al., 2000). The function of mammalian apoptosis is to selectively eliminate certain cells during development. This is done in order to maintain developmental balance and as a response to stress. Cells that are no longer needed or are damaged, will be self-destructed (Gilchrist, 1998). In plants, all the criteria defining mammalian apoptosis can never be met due to structural differences between animal and plant cells, and therefore it is here referred to as programmed cell death (PCD).

Apoptosis needs to be separated from necrotic cell death that occurs when cells are exposed to poisons, severe cold or heat, or traumatic injury leading to membrane and organellar damage. In contrast, apoptosis requires active cellular participation (Gilchrist, 1998). Animal cells undergoing apoptosis exhibit cell shrinkage, loss of contact to other cells, fragmentation of nuclear DNA (Gilchrist, 1998), activation of Ca^{2+} dependent endonuclease, nuclear deformations, formation of numerous micronuclei, and plasma membrane blebbing (Falcieri et al., 1994). Later, cells form apoptotic bodies that protect the adjacent cells from possibly toxic materials that could otherwise leak out, and are taken up by neighbouring cells and degraded within minutes to hours (Gilchrist, 1998).

The sequence of events leading to the onset of apoptosis in mammalians includes numerous steps. Cytochrome c release from mitochondria is activated through a voltage dependent anion channel, and when dATP is present, released cytochrome c can activate caspase family proteins. Activation of initiator caspases triggers a protease cascade that amplifies and executes the cell death signal. Loss of cytochrome c from the mitochondria may lead to ROS formation, which again might activate caspases and subsequently, apoptosis (Lam et al., 1999). Caspases are a family

of cysteine-dependent, aspartate-directed proteases which have crucial roles in the initiation and execution of mammalian apoptosis (Earnshaw et al., 1999).

In plant development PCD takes place at least during death of petals after fertilization, diploid parthenogenesis, development of tracheary elements, leaf senescence and in barley aleurone layer during germination (Gilchrist, 1998). PCD is also a central part of life cycle of perennial plants. Creeping perennials, such as clover, proliferate at apices and push out to new areas, while older parts of the plant age and die. A similar phenomenon takes place in trees, where dead material does not decompose, but remains in the wood (Thomas et al., 2000). Related to senescence, premature cell death was induced in transgenic tobacco plants with altered phenylpropanoid pathway regulation, suggesting a role for phenylpropanoid compounds in the regulation of plant PCD (Tamagnone et al., 1998).

Some of the features of mammalian apoptosis can be detected in plants also: DNA fragmentation can be induced by pathogens (Mittler et al., 1997; Gilchrist, 1998; Asai et al., 2000; Koch et al., 2000; Sasabe et al., 2000), by increasing intracellular [Ca^{2+}], H_2O_2, SA (O'Brien et al., 1998), and O_3 (Koch et al., 2000), during germination (Gilchrist, 1998), during senescence (O'Brien et al., 1998), and in root caps (Gilchrist, 1998). Other cellular features of plant PCD include vacuolisation of the cells (Mittler et al., 1997; Fath et al., 2000), loss of plasma membrane integrity (Fath et al., 2000) and increase in monomeric chloroplast DNA (Mittler et al., 1997). HR, which is a form of PCD in plants, differs from the developmentally triggered PCD by associated induced defence responses. Reactions involved in HR include ion fluxes, generation of ROS, protein synthesis, intact actin cytoskeleton, SA, protein kinases and an oxidative burst (Heath, M.C., 2000; Sasabe et al., 2000).

It has been suggested that the mitochondrial alternative oxidase (*Aox*) could be a plant specific cell death regulator by controlling the mitochondrial ROS production through the alternative respiration pathway (Lam and del Pozo, 2000). ROS are probable regulators of PCD in many plant systems (Jabs et al., 1996; Alvarez et al., 1998; Desikan et al., 1998; Mittler et al., 1998, 1999; Hückelhoven et al., 1999; Rao et al., 2000) and they affect plant gene expression (Levine et al., 1994; Desikan et al., 1998; Mittler et al., 1998).

1.5 Gene expression

1.5.1 Stress-induced genes

Distinct sets of genes are induced upon various stresses or other stimuli in plants. The most studied groups of genes related to stress responses include genes encoding PR-proteins (Warner et al., 1992), phenylpropanoid pathway enzymes (Kangasjärvi et al., 1994), ethylene biosynthetic enzymes, antioxidant enzymes, lipid metabolism enzymes (Kangasjärvi et al., 1994) and genes involved in the regulation of mitochondrial responses, such as *Aox* (Murphy et al., 1999). Three genes, phenylalanine ammonia lyase (*Pal*), chalcone synthase (*Chs*) and pathogenesis related protein

10 (*Ypr10*), often studied in relation to plant stress responses, and one gene that was first isolated in plants as an O_3- inducible gene, the mitochondrial phosphate translocator 1 (*Mpt1*), will be described in the following chapters. *Pal*, *Chs* and *Ypr10* are good markers for stress and ROS related gene expression and *Mpt1* opens up a new line in the study of the oxidative stress related processes in plants.

1.5.1.1 Phenylalanine ammonia-lyase (Pal)

Due to phenylpropanoid compounds' vast array of defence related functions in plants, PAL enzyme activity and gene -expression in stress situations has been intensively studied. PAL activity confers to the production of various phenylpropanoid compounds for plant protection, but it's main function in defence is to produce precursors for SA, signal molecule derived from a branch of the phenylpropanoid pathway (Figure 5), synthesis (Mauch-Mani and Slusarenko, 1996).

PAL level is increased either at the enzyme activity or mRNA level by numerous environmental cues presented in Table 4.

Table 4. PAL induction at the enzyme activity or mRNA level upon various stimuli. TMV = tobacco mosaic virus, SA= salicylic acid, JA= jasmonic acid.

External stimulus	Reference(s)
O_3	Rosemann et al., 1991; Eckey-Kaltenbach et al., 1994; Sharma and Davis, 1994; Sharma et al., 1996; Pääkkönen et al., 1998; Riehl Koch et al., 1998
Pathogen	Cui et al., 1996; Mur et al., 1996; Rajasekhar et al., 1999; Blilou et al., 2000
Elicitor (fungal or bacterial)	Edwards et al., 1985; Liang et al., 1989; Lois et al., 1989; Gowri et al., 1991; Kamada and Muto, 1994a, b; Marinelli et al., 1994; Pellegrini et al., 1994; Baillieul et al., 1995; Desikan et al., 1998; Sasabe et al., 2000
TMV	Pellegrini et al., 1994
Wounding	Liang et al., 1989; Lois et al., 1989; Pellegrini et al., 1994; Mur et al., 1996
UV-B irradiation	Kuhn et al., 1984; Lois et al., 1989; Kalbin et al., 1997; Logemann et al., 2000
Light	Liang et al., 1989; Asai et al., 2000
Ethylene	Ecker and Davis, 1987
JA	Gundlach et al., 1992
Reduced glutathione	Wingate et al., 1988
SA	Blilou et al., 2000

Role of SA in PAL induction is considered to be the potentiation of the response induced by some other cue (Mur et al., 1996; Shirasu et al., 1997). The role of ROS in mediating *Pal* expression has been studied in various plant systems with differing

results. H_2O_2 seems not to be involved in *Pal* induction in soybean (Levine et al., 1994) or tobacco suspension cell lines (Dorey et al., 1999; Sasabe et al., 2000), although it is clearly demonstrated that H_2O_2 alone is able to increasce *Pal* as well as *Gst* mRNA level in *Arabidopsis* suspension cell cultures (Desikan et al., 1998).

1.5.1.2 Chalcone synthase (Chs)

Chs expression is remarkably sensitive to UV- and blue light (Strid et al., 1994; Kalbin et al., 1997; Logemann et al., 2000; Loyall et al., 2000). Increase in the fluence rate of white light increases *Chs* mRNA level in *Arabidopsis*, as does blue light also. In some cases red light may also affect *Chs* transcript level (Jackson and Jenkins, 1995; Frohnmeyer et al., 1998).

In addition to UV-B, *Chs* up -regulation occurs as a response to O_3 (Rosemann et al., 1991), fungal elicitor (Loake et al., 1991; Kato et al., 1995), pathogens (Cui et al., 1996) and wounding (Creelman et al., 1992). Of the known signaling molecules, *Chs* is induced by methyl jasmonate (MJ) (Creelman et al., 1992), and by the reduced form of glutathione (Wingate et al., 1988; Loyall et al., 2000). Glutathione and the oxidative status of the cells are possibly involved in the transcriptional regulation of UV-B induced *Chs* expression (Loyall et al., 2000).

1.5.1.3 Pathogenesis related protein 10 (Ypr10)

PR-proteins are defined as proteins that are induced by pathogen invasion or by resembling situations. *PR* genes that have conserved sequences, but are not functionally designated, are called *Ypr*-genes (van Loon et al., 1994). PR10 (pathogenesis related protein 10) class proteins are classified as parsley PR1 -like proteins (van Loon et al., 1994). Genes encoding PR10 class proteins have been isolated so far in several plant species shown in Table 5.

Table 5. *PR10* class genes.

Plant species	Gene(s)	Reference
Potato	*Sth2, Sth21*	Matton et al., 1990
Asparagus (*Asparagus officinalis*)	*Aopr1*	Warner et al., 1992
Birch	*BetV1-Sc1, BetV1-Sc3, Ypr10*a, Ypr10*b*	Breiteneder et al., 1989
Pea	*I49*	Marrs, 1996
Bean (*Phaseolus vulgaris*)	*PR1, PR2*	Walter et al., 1990
Parsley	*PR1, PR2*	Rushton et al., 1996
Alfalfa (*Medicago sativa*)		Breda et al., 1996

PR10 class genes have been found neither in *Arabidopsis* nor tobacco. In most species *PR10* genes are encoded by multi- gene families (Meier et al., 1991; Després et al., 1995; Rushton et al., 1996; Truesdell and Dickman, 1997; Wang, C.-S. et al., 1999), whose members are often clustered (Crowell et al., 1992). Birch *Ypr10* genes encode proteins of the PR10 class that share structural but not likely functional similarity to the major birch allergen genes (Swoboda et al., 1995). Like *Pal* and *Chs*, *Ypr10* also responds to a wide array of environmental and developmental stimuli as shown in Table 6.

Table 6. Changes in *Ypr10* enzyme or mRNA levels upon external stimuli. SA= salicylic acid, ABA= abscisic acid, JA= jasmonic acid.

Stimulus	Reference(s)
Senescence	Crowell et al., 1992; Valjakka et al., 1999
Copper (Cu^{2+})	Utriainen et al., 1998
O_3	Pääkkönen et al., 1998
Pathogen	Crowell et al., 1992; Warner et al., 1992, 1993; Constabel and Brisson, 1995; Després et al., 1995; Midoh and Iwata, 1996; Truesdell and Dickman, 1997
H_2O_2	Crowell et al., 1992; Bi et al., 1995
JA	Moons et al., 1997
ABA	Moons et al., 1997; Wang, C.-S. et al., 1999
Salt stress	Moons et al., 1997
Methyl viologen	Crowell et al., 1992
SA	Crowell et al., 1992

Different *Ypr10* genes seem to respond to wounding differently, some are clearly up regulated (Crowell et al., 1992; Warner et al., 1992, 1993; Constabel and Brisson, 1995; Després et al., 1995; Truesdell and Dickman, 1997), while others are not affected (Midoh and Iwata, 1996). In birch *Ypr10* genes, differences are found even between different family members (Poupard et al., 1998).

1.5.1.4 Mitochondrial phosphate translocator (Mpt1)

The first plant mitochondrial phosphate translocator 1 (*Mpt1*) was isolated in birch as an O_3-inducible transcript by DDRT-PCR (Kiiskinen et al., 1997). *Mpt1* is a transmembrane protein on the inner mitochondrial membrane and it transports inorganic phosphate (P_i) into the mitochondrial matrix and carries most of the P_i required for ATP synthesis (Capobianco et al., 1991; Palmieri et al., 1993; Stappen and Kramer, 1994). Since ADP is phosphorylated to ATP by oxidative phosphorylation in mitochondria, availability of P_i within mitochondria is essential for the energy metabolism of the cell (Wohlrab, 1986). A single copy gene encodes *Mpt1* in birch. It shares considerable homology with phosphate carriers (P_ic) isolated from mammalians and yeast (Kiiskinen et al., 1997).

So far *Mpt1* homologs have been isolated from soybean cDNA libraries made of drought stressed plants [BG043543], apical shoots [AW099962], roots [AW102173]

and immature seed coats [AW186456]. It has also been found from potato tuber [BF153103], moss (*Physcomitrella patens*) [AW476902], and six day cotton (*Gossypium hirsutum*) fiber cDNA library [AI726701], as well as in the *Arabidopsis* genome project [AB007650].

1.5.2 Transcriptional regulation of stress related genes

In eukaryotes transcription is usually regulated by combinatorial control, meaning that several different proteins must bind DNA in a coordinated manner to achieve appropriate expression of the gene in question. Transcriptional regulation therefore consists of transcription factor(s) (*trans*- acting factor) and the corresponding binding site in the DNA (*cis*-element) (Wolberger, 1999). Not much is known about the transcriptional regulation of stress related plant genes. Some regulatory elements and trans- acting factors are, however, known and may be of importance in future work considering regulation of plant gene expression.

A class of transcription factors called WRKY have been isolated at least in tobacco (Yang et al., 1999) and parsley (Eulgem et al., 1999). They are induced by tobacco mosaic virus (TMV), SA (Yang et al., 1999), fungal elicitors (Eulgem et al., 1999), and wounding (Hara et al., 2000) via phosphorylation dependent activation mechanism (Yang et al., 1999). Binding sites for WRKY are found for example in the promoters of genes encoding tobacco basic chitinase (Yang et al., 1999) and parsley *PR1* (Eulgem et al., 1999). Organization of WRKY binding sites within promoter confers to efficient activity (Eulgem et al., 1999). In a massive gene -expression study on *Arabidopsis* SAR responses, 20 stress responsive genes clustered with *PR1*. Common for the promoters of the genes in this cluster was the existence of at least three WRKY binding sites on each of them (Maleck et al., 2000). Similarly, in an *Arabidopsis* mutant exhibiting constitutive SAR, mRNA levels of 16 cDNAs were elevated and their promoters carried WRKY binding sites (Petersen et al., 2000).

Numerous eucaryotic transcription factors respond to cellular redox state. These factors include AP-1 and 2, NF-κB, MTF-1 (metal response element binding transcription factor 1), and MAF (an oncogene family). Also *cis* -elements responsive to ROS, such as the antioxidant responsive element (ARE), have been described (Dalton et al., 1999; Delaunay et al., 2000). However, regulation of gene expression via these regulators does not only occur through ROS, for they respond to, or are fine tuned by, other cellular signals as well (Dalton et al., 1999). ARE was first described in the promoter of the rat *Gst* gene. ARE is responsive to H_2O_2 and phenolic antioxidants undergoing redox cycling. ARE may therefore be part of the signal transduction pathway leading to oxidative stress responses in eucaryotic cells (Rushmore et al., 1991). ARE motif was found from the promoters of three maize *Cat* genes, and it seems to be involved in the expression of *Cat1* during senescence related oxidative stress (Polidoros and Scandalios, 1999).

Mammalian metallothionein genes are regulated at the transcriptional level as a response to heavy metals through metal responsive elements (MREs) that occur in

the promoter as multiple copies (Koizumi et al., 1999). In mice with knocked out *CuZnSOD*, metallothionein expression was markedly induced, and the transcriptional activation occurred through MREs (Ghoshal et al., 1999), therefore the gene responded to the changed cellular redox balance.

More than 100 plant transcripts are regulated by light of different qualities. Numerous light responsive *cis*- elements are found in the promoters of many genes. Still, the assembly of elements varies greatly: Neither can any single element be found in all light regulated promoters nor do any single element confer to light induced expression all by itself (Terzaghi and Cashmore, 1995). Several light and UV-B responsive elements have been described in *Chs* promoters (Batschauer et al., 1996; Schäfer et al., 1997).

Ethylene responsive element binding factors (ERFs) are specific to plants. ERFs bind DNA motifs known as the GCC-box. Different ERF family proteins can either up- or down- regulate transcription by binding to the same GCC-box, and their responsive genes are differentially regulated by ethylene and abiotic stress conditions (Fujimoto et al., 2000). ERELEE4 binding site is also responsive to ethylene, but has been described to be related to the senescence process (Itzhaki et al., 1994). There are also numerous other stress, or signalling molecule responsive, regions in the promoters of different stress related genes (Dron et al., 1988; Goldsbrough et al., 1993; Rouster et al., 1997; Chen and Singh, 1999).

No clear, single stress related factors affecting plant transcription are known, although some of the primary defence responses are shared between stresses. It appears that the stress induced gene expression in plants is regulated through a complex network of transcription factors and their combinations binding to diverse mixture of stress related *cis* -elements in the promoter sequences. Nevertheless, some regulatory elements are recognised as responsible for certain responses, and the picture is getting clearer with the results of various EST and genome projects as well as with the transgenic approaches with deleted promoters.

2 Aims of the present study

The aim of this study was to reveal molecular stress responses occurring in the Silver birch, which is a common and commercially important deciduous tree species, covering large and ecologically diverse areas across the northern hemisphere. To reveal common factors underlying stress induced cell death, a model plant (Cat1AS tobacco) was used. The studies I-V were carried out to

1. Find possible common factors between biotic and abiotic stresses.
2. Reveal a possible stress-induced oxidative burst and its origin.
3. Study the relationships between the oxidative burst and stress induced gene -expression and cell death.

3 Materials and methods

3.1 Plant material

In birch (*Betula pendula*) experiments, either seed derived (II, III and V) or tissue culture derived material (I, additional UV-B irradiation data, clone 36) was used. In the tobacco study, transgenic lines carrying antisense *Cat1* gene (Cat1AS) and wild type SR1 *Nicotiana tabacum* plants (L.) were used (IV).

3.2 Growth conditions

Birch saplings were grown either in controlled climate chambers (I, II, III) or in greenhouse (III, V) under conditions simulating the weather in Finland in June (light: dark [22:2] photoperiod with daytime temperature 19°C and night temperature 12°C and relative humidity 55: 80% [day: night]). Tobacco plants were grown in low light conditions (80 µmol m^{-2} s^{-1}, 400-700 nm) with 14 h light/10 h dark at 25°C and 70% relative humidity. Mature pre-flowering plants were used.

3.3 Stress treatments

O_3 and wounding treatments were performed in computer controlled climate chambers (I, II, III). UV-B irradiations were either made in greenhouse (V) or controlled climate chambers (*Chs*, *Pal* and *Ypr10* expression studies in Figure 10), where birch saplings were exposed to 0 kJ m^{-2} (control) or 4 kJ m^{-2} of 300 nm (Caldwell) UV-B radiation for five hours. Philips TL 40/12 lamps covered with acetate filter to remove UV-C were used as the source of UV-B radiation. UV-B level was measured at plant level with Macam SR9010-PC spectroradiometer. Light level was 270-400 µMol m^{-2} sec^{-1}. Pathogen and *in planta* H_2O_2 production (glucose/glucose oxidase [G/GO]) treatments were performed in greenhouse (III). Tobacco plants were subjected to high light (HL)(1000 µmol m^{-2} s^{-1}) in a Fitotron chamber (IV).

3.4 Histology

Cell viability was studied in O_3 exposed birch by Evans Blue staining (I) and HL treated tobacco by Trypan Blue staining (IV). Light microscopy samples were collected from UV-B treated birch (V) and HL treated tobacco (IV).

Transmission electron microscopy (TEM) was subjected to samples from UV-B and O_3 treated birch (including $CeCl_3$ staining for subcellular H_2O_2 localisation)(II) and HL treated tobacco (IV).

H_2O_2 localisation with 3'3- diaminobenzidine (DAB) at the leaf level was made from O_3, pathogen, wound and G/GO treated birch (III) and HL treated tobacco (IV).

3.5 Enzyme activities

Enzyme activities of peroxidase (POX), GR and SOD were measured spectrofotometrically from O_3 treated birch leaves (I).

3.6 Polyamine concentrations

Concentrations of putrescine, spermidine and spermine were measured from O_3 exposed birch leaves by HPLC (High Performance Liquid Chromatogarphy) (I).

3.7 Isolation of genes

For obtaining gene specific probes or genomic clones, PCR with degenerate or specific primers (I, III) and cDNA and genomic library screens were performed (III).

3.8 Northern analysis

RNA was extracted and subjected to northern analysis with different probes. Treatments that were studied by northern method include O_3 (I, III), UV-B (V), pathogen (III), wounding (III), G/GO (III) and HL (IV). *In situ* hybridisations were made from UV-B treated birch leaves (V).

3.9 Lipid analysis

Membrane lipids and lipid peroxidation products were analysed from UV-B treated birch by gas chromatography (V).

3.10 Secondary metabolite analysis

Secondary metabolites were extracted and analysed by HPLC from UV-B exposed birch (V).

3.11 Inhibitor treatments

To unravel the enzyme complexes responsible for H_2O_2 production, effect of various inhibitors on the subcellular H_2O_2 accumulation in O_3 treated birch (II) and on the H_2O_2 accumulation in general in HL treated tobacco (IV) were studied. Also the involvement of various signalling intermediates in HL treated tobacco, were studied with the help of inhibitory chemicals (IV).

3.12 Sequence analysis

Sequence analysis was performed with GCG (Genetics Computer Group) software (I, III), and promoter elements were scanned with the plant *cis* -acting element database (III).

3.13 DNA fragmentation

DNA fragmentation was analysed from HL treated tobacco protoplasts by flow cytometry (IV).

3.14 Western analysis

Proteins were extracted from HL treated tobacco and subjected to western analysis with different antibodies (IV).

4 Results and discussion

4.1 Abiotic and biotic stresses induce H_2O_2 accumulation

As shown in various studies on plant stress physiology, accumulation of ROS as one of the earliest events is common to both biotic and abiotic stress –induced defence responses (Lamb and Dixon, 1997; Bolwell, 1999). In most cases ROS detected are either H_2O_2 or $\cdot O_2^-$ (Jabs et al., 1996; Schraudner et al., 1998). Both in birch and tobacco H_2O_2 was detected after stress treatments. Increase in the amount of H_2O_2 after stress treatments results either from increased H_2O_2 production or decreased detoxification or both. With the methods used in this study, it is impossible to distinguish which events are responsible for the increased amount of H_2O_2. Thus, the term "H_2O_2 accumulation" is used to describe the changes observed in the quantity of H_2O_2 in the experimental material.

4.1.1 H_2O_2 localisation

In birch $\cdot O_2^-$ accumulation was not detected after O_3 or pathogen treatments (data not shown), presumably due to its rapid dismutation to slightly less harmful form of ROS, H_2O_2. H_2O_2 accumulation was indeed detected in birch leaves after O_3, pathogen, wounding and G/GO treatment (III). Timing of the H_2O_2 accumulation coincided with lesion development, and H_2O_2 accumulation was most prominent surrounding the lesions, either in a halo- or spot-like manner (III). The O_3 induced H_2O_2 accumulation was also studied in more detail and at the subcellular level this H_2O_2 was detected first at the cell walls of palisade parenchyma cells (2h), and later both in the cell walls and on the plasma membranes at the same location (10h) (II).

In HL treated Cat1AS tobacco, H_2O_2 was detected in cells along leaf veins in patches (IV). Wild type SR1 tobacco cells also accumulated H_2O_2 as a response to HL, but the accumulation was much weaker and uniform than in the transgenic line (IV). H_2O_2 accumulation therefore appears to be a general stress response occurring upon various stresses both in birch and in tobacco.

4.1.2 Origin of the H_2O_2

The origin of the H_2O_2 burst in stressed birch and tobacco was studied with inhibitor treatments (II, IV). Diphenylene iodine (DPI), an inhibitor of flavin containing oxidases, was used to block the activity of the plasma membrane NADPH oxidase (II, IV). DPI can inhibit also other oxidases, but here only its effect on the apoplastic H_2O_2 production was studied, and low enough concentrations were used to avoid inhibition of peroxidases (Bolwell et al., 1998). Both in birch (II) and tobacco (IV), DPI was sufficient to partly block either the O_3 or HL induced H_2O_2 accumulation, clearly indicating a role for a plasma membrane oxidase in the initial oxidative burst upon

stress. The NADPH oxidase itself produces $\cdot O_2^-$ from molecular O_2, but according to a proposed model, the plant plasma membrane NADPH oxidase would act in tight connection with the EC-SOD (Ogawa et al., 1997). This way, the activity of NADPH oxidase would actually lead to accumulation of H_2O_2, when the superoxide produced would be instantly dismutated (Ogawa et al., 1997).

In birch, the O_3 induced H_2O_2 accumulation was partly inhibited by peroxidase inhibitors KCN and NaN_3 (II). This suggests a role also for cell wall peroxidases in the generation of O_3 induced oxidative burst. The existence of two H_2O_2 generating activities in birch apoplastic stress response was further substantiated by the two separate subcellular locations for the H_2O_2 accumulation, the plasma membrane and the cell wall (II). In other plant systems, either NADPH oxidase, or cell wall peroxidases seem to be the main sources of H_2O_2 during the oxidative burst (Bestwick et al., 1997; Bolwell et al., 1998).

4.1.3 Intracellular H_2O_2 accumulation

At 24 hours H_2O_2 accumulation in the O_3 -treated birch palisade parenchyma cells continued similarly as at two and 10 hours (II). In the mesophyll layer however, H_2O_2 was detected intracellularly in contrast to the earlier apoplastic accumulation (II). H_2O_2 was detected in the cytoplasm, peroxisomes, and mitochondria of the mesophyll cells, but not in the chloroplasts (II). Since chloroplasts have a central role in plant ROS and stress metabolism, two control experiments were carried out to reveal possible technical problems in localising chloroplastic H_2O_2. Also control samples with $CeCl_3$ but without standard EM- staining procedures were performed to ensure that the staining was distinguishable from possible EM- staining artefacts (data not shown).

First birch leaf segments were subjected to methyl viologen, that causes massive ROS production in the chloroplasts under HL conditions, and as a result H_2O_2 accumulated in the chloroplasts (II). Secondly, chloroplastic H_2O_2 accumulation was studied in O_3- treated birch clone (V5834), classified as O_3 -sensitive according to leaf damage. As shown in Figure 6B and D H_2O_2 accumulated in the chloroplasts of palisade parenchyma cells of these O_3 -sensitive plants at 10h as a response to O_3- treatment.

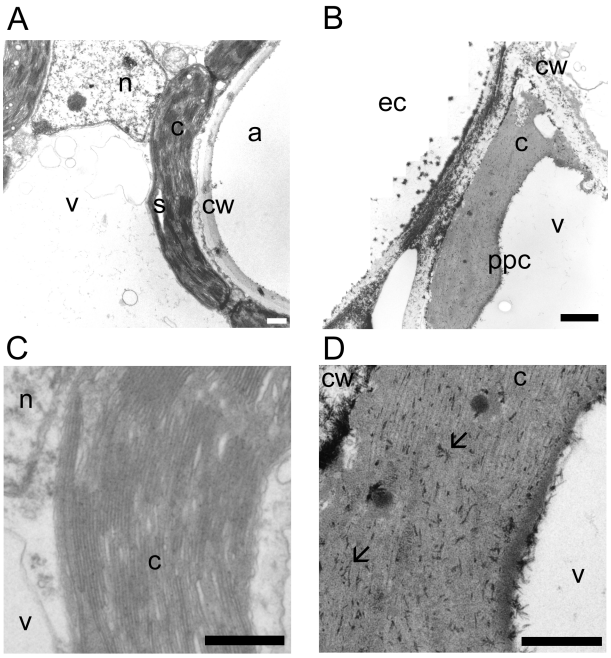

Figure 6. Chloroplasts of birch clone V5834 at 0h (A,C) and 10 h (B,D) after the beginning of an eight- hour O_3- exposure (C and D are close- ups of A and B, respectively). At 10 hours H_2O_2 (visualised with $CeCl_3$) can be seen as black precipitates in the cell walls of an epidermal cell (ec) and a palisade parenchyma cell (ppc) as well as inside the epidermal cell as spots (B). Chloroplastic H_2O_2 can be seen as small, scattered precipitates everywhere in the chloroplast of an O_3 treated cell (D, arrows). Bars 500 nm. a=apoplast, c=chloroplast, cw= cell wall, ec=epidermal cell, n=nucleus, ppc= palisade parenchyma cell, s=starch grain, v=vacuole.

It therefore seems that chloroplasts are highly tolerant to oxidative stress due to extremely efficient scavenging mechanisms in this organelle, which is exposed to radicals continuously during normal metabolism (Asada, 1999). Thus chloroplastic ROS accumulation as a response to stress may at least partly define plant's susceptibility to oxidative stress. Also the result of methyl viologen treatment leading to H_2O_2 accumulation in birch chloroplasts, as well as the appearance of H_2O_2 in the chloroplasts of O_3- sensitive birch clone suggest, that when a drastic enough oxidative stress is subjected to chloroplasts, even its efficient scavenging ability can be exceeded.

Still, the cytoplasmic scavenging mechanisms seem to be more important under high O_3 peaks than the chloroplastic ones (Tepperman and Dunsmuir, 1990; Pitcher and Zilinskas, 1996; Torsethaugen et al., 1997). Also the peroxisome, and CAT therein, may play an important role in detoxification of H_2O_2 produced in the other cellular compartments, especially the cytoplasm, since this organelle may act as a sink for H_2O_2 detoxification (Willekens et al., 1997).

4.1.4 Markers of ROS accumulation

Increase in the activities, or in the protein or mRNA levels of antioxidant enzymes and their respective genes, can also be considered as markers for the appearance of ROS in the plant tissue. Enzyme activities of SOD, peroxidase, and GR increased in O_3-treated birch leaves, and this rise was in all cases more prominent in the O_3-sensitive birch clone (I). Similarly, the mRNA levels of the genes encoding antioxidant enzymes *Apx, CuZnSod, Gst* and *Cat* were increased upon O_3-treatment in birch (III). Induction in the enzyme activities and increase in the mRNA levels of ROS detoxifying enzymes suggests that ROS accumulate in the O_3-treated tissue in concentrations that need to be scavenged actively. Also the level of free putrescine, which can act as an antioxidant (Bors et al., 1989; Evans and Malmberg, 1989; Rowland-Bamford et al., 1989), increased in the O_3-sensitive birch clone (I). Higher activities of antioxidant enzymes and polyamine levels in the O_3-sensitive birch clone are likely to reflect higher ROS concentrations, rather than more efficient scavenging efficiency, since more severe damage was detected in these plants.

The artificial H_2O_2 generating system, G/GO, increased the transcript levels of genes encoding ROS detoxifying enzymes (III), further justifying their use as markers for ROS accumulation in plant tissue. Likewise, in the HL treated Cat1AS tobacco plants, protein levels of APX and GPX increased rapidly after the onset of HL treatment, in addition to the respective mRNA levels (IV). Timing of the increase in enzyme activities (I, IV), followed that of the mRNA accumulation (III, IV), suggesting that the increased enzyme activities are probably not meant for detoxification of the primary oxidative burst on the plasma membranes and cell walls, but more likely they are directed to scavenge the later accumulating intracellular ROS occurring at the same time with the appearance of visible injury (II, III). This is consistent with the suggestion of Foyer et al (1994), that increases in ROS detoxifying enzyme activities is not related to protecting the tissue under stress from injury, but is rather a response to the damage. Also cell wall thickness and alkalinization confer to apoplastic ROS detoxification (Moldau, 1998).

4.2 Stress-induced H_2O_2 accumulation and cell death are closely linked

$\cdot O_2^-$ has a regulatory role in the stress induced cell death in *Arabidopsis* (Jabs et al., 1996; Overmyer et al., 2000), and H_2O_2 similarly in tobacco (Schraudner et al., 1998) and *Arabidopsis* (Alvarez et al., 1998). In mammalian apoptosis, ROS are claimed to be one of the main initiators of the process (Gilchrist, 1998). Especially, ROS produced in the mitochondria in the early phase contribute to the ongoing of other mitochondrial responses leading to apoptosis (Green and Reed, 1998).

4.2.1 Tissue damage

O_3 induced tissue damage was detected in birch as brown necrotic lesions earliest at 10 h or latest at 48 h (I, III). Membrane damage in the leaves could also be de-

tected by vital staining the leaves with Evans Blue. This experiment showed that the permeability of the O_3 treated cells increased prior to cell death, therefore indicating membrane damage possibly leading to leakage of cellular constituents and subsequent cell death (I). Membrane damage was detected both in the sensitive and tolerant birch clones in a similar extent, but only in the sensitive clone clusters of dead cells were detected later, combined with large visible lesions (I). In birch, O_3 injury and H_2O_2 accumulation correlated spatially (III). H_2O_2 accumulated at the edges of damaged tissue, as well as in tissues surrounding it (III).

Similarly as O_3, other stresses have an ability to induce H_2O_2 accumulation at the sites of injury. In birch H_2O_2 accumulated at the sites subjected to wounding or pathogen infiltration (III). Pathogens were applied to birch leaves by injecting the bacterial suspension with a syringe without a needle. As in the O_3 treated tissue, in the pathogen treated leaves H_2O_2 accumulated at the edges of injection sites, where the damage formed, and also in tissue surrounding the lesions (III).

In subcellular H_2O_2 localisation studies of O_3-treated birch (II), H_2O_2 was first detected in the cell walls and on the plasma membranes of palisade parenchyma cells. Later similar accumulation was seen in the cell walls and plasma membranes between palisade parenchyma cells and epidermal cells (II, Figure 7A). Subsequent cell death at 24h occurred mainly in the palisade parenchyma and epidermal cells (I, II, III).

Similarly in barley leaves infected with fungi, H_2O_2 accumulation began in the palisade parenchyma cells underlying the attacked epidermal cell (Thordal-Christensen et al., 1997; Vanacker et al., 2000). H_2O_2 accumulation was especially prominent in the cell walls and plasma membranes adjacent to the attacked epidermal cell (Figure 7B). As in the case of O_3, H_2O_2 accumulation was later detected in the epidermal cells (Figure 7B). Later, cell death took place in the cells accumulating H_2O_2 (Thordal-Christensen et al., 1997; Vanacker et al., 2000).

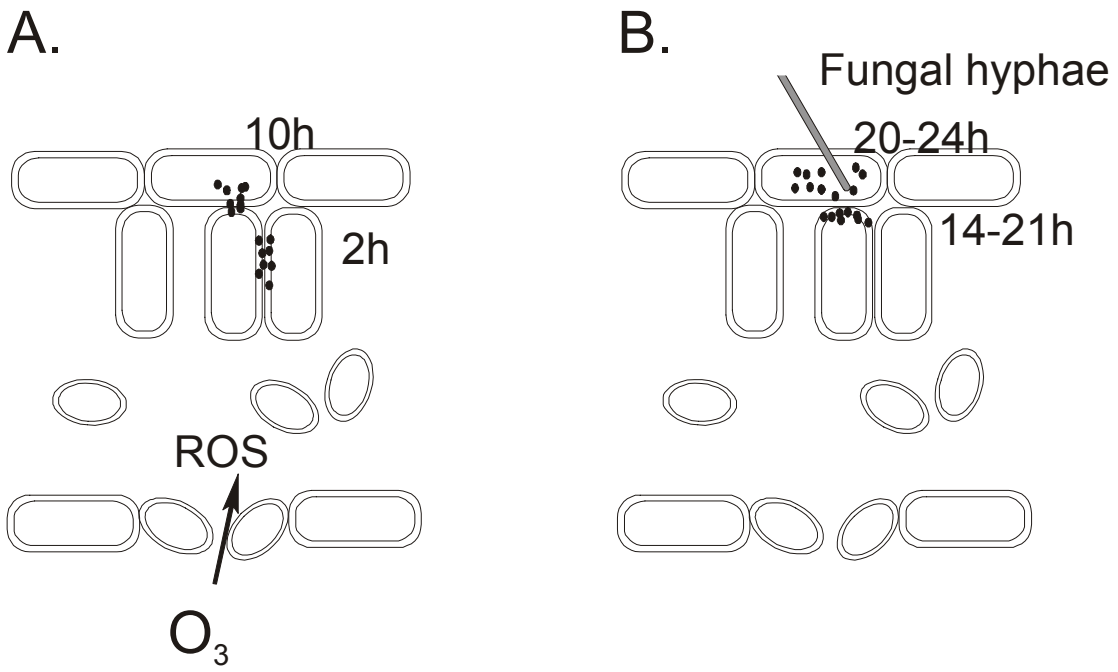

Figure 7. Model describing the advancement of O_3- (A) and fungal penetration (B) induced H_2O_2 accumulation in plant tissue. In both cases H_2O_2 first accumulates in the mesophyll cells and later spreads to the adjacent epidermal cells. Subsequent cell death takes place at the sites of H_2O_2 accumulation (II) (Thordal-Christensen et al., 1997; Vanacker et al., 2000). Black spots represent H_2O_2.

Similarities in the H_2O_2 accumulation pattern and subsequent cell death in O_3-treated birch (II) and barley infected with a fungus (Thordal-Christensen et al., 1997; Vanacker et al., 2000) are remarkable (Figure 7). In both cases H_2O_2 accumulation was first detected in the mesophyll cell walls, from where it spread into the adjacent epidermal cells. Later, cells accumulating H_2O_2 die in both plant systems. It is of utmost interest that two different plant systems and stress situations behave in such a coordinated manner during the events leading to cell death. It suggests that the onset of stress induced cell death in plants is a controlled physiological response, and can be activated by ROS.

4.2.2 Role of mitochondria in the process of cell death

Subcellular H_2O_2 localization revealed H_2O_2 accumulation in the mitochondria of spongy mesophyll cells at 24h, at the same time as the visible injury appeared (II). Concomitantly, disintegration of the mitochondrial cristae was detected (II). Degradation of the cristae has been observed earlier in O_3-treated birch (Pääkkönen et al., 1995a) as well as in pathogen treated parsley (Naton et al., 1996). Similar structural damage has been reported in the mitochondria of HL treated Cat1AS tobacco plants (IV).

As further evidence pointing at the direction of ROS' central role in the mitochondrial stress response, changes in the expression of genes encoding mitochondrial

proteins were detected (IV, V). Transcript levels of *Mpt1* rose strongly in O_3 treated birch (Kiiskinen et al., 1997), and a similar rise was seen in the levels of *MnSod* and *Aox* in the HL treated tobacco (IV). Increase in the *Aox* mRNA level is a good indicator of altered mitochondrial redox balance, since stress conditions tend to direct the mitochondrial electron flow through the alternate pathway, when the respiratory pathway is impaired (Vanlerberghe and McIntosh, 1996). Increase in the mRNA level of *MnSod* can be considered as a marker for accumulation of $\cdot O_2^-$ within the mitochondria. The role of *Mpt1* in the O_3 induced stress response is still unknown, but it seems likely that it responds to the oxidative balance of the mitochondria, which as such may represent the cell's faith at the onset of PCD.

4.2.3 Markers of PCD

In HL treated Cat1AS tobacco H_2O_2 accumulated mainly along the leaf veins (IV). Vital staining with Trypan blue revealed a pattern of cell death that correlated with the H_2O_2 accumulation pattern (IV). DPI and another inhibitor of flavin containing oxidases, quinacrine (Q), were able to block the HL induced H_2O_2 accumulation partly, and in a similar fashion the HL induced cell death was inhibited by these two chemicals, suggesting a central role for the NADPH oxidase derived oxidative burst in the initiation of the cell death process in HL treated tobacco (IV).

Treatment with cycloheximide (CHX), an inhibitor of protein synthesis, was also able to prevent cell death of the HL treated Cat1AS tobacco cells, indicating that the HL induced cell death is an active process, requiring *de novo* protein synthesis (IV). Signalling events required for the HL induced oxidative cell death included Ca^{2+} release, G- protein, protein kinase activity and ion fluxes (IV).

Nuclear condensation and DNA laddering are well known features in the mammalian apoptosis (Gilchrist, 1998). Similar nuclear events are seen e.g. in pathogen treated tobacco (Sasabe et al., 2000) and O_3-treated hybrid poplar (Koch et al., 2000). In HL treated Cat1AS tobacco nuclei were deformed, and their chromatin was fragmented and condensed as shown by TEM and flow cytometry (IV).

4.2.4 H_2O_2 is a crucial component of the cell death process

In birch, injection of the H_2O_2 generating system G/GO into the leaves resulted in visible injury, closely resembling that induced by O_3 or pathogen treatment (III). Just as in the two stresses mentioned, in G/GO treated leaves H_2O_2 accumulated in the vicinity of the edges of lesions as well as in tissue surrounding them, although in a spotted like manner (III). Thus exogenous H_2O_2 alone is able to induce endogenous H_2O_2 production, leading to cell death substantiating strongly H_2O_2's involvement in the stress induced cell death process. G/GO treatment resulted in biphasic H_2O_2 accumulation within birch tissue (III), the first one arising from the reaction between G (glucose) and GO (glucose oxidase), and the second one, induced by the first, and produced by the cells endogenously. This biphasic H_2O_2 build up in the tissue is re-

flected by the biphasic rise in the mRNA levels of the genes encoding ROS responsive enzymes (III). Biphasic H_2O_2 accumulation is also required in the onset of cell death in O_3-treated tobacco (Schraudner et al., 1998). Also in Draper's (1997) model biphasic H_2O_2 and SA accumulation are required for cell death initiation. *Pal* transcript accumulation also occurs in two phases upon oxidative stress, suggesting that SA has a role with H_2O_2 in the cell death regulation (III).

It is possible that in plants similar kinds of events take place in the course of action of PCD, as happen in the onset of mammalian apoptosis. Some of the features of mammalian apoptosis occur in stress induced cell death process in birch and tobacco, and H_2O_2 clearly has an active role in the onset of PCD both in birch and tobacco.

4.3 Stress-induced gene expression can be mediated by H_2O_2

4.3.1 Isolation of genes

Pal and *Chs* were chosen to be isolated from birch due to their well known stress responses in other plant species (Lois et al., 1989; Loake et al., 1991; Rosemann et al., 1991). Screening of 75 000 plaques from a two hour O_3- induced cDNA library resulted in the isolation of *Chs1* cDNA of 1522 bp in length [Y11022]. 100 000 plaques were screened for isolation of *Pal* cDNA clones. Clone lengths varied from 1.4 kb to 2.1 kb. Both 3' and 5' ends of the clones were sequenced to confirm they encoded *Pal*. None of the clones contained a full- length cDNA due to DNA secondary structures that apparently hindered cDNA synthesis during library preparation.

4.3.2 Organisation of genes

Southern analysis revealed that birch *Pal* is encoded by five to seven genes, *Chs* by one to two genes and *Ypr10* and pollen allergen genes together by at least nine genes (data not shown). Both *Pal* and *Chs* have been shown to occur in tandemly linked clusters in the genomes of several species (Howles et al., 1994, 1995; Akada and Dube, 1995; Liao et al., 1996). From three to four *Pal* genes were clustered in the birch genome (III). In similar PCR analysis with *Chs* no clustering was detected. Thus *Chs* is either encoded by one gene or if there are two, they are not clustered. As *Pal*, two birch *Ypr10* genes were clustered (III).

4.3.3 Gene expression

ROS are well known regulators of eukaryotic gene expression (Dalton et al., 1999). Since many oxidative stresses induce a vast array of defence related genes (Kangasjärvi et al., 1994), it is of interest whether stress- induced accumulation of ROS affects the activation of gene expression.

The stress-induced alterations in gene expression patterns of various birch and tobacco genes are presented in Table 7 and Figures 8, 9 and 10.

Table 7. Stress-induced changes in the gene expression patterns of defence related genes in birch and tobacco.

Gene	O_3	Pathogen	Wounding	G/GO	UV-B	HL
Pal	↑ (I, III)	↑ (III)	↑ (III)	↑ (III)	↑ (Fig. 10.)	
Ypr10	↑ (III)	↑ (III)	- (III)	↑ (III)	- (Fig. 10.)	
Chs	↓ (Fig. 9.)		↑ (Fig. 8.)		↑ (V, Fig. 10.)	
Apx	↑ (III)			↑ (III)		↑ (IV)
Gst	↑ (III)			↑ (III)		
CuZnSod	↑ (III)			↑ (III)		
MnSod						↑ (IV)
Cat	↑ (III)			↑ (III)		
Gpx						↑ (IV)
Aox						↑ (IV)
Rubisco (RbcS)	↓ (I)					
Mpt1	↑ (Kiiskinen et al., 1997)	↑ (III)	- (III)	↑ (III)		

In general, the treatments that induced H_2O_2 accumulation within plant tissue also lead into increased levels of mRNA of most of the studied genes. Of the H_2O_2 accumulation inducing stresses, wounding appeared the only one exhibiting differential gene expression patterns. For gene expression studies, leaves were wounded by piercing the whole leaf area with needles (III). *Pal* and *Chs* mRNA levels clearly increased in response to wounding (III, Figure 8), whereas *Ypr10* and *Mpt1* mRNA levels didn't respond, despite their responsiveness to O_3, a pathogen and G/GO (III).

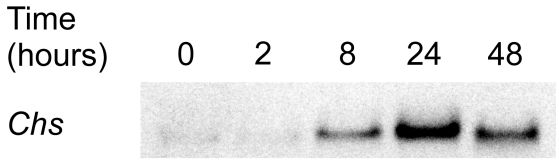

Figure 8. Northern analysis of *Chs* expression upon wounding. Wounding was performed as described in IV. *Chs* mRNA level increases at eight hours, reaches a maximum at 24 hours, and declines slightly at 48 hours.

The wound response may therefore induce additional signalling components conferring to the inhibition of *Ypr10* and *Mpt1* expression or an additional unknown component is required for the activation of *Pal* and *Chs* in the wound response. It may be that *Pal* activation can also occur through an H_2O_2 independent regulatory pathway in the wound response. There are studies concerning *Pal* expression and its un-relatedness to the oxidative burst in other plant systems (Levine et al., 1994). It is also difficult to know, whether a certain concentration of H_2O_2 is needed for the up regulation of gene expression. If this is the case the level may show a discrepancy between genes leading to differential gene- expression upon various oxidative stresses.
Rubisco small subunit *(RbcS)* (I) and *Chs* mRNA (Figure 9) levels declined in O_3 treated birch leaves.

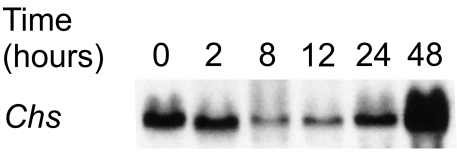

Figure 9. Northern analysis of *Chs* expression upon O_3 treatment. Birch saplings were exposed to 150 nL L^{-1} O_3 for eight hours, and samples were collected at the indicated time points (III). *Chs* mRNA level decreases first and rises to a level higher than background by the end of the experiment.

As a photosynthesis related gene, *RbcS* down-regulation is likely to occur with the decrease in the overall photosynthetic activity in O_3 exposed plants (Pääkkönen et al., 1996). Regulation of *Chs* expression has been thoroughly studied in plant-light responses (Martin, C.R., 1994). Although UV-B induced PR-1 expression in tobacco was postulated to occur via ROS signalling (Green and Fluhr, 1995), the regulation of *Chs* transcription occurs through several light (Strid et al., 1994) and elicitor responsive as well as tissue specific promoter elements (Martin, C.R., 1994). While *Chs* was not induced by O_3, *Ypr10*, which is induced by H_2O_2, was not induced by UV-B irradiation in birch (Figures 9 and 10, respectively), while *Chs* (II, Figure 10) and *Pal* (Figure 10) mRNA levels increased. It thus appears that *Ypr10* mRNA level responds specifically to changes in ROS, whereas *Pal* and *Chs* mRNA level can be altered via other regulatory pathways also.

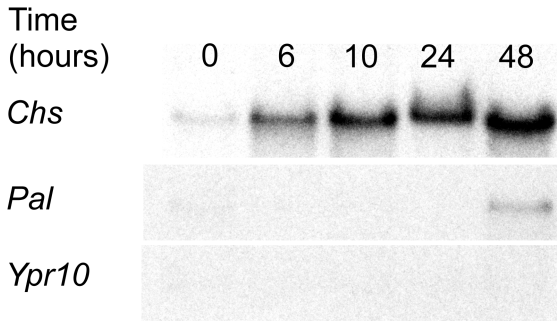

Figure 10. Northern analysis of *Chs*, *Pal* and *Ypr10* expression upon UV-B irradiation. Saplings of clone 36 were subjected to 4 kJ m^{-2} of UV-B irradiation for five hours, and samples were collected at the indicated time points. *Chs* mRNA level increases already at six hours and continues to increase until the end of the experiment. *Pal* mRNA level increases slightly at 48 hours, whereas *Ypr10* mRNA level is not affected by UV-B treatment.

Pal promoter regulatory elements contain similar light responsive motifs as described for *Chs* (Wu et al., 1996; Murakami et al., 1997), and their expression upon stress is light dependent and coordinated (Leyva et al., 1995). Light regulation has not been proposed for *Ypr10* so far.

The mRNA levels of genes encoding ROS detoxifying enzymes increased consistently upon oxidative stress both in birch (III) and tobacco (IV). In the case of HL treated Cat1AS tobacco plants, the light and ROS regulated gene expression were closely linked, because oxidative stress was applied by light. Since the HL treatment itself results in a massive burst of H_2O_2 produced by the tobacco cells, ROS probably play a role in the regulation of the genes encoding antioxidant enzymes. Inhibiting NADPH oxidase did not affect the protein levels of several defence related proteins (PR-1a, PR-N, GPX, APX and HSP 17.6), suggesting that the H_2O_2 produced by NADPH oxidase is not involved in the transcriptional and/or translational regulation (IV). It does not, however, exclude the possibility that ROS derived from other cellular sources could act as an inducing agent.

As ROS production in mitochondria has been considered as a trigger in the onset of apoptosis (Gilchrist, 1998; Lam et al., 1999), the increase in *Mpt1* transcript levels upon oxidative stress could be related to the disruption of mitochondrial redox balance during stress, since P_i transport may occur electrogenically (Wohlrab, 1986). On the other hand, the increase may also reflect the changes in energy metabolism of the cells under stress (i.e. increased ATP requirement) (Umeda et al., 1994; Robertson et al., 1995), or simply the degradation of mitochondria in stressed cells (II, IV) could lead to the generation of new mitochondria and the components needed.

4.3.4 Promoter analysis

Promoter structure has been thought to become a shortcut to understanding of regulation of gene expression in plants (Somerville, 2000). Such analysis have so far

successfully been performed with the *Saccharomyces cerevisae* complete genome and extensive expression data (Zhu and Zhang, 1999). In plants, the EST projects are still ongoing and the experimental data on regulatory promoter *cis*-elements is still rather limited.

The promoter structures of O_3 induced birch genes were studied with their non-O_3 induced counterparts (*Pal* and *Ypr10* [III], *Mpt1* is a single copy gene in birch [Kiiskinen et al., 1997]). The promoter of O_3-inducible *Pal1* carries ARE. It was not found in any other promoter studied here, nor was it found in the *Pal* promoters of other species either. ERELEE4 (Itzhaki et al., 1994) was not detected in the promoters of the O_3-inducible genes *Pal1*, *Ypr10*b*, and *Mpt1*, whereas it was found in the promoters of *Pal2* and *Ypr10*a*, the non O_3-inducible genes. This particular element has been described to be involved in the senescence process. Another ethylene responsive element, the GCC-box, has a role in regulating stress induced, or suppressed gene expression. It may therefore act either as a negative or positive regulator of gene- expression (Fujimoto et al., 2000). Since ERELEE4 is only present in the promoters of non O_3-inducible genes and not in the O_3-inducible genes, it may have a role as a possible negative regulator binding site in the birch O_3-response. This is especially intriguing since ethylene emissions increase markedly in O_3-treated birch (Vahala, Ruonala, Tuominen and Kangasjärvi, in preparation). Another distinction in the promoters of O_3- and non O_3-inducible promoters was the existence of the combination of MRE and auxin/SA-responsive element in the promoters of O_3-inducible promoters (III). Since MRE binding factors respond to redox changes (Ghoshal et al., 1999) and SA is a central component in inducible plant defence responses (Draper, 1997), combinatorial regulation via these two elements may be one of the factors that regulate ROS dependent gene expression in birch.

Regulation of stress induced gene expression is likely to occur through complex protein interaction and DNA- binding events that share common features, but in specific combinations lead to the appropriate response.

4.4 ROS independent stress responses

UV-B irradiation was studied as an abiotic stress possibly not acting via ROS signalling. Very high UV-B-irradiation caused chlorotic lesions in the birch leaves within 10 d from the beginning of the exposure, combined with severe membrane damage. By the 12^{th} day of the experiment more than 50% of the mitochondria had disintegrated. Lipid composition changed only slightly and no lipid peroxidation products were detected (V). Concentrations of quercitin-3-glycoside and of three unknown flavonoid compounds increased as a response to UV-B irradiation (V).

Increases in the concentrations of phenolics were reflected by increase in the mRNA levels of *Chs* (V) and *Pal* (data not shown) in UV-B treated birch, and *Chs* expression was localised in the upper mesophyll cells (V). In other plant systems *Chs* expression was localised to the mesophyll cells (Knogge and Weissenböck, 1986)

suggesting that flavonoids for UV-B screening can be synthesised elsewhere, and transported to the epidermal cells, which is the most likely location for their action.

Although the UV-B irradiation experiment described here (V) was performed with unrealistically high UV-B dose, it does give valuable information about ROS independent stress responses. It seems likely that in birch, UV-B regulates *Pal* and *Chs* transcript levels by ROS independent pathways. In some cases ROS regulation of gene expression has been shown as a response to UV-B irradiation (Green and Fluhr, 1995). In the case of birch however, other regulatory pathways are more likely candidates for the induction in gene expression.

The disintegration of mitochondria, that was considered a result of ROS accumulation in this compartment in the stress situations discussed earlier, appeared in the UV-B irradiated birch leaves after severe visible damage detectable in the leaves. The mitochondrial destruction may therefore be a result of the UV-B induced chlorosis, independent of ROS, although their involvement cannot be ruled out. It also appears that in this plant system the ROS pathway is not activated in response to UV-B stress, indicated by the lack of induction in the expression of the ROS responsive gene *Ypr10* and the lack of increase in the levels of lipid peroxidation products in the leaves.

4.5 Concluding remarks

Biotic and abiotic stresses in birch and tobacco are able to set off reactions that lead to H_2O_2 accumulation in plant tissue. H_2O_2 produced by the plant cells was able and present in sufficient amounts to induce cell death. This was seen to occur in all the cases where stress treatment lead to H_2O_2 accumulation. In the wounding treatment, cell death occurred only at the wound site, related to the H_2O_2 localization, as was the case in other stresses also: cell death and H_2O_2 accumulation were always spatially closely related. H_2O_2 induced cell death could also be induced by an H_2O_2 generating system, therefore making this cell death process directly dependent on the endogenous H_2O_2 production. In tobacco, cell death was shown to be an active process, therefore suggesting it being a form of PCD in plants. In birch the involvement of NADPH oxidase and mitochondrial ROS accumulation propose a possibility of PCD in the oxidative stress responses.

In addition to its central role in inducing cell death, H_2O_2 appears to be an efficient regulator of plant gene expression. mRNA levels of several stress related genes increase as a response to H_2O_2, and H_2O_2 accumulating stresses. UV-B was already postulated not to involve ROS signalling, and the *Pal* and *Chs* activation seen in this stress situation, is likely to occur through a ROS independent pathway.

Pal and *Chs* are induced upon wounding, whereas *Ypr10* and *Mpt1* are not. There may be additional signal components than H_2O_2, activated by wounding, that confer to the induction of *Pal* and *Chs* upon this stress. These additional factors may also lead to the inhibition of *Mpt1* and *Ypr10* expression. Also, the threshold for H_2O_2

level needed for different genes may vary, resulting in differential gene expression in some stress situations. Summary of the conclusions is presented in Figure 11.

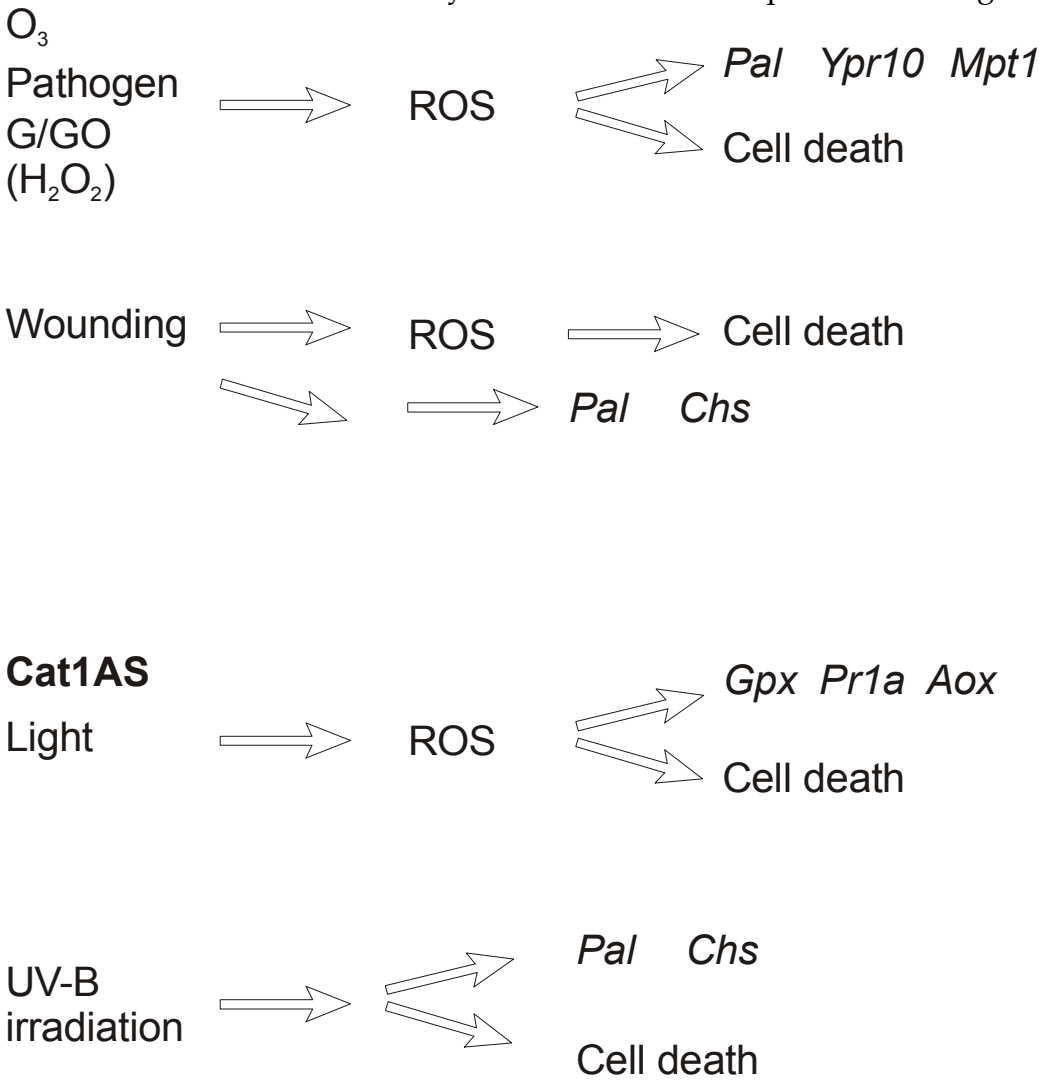

Figure 11. A schematic representation of stress induced ROS accumulation, gene expression and cell death in birch and tobacco. ROS accumulation is an integral part of the cell death process induced by various biotic and abiotic stresses. In some of the stresses, ROS also confer to the signalling involved in the induction of gene expression. On the other hand, the same genes can also be activated via ROS independent signalling pathways. *Aox*= alternative oxidase, Cat1AS= catalase1 antisense plants, *Chs*= chalcone synthase, *Gpx*= glutathione peroxidase, *Mpt1*= mitochondrial phosphate translocator1, *Pal*= phenylalanine ammonia-lyase, *Pr1a*= pathogenesis related protein 1a, ROS= reactive oxygen species, and *Ypr10*= pathogenesis related protein10.

Finding out the mechanisms of formation of the O_3 injury may help in dissecting the general pathological events taking place in plants under stress. Similarities in the stress responses caused by biotic and abiotic stress factors may be important in the future when agronomy and forestry have to deal with ever increasing concentrations and novel types of air pollutants. By showing how air pollutants, pathogens

and other environmental factors affect trees, it may become easier to convince people of the importance of environmental regulations and thresholds.

5 Acknowledgements

This work was started at the University of Kuopio, in the graduate program of Environmental physiology, Molecular biology and Ecotechnology in 1995 at the Department of Ecology and Environmental Science, and finished in the Institute of Biotechnology, University of Helsinki during 1998-2001. I wish to thank Professor Lauri Kärenlampi for providing the facilities at the University of Kuopio. In the University of Helsinki I wish to thank Professor Tapio Palva and the Forest Biotechnology program for the excellent working facilities both in the Institute of Biotechnology and at the Division of Genetics. I also wish to thank Docent Pekka Heino for his guidance and help.

I wish to thank the advisors of this work Professor Jaakko Kangasjärvi and Professor Tapio Palva. Jaakko has advised both my MSc thesis and PhD thesis and with his help I've grown to think like a scientist. Jaakko has always shared his wide knowledge on plant sciences with his students and has thus enabled a molecular biologist like me to finish a thesis concerning both plant physiology and molecular biology. Tapio's valuable comments on my work and his wide knowledge and experience have helped me in many occasions.

I am grateful to the official pre-examiners of this thesis, Docent Elina Oksanen and Docent Kurt Fagerstedt for their careful examination and the comments for improving the manuscript.

I want to thank our collaborators in the University of Gent, Belgium, where the work for the tobacco manuscript was done. There at the "Vakgroep Moleculaire Genetica and Departement Plantengenetica" I especially want to thank Professor Dirk Inzé for the opportunity to work in one of the leading plant molecular laboratories in the world. I also wish to thank Dr Frank van Breusegem and Dr James Dat (currently in Université de France-Comté) for teaching me the tobacco work.

I am grateful for all the people I ever worked with. I began my work with MSc Jaana Tuomainen, PhD Merja Utriainen, MSc Maarit Sillanpää, MSc Markus Kiiskinen, BSc Kirk Overmyer, MSc Minna Korhonen, and PhD Leila Kauppinen at the university of Kuopio. Thanks for being good co-workers and friends. I also wish to thank Professor Sirpa Kärenlampi, PhD Anu Wulff, PhD Seija Anttonen, and Docent Reijo Karjalainen at the university of Kuopio.

I wish to thank my colleagues Ph.Lic. Annikki Welling, PhD Hannele Tuominen, PhD Reetta Kettunen, MSc Sari Tähtiharju, MSc Jorma Vahala, BSc Reetta Ahlfors, MSc Ilkka Tamminen, and MSc Tuula Puhakainen at the Institute of Biotechnology and at the division of Genetics. It has been a great pleasure to work and party with people so smart and happy. I also want to thank the whole staff at the Division of Genetics.

MSc Airi Tauriainen, Mirva Lehtinen and Sari Eräluoto I want to thank for their excellent technical help.

I want to thank all my friends for great time and company, not forgetting all those of you who babysat or otherwise helped us during these years.

I wish to thank my family, my parents Irma and Tapio Pellinen and my brother Oku Pellinen for their support and confidence during this work. I also want to thank the Ruippo family for their support. Most of all I want to thank my husband Pekka, he has helped me through the hardest times when I've wanted to give up. And it certainly is handy to have your personal system administrator around 24 hours.

Thanks to Lotta and Anna for the joy you bring!

This study has been financially supported by the graduate programs Environmental physiology, Molecular biology and Ecotechnology (University of Kuopio) and ENSTE (Åbo Academi) and, Niemi- säätiö.

Kuopio

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