Biochemical Mechanisms of Plant Defense A Central Role for Reactive Oxygen Species

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Abstract

In general mechanics stress is clearly defined as the point or degree of bending of an elastic system at the very point of just symptomless reversibility and irreversible deformation or break. In medicine and botany, stress is supposed to indicate all situations beyond normal, defined by the observer. All organs of higher plants (with some exceptions) perform aerobic metabolism and are thus subject to activated oxygen species. Oxygen oversaturation and thus oxygen stress may occur under various different conditions. Since most abiotic and biotic stress situations in plants result in the accelerated production of ROS oxidative stress is a common signaling event in plant stress and redox regulation therefore plays a central role in the stress signaling network (PASTORI & FOYER 2002). In this review basic reactions operating during stress and defence will be discussed where certain prooxidative situations and antioxidative processes in plants will be dealt with.

Keywords: reactive oxygen; phytotoxins; oxidative burst; redox signaling

RULES AND PATHWAYS OF OXYGEN ACTIVATION IN PLANTS

Oxygen in the triplet ground state ($^3O_2$) has to be activated in order to react with atoms or molecules in the "normal" singlet ground state thus circumventing spinforbidden reactions. The most important reactions of oxygen activation are briefly adressed in the following.

Photodynamic reactions

Oxygen can be activated by photodynamic reactions where superoxide or singlet oxygen may be generated.

Photodynamic reactions undergoing charge separation within the excited pigment are called photodynamic reaction type I (where P represents a pigment in its ground singlet state and P^* its activated triplet form; $_+P$ represents a photooxidized, i.e. bleached pigment):

$$P + \text{light} \rightarrow P^* \quad \text{(pigment activation)}$$

$$P^* \rightarrow {}_+P^- \quad \text{(charge separation)}$$

$${}_+P^- + O_2 \rightarrow {}_+P + O_2^{\cdot -} \quad \text{(superoxide formation)}$$

In a photodynamic reaction classified as type II singlet oxygen is formed:

$$P + \text{light} \rightarrow P^*$$

$$P^* + {}^3O_2 \rightarrow {}^1O_2 + P \quad \text{(exciton transfer forming singlet oxygen)}$$

Light independent oxygen activation

In the presence of appropriate reductants (E) with high affinity for oxygen (negative redox potential: E_0' of the redox pair O_2/O_2^- = –330 mV), superoxide may be formed from atmospheric oxygen:

$$E + O_2 \rightarrow E^+ + O_2^{\cdot -}$$

Superoxide dismutates at neutral pH in aqueous media with a rate constant $k = 2 \times 10^5$ l/M.s, yielding hydrogen peroxide:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2\,H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide in turn may be reduced by the certain electron donors yielding the highly reactive hydroxyl radical (·OH, redox potential close to +2V):

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

OXYGEN ACTIVATION IN PLANTS

Abiotic stress factors inducing oxidative stress in plants

Ozone and SO_2 as air pollutants and the increase of UV-B as an effect of the decrease of the stratospheric ozone layer and radioactive deposition must be seen as potentially toxic for plants. Radioactive deposition is no longer an immediate toxicological threat to plants and can only be seen as a potential impact to the consumers (animals; humans: "damage" in the sense of monetary losses). UV-damage in plants is not much of a problem due to the plant's capacity to establish protective systems on the basis of a complex set of UV-absorbant phenolics (JANSEN *et al.* 2001). SO_2 in most regions of the industrial world (western hemisphere) due to binding with CaO (forming gipsum) after the burning process in the power plants is also no longer a serious problem; in former times, when SO_2 concentrations in industrial regions or in their downstream exhaust could reach up to 1000 $\mu g/m^3$ it has caused serious pathological problems in both animals and plants (HIPPELI & ELSTNER 1996a,b).

Ozone, however, may reach concentrations in the atmosphere which urges the plants to react and seems to be of higher relevance to plants as compared to animals and man (ELSTNER 1996). Ozone enters the plant through the stomata and induces the production of reactive oxygen species in an aqueous environment (SANDERMANN 1996). Ozone also reacts with cell wall components like phenolics thereby inducing the production of ROS (RAO *et al.* 2000). After ozone treatment of wheat leaves a decrease of the photochemical capacity was observed along with a stable EPR signal which had similarities to the ubisemiquinone radical. This EPR signal seemed to be associated with permanent leaf damage (REICHENAUER & GOODMAN 2001).

Oxygen activation by phytotoxins

Phototoxins

Production of ROS is observed after illumination of cercosporin, a perylenequinone toxin produced by several phytopathogenic *Cercospora* species e.g. *C. beticola* and *C. kikuchii*. Cercosporin (Cerc) mainly seems to induce the formation of singlet oxygen and superoxide in photodynamic reactions both of type I and type II (YOUNGMAN *et al.* 1983; DAUB & HANGARTER 1983; YOUNGMAN & ELSTNER 1984):

$$Cerc + light \rightarrow Cerc^* \text{ (triplet state)}$$

$$Cerc^* + {}^3O_2 \rightarrow Cerc + {}^1O_2 \text{ (type II reaction)}$$

or:

$$Cerc^* \rightarrow {}_+Cerc^-$$

$${}_+Cerc^- + O_2 \rightarrow {}_+Cerc + O_2^{\bullet -} \text{ (type I reaction)}$$

Under illumination cercosporin induces lipid peroxidation in plant cells (CAVALLINI *et al.* 1979; DAUB 1982) followed by changes in membrane structure. Singlet oxygen quenchers like DABCO (diazabicyclooctane) delayed killing of cells by cercosporin (DAUB 1982).

Reductive oxygen activation by phytotoxins

An important field of research on oxygen activation during host-pathogen interactions concerns the investigations on reaction mechanisms of toxins produced by pathogenic fungi and bacteria. Several such toxins have been shown to act as redox cyclers where the quinoid derivatives dothistromin and dihydrofusarubin have to be mentioned in this context (HEISER *et al.* 1998). In other cases bacterial or fungal toxins introduced into the plant react in analogy to certain herbicides such as the "quat"dyes: As recently documented, naphthazarin toxins such as dihydrofusarubin produced by certain strains of *Fusarium solani* induce redox cycling and superoxide production with similar kinetics as methylviologen after reduction by photosystem I (ALBRECHT *et al.* 1998) or by certain NAD(P)H oxidoreductases (such as diaphorases, ROHNERT *et al.* 1998). These redox reactions are responsible for the observed bleaching and necrotization of the treated plants.

Figure 1 shows the formation of ROS by phytotoxins as recently reviewed by HEISER *et al.* (1998).

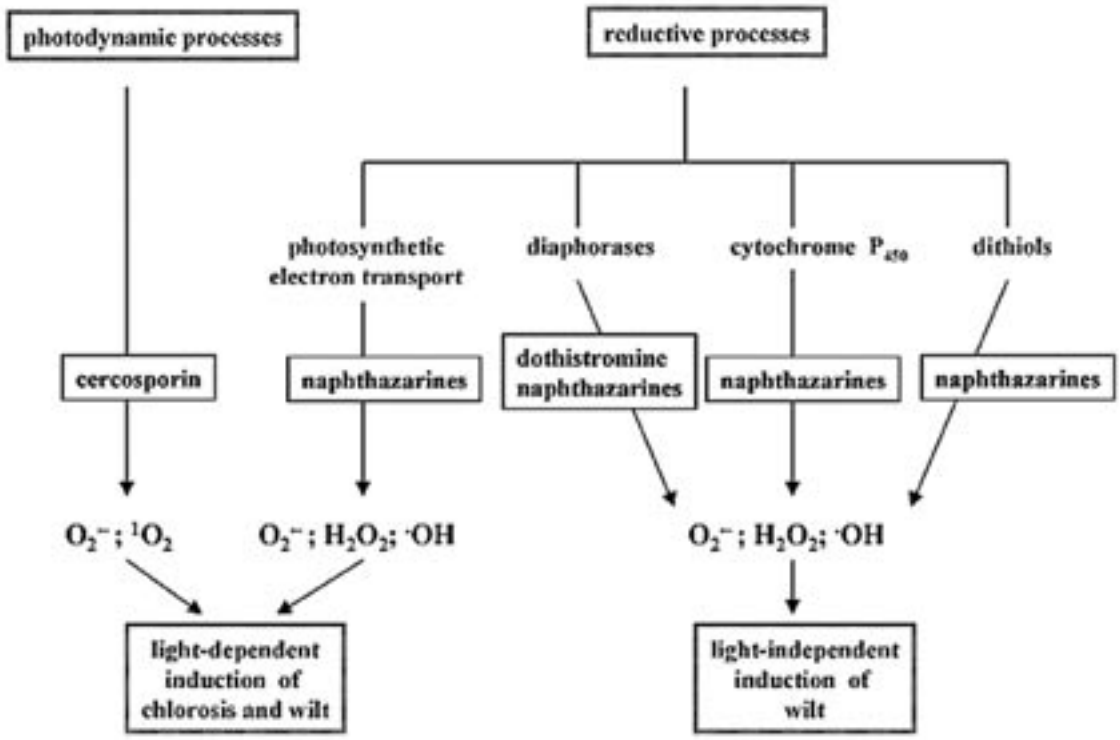


Figure 1. Mechanisms of oxygen activation by a number of phytotoxins (from HEISER *et al.* 1998)

The oxidative burst

The production of active oxygen species (AOS) in plants in response to pathogen infection was first reported by DOKE (1983) in *Phytophthora infestans*-infected potato tubers. In the following years induction of the oxidative burst was recognized as a central mechanism in plant signalling pathways leading to defense activation (GRANT *et al.* 2000; MEHDY 1994). The mechanism by which AOS are produced in plants after pathogen attack is still under discussion. Several authors have described a NADPH-dependent oxidase system, similar to that present in mammalian neutrophils, which reduces oxygen in a one-electron step leading to the formation of superoxide (KELLER *et al.* 1998; SAGI & FLUHR 2001).

The signaling cascade finally resulting in activation of the NADPH-oxidase starts with the recognition of the pathogen by the host cell. This recognition event is mediated by elicitors which are released by the pathogen (exogenous elicitors) and include structural components or metabolites of the pathogenic organism. Besides the pathogen-derived elicitors also plant constituents released from the cell wall in response to pathogen infection, socalled endogenous elicitors, may induce defense responses. After binding of the elicitor to a receptor protein which in most cases is located in the plant plasmalemma the signaling cascade is activated. Depending on the plant species and/or the attacking pathogen different signaling molecules are involved in induction of defense mechanisms. OKADA *et al.* (2002) only recently demonstrated the existence of high-affinity binding proteins for N-acetylchitooligosaccharide elicitor in the plasma membranes of wheat, barley and carrot cells. This elicitor stimulated several defense responses in cell suspension cultures of different plant species including changes in membrane potential and ion flux, the expression of defense-related genes and the production of ROS (OKADA *et al.* 2002).

Components of the signaling network finally resulting in the activation of the NADPH-oxidase are Ca^{2+} influx, alkalinization of the apoplast and protein phosphorylation cascades. Several observations indicate a positive feedback regulation of NADPH-oxidase activation through H_2O_2 (VRANOVA *et al.* 2002). How the components of this signaling network work together in the induction of the oxidative burst is not fully understood but is a matter of intensive investigations.

Besides the activation of oxygen through the NADPH-oxidase another possibility is the generation of hydrogen peroxide by peroxidase enzymes located in the cell wall (BOLWELL & WOJTASZEK 1997). Plant cells, in contrast to animal cells contain more or less rigid cell walls which, together with the intercellular space comprise the apoplastic compartment. Oxygen activation in this extracellular space include both products derived from the NADPH-oxidase of the plasmatic membranes and the products of cell wall intrinsic enzymes such as peroxidase(s) (ELSTNER 1987; ELSTNER & OßWALD 1994; ASARD *et al.* 1998). These peroxidases produce hydrogen peroxide at the expense of NAD(P)H in a manganese catalyzed reaction (ELSTNER 1991) allowing an extremely complex crosslinkage of C6–C3 phenylpropanoids forming lignin (HATFIELD & VERMERRIS 2001) as an essential element of woody plants and generally in pathogen defence.

It is probable that different plant species developed different ways of AOS generation and that several mechanisms exist simultaneously in the same plant.

Internal metabolic events in green plants forming ROS

Stressors such as cold, drought and many others cause internal oxygen activation via feed-back of metabolic blocks to the photosystems. External stress always has a change of internal metabolism as consequence, mediated by a complex interaction of hormones which are synthesized de novo or released from internal stores. Adaptation to drought, for example, is counteracted by stomatal closure. This event is initiated by the hormone abscisic acid and mediated by H_2O_2 produced via a NADPH oxidase (ZHANG *et al.* 2001).

Green plants have adapted to extremely different environmental conditions. Since normally plants, in contrast to animals, cannot escape, they have either to adapt or to die. Dependent on the strength of these abiotic or biotic impacts, several symptoms are expressed indicating the deviation from normal metabolic conditions. Most of these visible or measurable symptoms have been shown to be connected with oxygen activation (ELSTNER 1982, 1987; ELSTNER & OßWALD 1994; HIPPELI & ELSTNER 1996a,b) where principally a transition from heterolytic (two electron transitions) to increased homolytic (one electron transitions) reactions is observed. Homolytic reactions create free radicals. We thus address these situations as "oxidative stress". These stress situations are generally counteracted by a parallel increase of radical scavenging processes or by pre-existing or de novo synthesized compounds which function in detoxification of deleterious ROS and thus metabolic control within certain limits. At advanced or prolonged stress conditions this control may be gradually lost and chaotic radical processes may dominate. Finally, lytic processes induce cellular and tissue decompartmentalizations yielding visible necrosis (Figure 2).

EFFECTS OF REACTIVE OXYGEN SPECIES IN PLANTS

Deleterious effects

Oxidative processes occur if ROS are produced at a cellular level and the detoxification system is overloaded or exhausted. H_2O_2 is moderately active and may inactivate enzymes by oxidating their thiol groups. The most reactive activated oxygen species is the hydroxyl radical that is formed from H_2O_2 by reduction by metal ions (Fe^{2+}, Cu^{+}). The hydroxyl radical can potentially react with all biological molecules.

The peroxidation of unsaturated fatty acids is induced by hydroperoxyl radicals (HO_2, formed by protonation of the superoxide radical in aqueous solution) or by OH-radicals (YOUNGMAN & ELSTNER 1981; WINTERBOURN 1999) primarily yielding lipid radicals via abstraction of a hydrogen atom. Singlet oxygen in contrast to atmospheric oxygen is not subject to the spin rule and reacts rapidly with fatty acid molecules (LH), especially at double bonds, producing lipid hydroperoxides.

$$LH + {}^1O_2 \rightarrow LOOH$$

LOOH in turn can be reduced by one electron donors (E^- representing reduced transition metal ions like Fe^{2+} or Cu^{+}, semiquinones, heme- and nonheme proteins, isoalloxazines or pteridines) yielding alkoxyl ($LO\cdot$)-radicals:

$$E^- + LOOH \rightarrow E + LO\cdot + OH^-$$

These $LO\cdot$ radicals may initiate chain reactions thus reacting further cooxidizing other molecules, for example by initiating cooxidative bleaching of pigments.

Induction of defense responses

In response to elicitor binding the induction of a wide range of defense responses is reported in plants (reviewed by HUANG 2001). These induced defense responses include the production of phytoalexins and

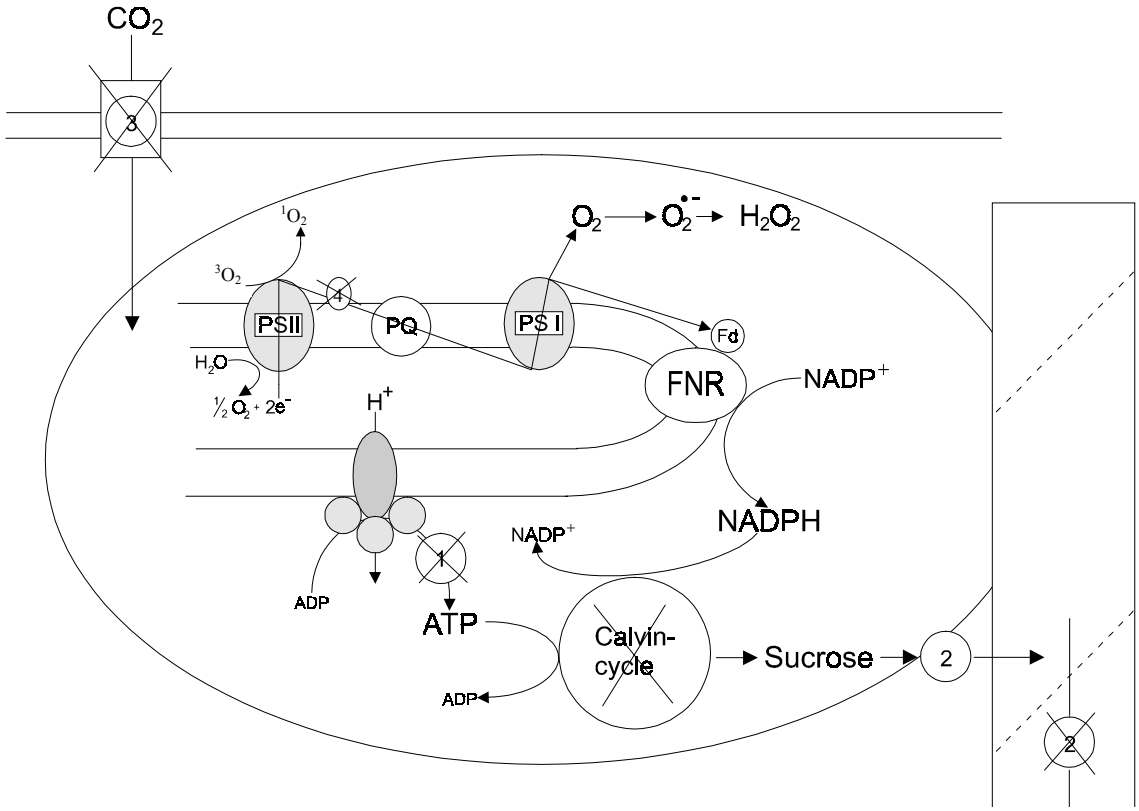


Figure 2. Metabolic, regulatory and morphological changes during stress development and stress management in green plants (from ELSTNER & OßWALD 1994).

pathogenesis-related proteins as well as the fortification of the cell wall by the formation of papillae, induced lignification or suberinization. Reactive oxygen species formed during the pathogen-induced oxidative burst are involved in the induction of defense responses in several ways (MEHDY 1994; HUANG 2001):

- Cross-linking of cell wall structural proteins
  In soybean cells it could be shown that H_2O_2 induces the rapid insolubilization of hydroxyproline-rich glycoproteins (HRGPs) in the cell wall, a reaction which contributes to plant resistance against invading pathogens (BRADLEY *et al.* 1992; BRISSON *et al.* 1994).
- Induction of defense-related genes
  For several plant species it could be shown that defense responses like phytoalexin accumulation are abolished by inhibitors of ROS formation (HUANG 2001). The induction of defense responses by ROS is believed to be mediated by transcriptional activation of defense-related genes as outlined in chapter Antioxidative strategies of plants.
- Induction of the hypersensitive response
  Several reports indicate a causal involvement of ROS in the induction of hypersensitive cell death.

SIGNALING THROUGH AOS

Since the accelerated production of ROS is the best documented common response of plants to several biotic and abiotic stress situations (PASTORI & FOYER 2002) signaling events in plants induced by ROS have become the focus of interest of several research groups in the last few years. LEVINE *et al.* (1994) demonstrated that H_2O_2 is a diffusible signal in the induction of defense related genes in plants, a finding which was affirmed by several reports in the following years (WU *et al.* 1997; CHAMNONGPOL *et al.* 1998). Also superoxide is discussed as signaling molecule in several plant-pathogen interactions (JABS *et al.* 1997).

Several studies implicated a causal involvement of ROS in hypersensitive cell death induction. LEVINE *et al.* (1994) showed that treatment of soybean cells with H_2O_2 resulted in a dose dependent cell death induction. Inhibition of catalase by aminotriazole enhanced cell death in soybean cells inoculated with avirulent *Pseudomonas syringae* pv. *glycinea*. During the last years several reports appeared which questioned the direct involvement of ROS in the hypersensitive

response. GLAZENER *et al*. (1996) demonstrated that a mutant of *Pseudomonas syringae* pv. *syringae* (hrmA) showed a decreased ability to induce plant cell death but induced an oxidative burst comparable to wild type bacteria. PIEDRAS *et al*. (1998) showed that an oxidative burst was induced in tomato cells with Avr9 elicitor from *Cladosporium fulvum* but no cell death induction took place. Several other reports exist which demonstrate that elicitors or pathogens trigger an oxidative burst in plant cells without inducing cell death (DEVLIN & GUSTIN 1992; JABS *et al*. 1997). Looking on these results the role of ROS in cell death induction strongly seems to depend on the plant and the pathogen studied.

The conversion of the ROS signal into a cellular response might be achieved through redox-sensitive proteins which are reversibly oxidized/reduced and therefore modulated in their activity by ROS. This is the case for several enzymes of the Calvin cycle as well as for glucose-6-phosphate dehydrogenase where thiol groups are oxidized by ROS. Another example for a redox-sensitive protein is the cytochrome bf-complex in the thylakoid membranes. The redox state of the Fe-S-protein associated with the cytochrome bf-complex modulates the activity of a kinase responsible for the phosphorylation of the light-harvesting complex from photosystem II (VENER *et al*. 1998).

Besides the modulation of enzyme activity the ROS signal may be converted into cellular responses by regulation of gene expression. Oxidative stress-responsive promoter elements have yet been identified in several plant species (reviewed by PASTORI & FOYER 2002). For example SANDERMANN *et al*. (1998) found an ozone-responsive region in the promoter of the stilbene synthase gene in grapevine. RYALS *et al*. (1997) described a protein in *Arabidopsis* with high homology to the mammalian redox-regulated IκB transcriptional repressor indicating a possible role for NFκB-transcription factor mediated gene induction in response to a ROS signal.

ANTIOXIDATIVE STRATEGIES OF PLANTS

Stress avoidance

Every episode during the stress cascade (Figure 2) is characterized by the balance between pro- and anti-oxidative capacities. In the beginning the plant tries to minimize electron pressure arising from photosystem II (PSII) on an organizational level by:

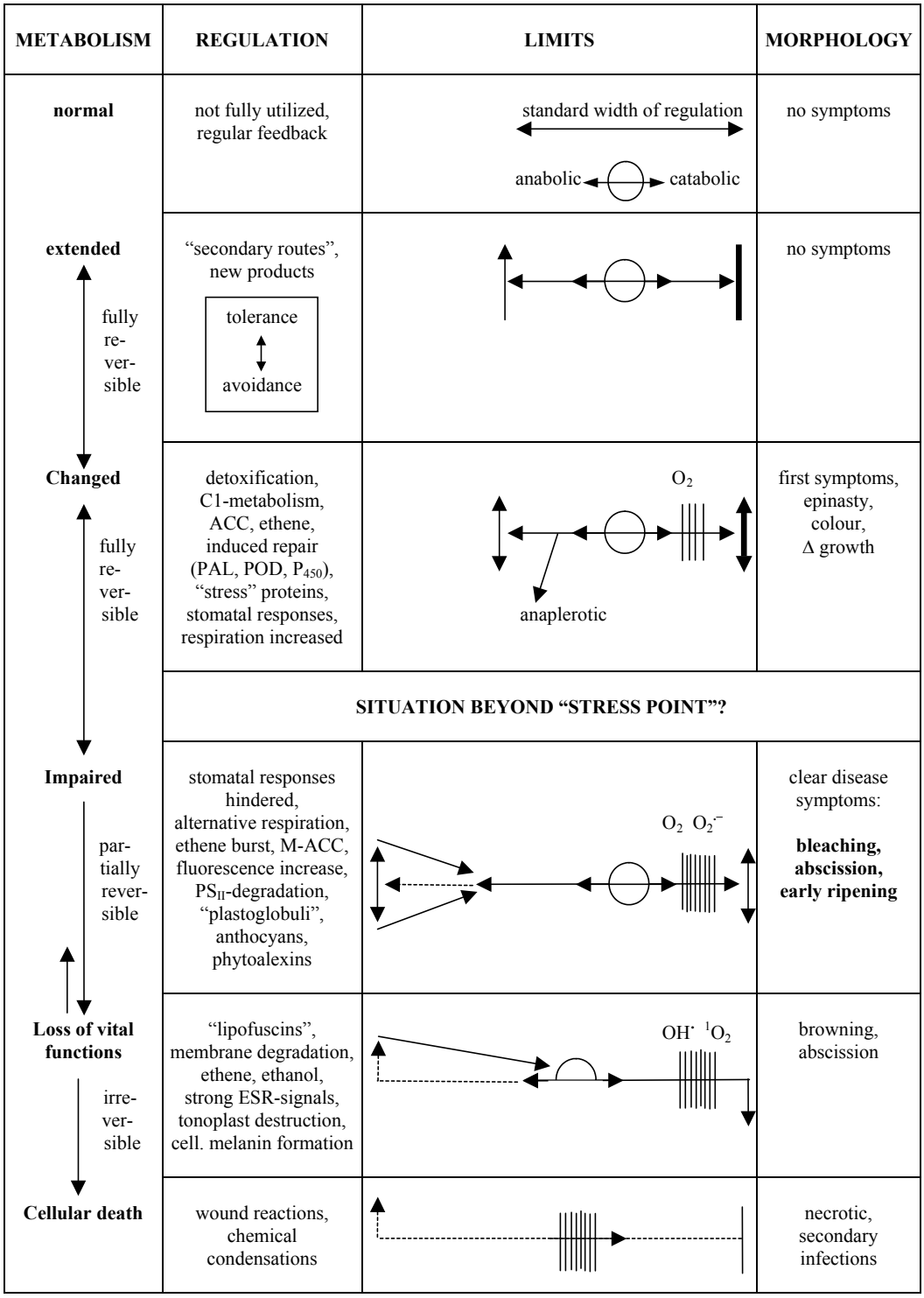
- reducing the optical diameter i.e the light harvesting systems (LHCII) of PSII by transfer and integration of the chlorophyll-protein complex into photosystem I (ALBERTSSON 2001) and by non-photochemical quenching (MÜLLER *et al*. 2001)
- induction of photorespiration, connecting several cellular compartments by "carbon-idling" and enhancement of C-1 metabolism (ELSTNER 1987; COSSINS 1987; HANSON *et al*. 2000)
- H_2O_2 cycling via the "Beck-Halliwell-Asada-cycle" (FOYER *et al*. 1997)
- the xanthophyll cycle (MÜLLER *et al*. 2001; DEMMIG-ADAMS & ADAMS 1996; POLLE 2001) protecting PSII
- photoinhibition by inactivating the electron-"outlet" of PSII via radical-induced degradation of the D-protein (MELIS 1998), and
- via the novel and very recently decribed plastid terminal oxidase (quinone-oxygen oxidoreductase) resembling the cyanide-resistant alternative oxidase linked to carotene desaturation which is responsible for chlororespiration (CAROL & KUNTZ 2001).

Both photoinhibition and chlororespiration are tuned by the redox-state of the plastoquinone-cycle which seems to play the dominant, pivotal role in the feedback regulation of the high fidelity-functioning of the photosystems and thus the security of the whole thylakoid system.

- In the same context, alpha tocopherol molecules close to the P680-moiety of photosystem II seem to play a pivotal role in the protection of the D1 shield protein from light dependent damage via singlet oxygen. Tocopherol concentration in chloroplasts increases in a light dependent manner and during senescence. When tocopherol biosynthesis is blocked by pyrazolinat-herbicide plants become extremely light-sensitive and severe bleaching, degradation of D1 protein and finally tissue necrosis is observed (TREBST *et al*. 2002).

Enzymatic protection

Plants possess a very effective enzymatic antioxidative system that allows protection of cellular components from oxidative damage. Enzymes which detoxify ROS are superoxide dismutase, catalase, ascorbate- and glutathione-peroxidase and dehydroascorbate reductase acting in different compartments of the plant cell. The mode of action of these antioxidant enzymes is excellently reviewed by several authors (e.g. FOYER & MULLINEAUX 1998) and therefore should not be dealt with in this context.



Fd = ferredoxin; FNR = NADPH-ferredoxin-oxidoreductase; PQ = plastochinone pool; PS I = photosystem I; PS II = photosystem II (from HEISER *et al.* 1998)

Figure 3. Formation of ROS via inhibition of the Calvin cycle, induced by stomatal closure (3), a block in phloem loading (2) (often observed with virus infections) or uncoupled electron transport leading to ATP-deficiency (1) or inhibition of electron transport by herbicides (4). After the Calvin cycle is blocked reoxidation of NADPH is decreased and electrons are transferred to molecular oxygen at photosystem I leading to superoxide formation, or to $^1O_2$ formation in photosystem II

Roles of phenolics as antioxidants

Several non-enzymatic antioxidants are effective in protecting plant cells agains oxidative damage. One of the most important group of plant antioxidants are the phenolics, ubiquitarily found in the plant kingdom. Among the approximately 50 000 secondary plant metabolites phenolics represent the largest group. Today we know that phenolics contribute to the overall fitness of plants (KIRÁLY 2000) with ascertained multifold functions such as insect attraction by colors, protection against pathogens or competitive neighbour plants, to mention just a few (BECKMAN 2000). Since phenolics are composed of one or more benzene rings containing various substituents, especially hydroxyl groups, their biochemical activities are extremely broad-ranged. Depending on the neighbouring substituents of a phenolic hydroxyl group, their antioxidative properties are outstanding and comprise all known mechanisms. This is of special importance not only for plants themselves but also for humans and animals where they act as vitamins and/or protectants against oxidative stress (DECKER 1995; STADLER *et al.* 1995; RAKOTOARISON *et al.* 1997). Structure activity relationships of flavonoids in protection against lipid peroxidation have been thoroughly investigated by HEIJNEN *et al.* (2002).

Principally there are two types of phenolics found in plants: those which are constitutively present (preformed defence molecules) and those which are newly synthesized after an elicitation process e.g. after infection. During and after infections, de novo synthesis of phenolics as well as phenol-oxidizing enzymes (phenoloxidases, peroxidases) play a pivotal role in defence in at least a triplicate way:

- the function in wound healing and active defence involves the formation of barriers by melanin-type polymers by phenoloxidases and superoxide production by a cooperative phenolase – photosystem I – dependent redox cycling of o-dihydroxy phenylpropanes such as DOPA or dopamin
- the formation of phytoalexins from basic phenylpropanes such as cinnamic acid or phenylalanin and following hydroxylation(s) producing polyphenols and corresponding quinones
- the action of o-quinones as NH_2- or SH- acceptors via a Michael-type addition thus inactivating vital functions of invading microorganisms (ELSTNER *et al.* 1996).

When phenol metabolism is impaired, for example by *Helminthosporium oryzae* toxin, part of the plant resistance against the pathogen is lost (VIDHYASEKARAN *et al.* 1992).

CROSS-TOLERANCE

Several authors (c.f. PELL & STEFFEN 1991) reported on ecological factors and developmental processes connecting stress and oxygen activation. The most important notion is that all these impacts underly feedback to the chloroplast: independent on the site of transport- or metabolic "block", be it in the roots (salt, drought, mineral deficiency), in the transport system (xylem or phloem blocks by infections), in phloem loading (photooxidants, infections), limitations in CO_2 fixation (stomatal closure, lack of Calvin cycle activities due to enzyme inhibition) or in photosynthetic electron transport itself. As a consequence photosynthetic oxygen activation and/or photodynamic processes are provoked, leading to a chain of reactions as outlined by HIPPELI and ELSTNER (1996b) (Figure 3). ROS are, therefore, key components in cross-resistance phenomena. For example ozone which has been shown to act as an abiotic elicitor inducing oxidative stress has been used as a tool for analyzing stress responses in terms of predisposals to pathogen attack (SANDERMANN *et al.* 1998; LANGEBARTELS *et al.* 2000). Some plants have developed protective devices (scavenger systems) which react in the gaseous phase rendering the plant more or less resistant: Isoprene emission, which is observed in many plants under atmospheric conditions favouring tropospheric ozone formation, is such an example of the synthesis of a volatile antioxidant molecule protecting against ozone damage (LORETO *et al.* 2001). Thus, abiotic stressors/effectors may lead to signals within the plant opening metabolic pathways yielding cross-resistance for example tolerance/resistance against pathogens such as virus, bacteria or fungi in the plant, where calcium and ROS have been identified as key messengers of the abiotic signal chain (BOWLER & FLUHR 2000).

For H_2O_2 it has been shown that it interacts with several other signaling systems like plant hormones or Ca^{2+} (PASTORI & FOYER 2002). This observation supports the pivotal role of H_2O_2 in a common stress signaling network.

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