Chapter 5

ROLE OF SALICYLIC ACID IN THE INDUCTION OF ABIOTIC STRESS TOLERANCE

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Abstract: Investigations on compounds capable of reducing the stress sensitivity of crops are of great importance from both the theoretical and the practical point of view. In terms of stress physiology, salicylic acid was first demonstrated to play a role in responses to biotic stress. However, it was gradually found to have more and more effects that could be of importance for other stress factors, and a great deal of evidence has accumulated in recent years suggesting that salicylic acid also plays a role in responses to abiotic stress effects (such as low and high temperature, UV-B irradiation, ozone, heavy metals, etc.). Most papers, on this subject, have reported on the protective effect of exogenous salicylic acid against abiotic stress. When applied in satisfactory concentrations salicylic acid may cause a temporary low level of oxidative stress in plants, which acts as a hardening process, improving the antioxidative capacity of the plants and helping to induce the synthesis of protective compounds such as polyamines. Numerous mutant or transgenic plants are now available in which the salicylic acid metabolism has been modified in some way. These allow us to obtain a more accurate picture of the endogenous effect and role of salicylic acid. Evidence now suggests the existence of a regulatory defence mechanism in which salicylic acid plays an important role, but which is not stress-specific, apparently functioning against many different stress factors. This chapter provides a review of the effects exerted by salicylic acid and related compounds in relation to abiotic stress tolerance.

Key words: Abiotic stresses, oxidative stress, salicylic acid, signal transduction.

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1. INTRODUCTION

The name of salicylic acid (SA) is derived from the word *Salix*, the scientific name of the willow tree. Both the American Indians and the ancient Greeks knew that the leaves and bark of willow trees could be used as a painkiller and antipyretic (one has the feeling that they discovered everything, but their knowledge lost and we have to start again from the beginning). Salicilin, the glucoside of salicylic alcohol, was isolated from willow bark in 1828. Acetyl SA, commercially known as aspirin, is one of the best known "antistress compounds" used by human beings.

SA is generally present in plants in quantities of a few μg/g fresh mass or less (Raskin *et al.,* 1990), either in a free state or in the form of glycosylated, methylated, glucose-ester or amino acid conjugates (Lee *et al*., 1995). It can be detected in the largest quantities in thermogenic flowers at flowering, or after pathogenic infection (Raskin, 1992). The biosynthesis of SA starts from phenylalanine and follows one of two known paths of synthesis, one of which involves trans-cinnamic acid and the hydroxylation of benzoic acid (BA). Feeding tobacco leaf tissue with putative precursors showed that only BA was capable of increasing tissue levels of SA, suggesting that BA is a direct precursor of SA in tobacco (Raskin, 1995). However, later results indicated that benzoyl glucose, a conjugated form of BA, is more likely to be the direct precursor (Yalpani *et al.,* 1993; Chong *et al.,* 2001). The cinnamic acid-derived synthesis of SA also takes place in cucumber, potato and rice. The other possible pathway is the formation of ortho-coumaric acid (ortho-hydroxy-cinnamic acid; oHCA) from trans-cinnamic acid, followed by a chain-shortening reaction leading to SA**.** The latest results show that higher plants may produce pathogen-induced SA from isochorismate, a biosynthetic pathway typical of bacteria (Wildermuth *et al.,* 2001).

The first plant physiological processes in which SA was reported to play a role were growth regulation (DeKock *et al.,* 1974) and flower induction, which was demonstrated in a long-day *Lemna gibba* L. strain (Cleland, 1974; Cleland and Ajami, 1974). Since then both endogenous and exogenous SA have been shown to have various effects (Raskin, 1992), but these cannot always be generalised, as the studies were carried out on various plant species in various systems (from the whole plant to cell suspensions). It has been demonstrated that SA and other phenolic compounds may influence the uptake of numerous ions. In the case of barley the uptake of phosphate (Glass, 1973) and potassium ions (Glass, 1974a) was inhibited in the presence of SA, probably due to the depolarisation of cell membranes (Glass, 1974b). There was a substantial decrease in transpiration in bean leaves after treatment with 1 or 10 mM SA (Larque-Saavedra, 1978, 1979). It has been demonstrated that SA and related compounds are capable of

inhibiting the abscisic acid (ABA) induced stomatal closure (Rai *et al*., 1986). Long-term SA treatment was observed to reduce the quantity of the Rubisco enzyme in barley plants, thus inhibiting photosynthetic activity (Pancheva and Popova, 1998). When wheat plants were treated with SA for seven days, it was found that, while low (0.05 mM) concentrations of SA promoted photosynthesis, higher quantities (0.5–1.0 mM) inhibited photosynthetic activity, principally due to the inhibition of PSI electron transport and to a reduction in the level of cytochrome f_{554}. No effect was observed, however, when isolated thylakoids were treated with SA (Sahu *et al*., 2002).

SA was able to stimulate the adventitious root primordia of bean plants (Kling and Meyer, 1983), while in maize shoots the *in vivo* nitrate reductase activity was found to increase after treatment with 0.01–0.1 mM SA (Jain and Srivastava, 1981). It is probable, however, that this increase in activity is an indirect effect arising from the inhibition of enzyme inactivation. Another well-known effect of SA is that it increases the temperature of certain thermogenic plants. The production of heat by plants was first described by Lamarck in 1778 in a study on *Arum* species, but since then many cases have been reported, mainly in members of the Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae, Nymphyaceae and Palmae families (Meeuse and Raskin, 1988). In some flowering *Arum* species, the oxygen uptake when heat production is most intense may be as great as the oxygen consumption of a flying hummingbird (Lance, 1972). Heat production in these species serves principally to aid the exudation of perfumes. During certain parts of the flowering period the temperature of the flower may rise by as much as 12°C. It was demonstrated in the species *Sauromatum guttatum* S. that the calorigenic substance responsible for the induction of heat production was identical to SA (Raskin *et al*., 1987). Evidence was found to show that the increase in alternative oxidase expression as the result of SA played a role in the induction of thermogenesis (Rhoads and McIntosh, 1992). However, SA was also shown not only to induce cyanide-resistant respiration but also to block electron flow from the substrate dehydrogenases to the ubiquinone pool in isolated mitochondria, and acted as an uncoupler of the mitochondrial electron transport chain (Norman *et al.,* 2004).

The role of SA in the signal transduction processes of biotic stress tolerance has already been widely studied. It is involved in the development of the hypersensitive reaction (HR): in tobacco leaves infected with tobacco mosaic virus and there is an increase in the level of endogenous SA in the necrotic lesion and surrounding tissues (Enyedi *et al*., 1992). The external application of SA induces the expression of pathogenesis-related (PR) proteins in tobacco (Malamy *et al*., 1990; Yalpani *et al*., 1991) and in rice

(Rakwal *et al*., 2001). A large body of evidence indicates that SA is also required for the development of systemic acquired resistance (SAR). The level of endogenous SA increased in cucumber plants when acquired resistance developed (Métraux *et al*., 1990). Transgenic tobacco plants, incapable of accumulating SA due to the presence of a salicylate-hydroxylase enzyme gene (*NahG*) of bacterial origin, were unable to develop systemic acquired resistance (Gaffney *et al*., 1993). Nevertheless, SA does not appear to be the signal molecule transported from the site of infection to more distant tissues, though the accumulation of SA in the given tissues is essential if SAR is to develop (Vernooij *et al*., 1994).

There is an increasing body of evidence suggesting that SA is involved not only in biotic stress, but also in abiotic stress. Most work has been done on the protective effect of exogenously applied SA. A summary will be given in the next chapter of the relationship between SA and various abiotic stress factors, followed by a summary of the known physiological and biochemical effects of SA that may explain the change in stress tolerance.

2. STUDIES ON THE EFFECT OF EXOGENOUS SALICYLIC ACID

2.1 Toxic metals

Heavy metal ions play an important role in many metabolic processes, making them essential in trace element quantities for the metabolism, growth and development. Heavy metals are only able to exert any stimulatory or inhibitory effect on plants if they are present in a form available to the plants. Availability is influenced by a range of abiotic and biotic factors. The abiotic factors include the ionogenicity of the toxic metal, its solubility in water, and its ability to form complexes, and the pH and redox potential of the soil. The biotic parameters include the protons and organic acids (e.g. citric acid, amino acids) exuded into the rhizosphere from plant roots, the symbiosis of higher plants with mycorrhizal fungi, and the quantity of humic acids and humin present in the soil as a result of organic matter decomposition. Problems only arise when the cells encounter a higher concentration of heavy metal ions, which cause cell damage. One aspect of heavy metal toxicity is the inactivation of biomolecules, either through the blockage of functional groups or through the exchange of vital ions. A further source of danger is the autooxidation of the heavy metals and the formation of reactive oxygen species (ROS), which may also damage the cells due to the Fenton reaction.

Both plants in natural ecosystems and cultivated crops can be divided into two major groups, based on the ability or inability of the plants to adapt to heavy metal ions. In plants, sensitive to heavy metals and unable to adapt, cell damage, or in severe cases cell death, may be caused by a number of mechanisms: i) certain heavy metal ions, readily exchanged, for the essential metal ions in the active centre of enzymes (e.g. Mg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}), thus inhibiting the regulatory role of the enzymes, ii) they may prevent the maturation of nucleic acids by inhibiting the splicing process, iii) heavy metal ions may form free radicals, leading to the lipid peroxidation of cell membranes thus cause them to lose their ion permeability. Plant species tolerant of heavy metal ions have various mechanisms to ensure their survival. These include the inhibition of metal ion uptake, biochemical or enzymatic modifications of the root surface, the binding of metal ions to cell wall sections, the transportation of metal complexes to the vacuoles for storage, the formation of less toxic compounds by means of methylation, and the excretion or secretion of heavy metal ions. Some of the most important compounds involved in heavy metal tolerance are metal-binding proteins and phytochelatins. The former are cysteine-rich, low-molecular-mass proteins, found mostly in animals, capable of neutralising toxic heavy metal ions by forming mercaptide complexes, while the latter are peptides occurring in plants and yeasts and contain glutamic acid in a γ-bond, allowing them to form thiolate bonds with heavy metal ions and thus protect plant cells from damage. Phytochelatins are important not only due to their metal-binding capacity, but also because they transport the bound metal ions from the cytoplasm into the vacuoles, where they can be stored as non-toxic form, bound to organic acid ligands.

One of the earliest works to report on the protective effect of SA against abiotic stress factors, dealt with heavy metals. SA application at a concentration of 0.1 or 0.2 mM reduced the inhibitory effect of Pb^{2+} and Hg^{2+} on the seed germination and seedling growth of two rice (*Oryza sativa* L.) cultivars (Mishra and Choudhuri, 1997). SA increased the fresh and dry mass of shoots and roots in both cultivars under heavy metal stress conditions. The higher concentration of SA was more effective, as evident from a better recovery from metal-induced growth inhibition. SA moderated the inhibitory effect of lead on the activity of the nitrate reductase enzyme in maize (*Zea mays* L.) plants (Sinha *et al*., 1994). SA-induced aluminium tolerance was also reported in *Cassia tora* L. plants, while Al increased the SA concentration in the roots (Yang *et al.,* 2003). Increased citrate efflux due to the SA treatment was associated with decreased inhibition of root growth and of Al content in the root tips. The results suggest that exogenous SA was able to confer Al tolerance by increasing the citrate efflux, not by increasing the citrate synthase activity and citrate concentration in the root

tips, which remained unaffected by the treatment (Yang *et al.,* 2003). This suggests that SA has some role in the tolerance of plants to heavy metal stress, though other authors found no increase in the endogenous SA content as the result of Cd treatment, or any difference in the SA content of sensitive and resistant plants of *Salix viminalis* in the control (Landberg and Greger, 2002).

The preliminary treatment of barley plants with SA was found to prevent the lipid peroxidation induced by 25 μM cadmium, thus increasing the fresh shoot and root mass. However, this protective effect was not due to an increase in the antioxidant capacity. The increased activity of antioxidant enzymes observed in untreated plants during cadmium stress could not be detected in plants, preliminarily treated with SA (Metwally *et al*., 2003). Other results indicate that, although SA reduced the cadmium uptake of the roots, the compound itself stressed the plants; so preliminary treatment with SA could aggravate the damaging effect of cadmium (Pál *et al.,* 2002). At the same time, the endogenous SA content exhibited a concentration-dependent increase in maize plants, treated with cadmium (Pál *et al*., 2005). Higher cadmium concentrations (0.025 mM or more) triggered an approximately 3-fold accumulation of free and conjugated BA and SA, with a higher amount in the bound form. However, the accumulation was not of the same magnitude for SA and BA. The accumulation of conjugated forms of BA indicates the rapid conversion of free forms to conjugated forms. The large-scale accumulation of bound BA can also be explained by the lower rate of SA biosynthesis. The accumulation of free and bound oHCA was also observed in Cd-treated leaves. Among the phenolic compounds the highest accumulation was found in the case of bound oHCA in the leaves. Since oHCA has been demonstrated to have antioxidant properties (Foley *et al.,* 1999), these results, therefore, suggest that the increase in the oHCA content was induced independently of the SA biosynthesis, but may play a role in the antioxidative response to cadmium. The increased endogenous SA levels in the leaves of maize seedlings may be associated with the oxidative stress observed in the leaves of Cd-stressed plants, suggesting a role for SA in the response of maize to cadmium.

Worldwide, several hundreds plant species are now known to hyperaccumulate various trace elements, including heavy metals in their shoots when growing in their native habitats (Freeman *et al*., 2005). The extraordinary ability of these plants to hyperaccumulate Ni/Zn makes them an ideal source of genetic material for the development of both mineral nutrient-fortified crops and plants suitable for the phytoremediation of metal-polluted soils and waters (Guerinot and Salt, 2001). Heavy metal tolerance is often correlated with intracellular compartmentization (Brune *et al*., 1995). Nickel hyperaccumulation is usually due to a highly efficient

pumping system that transfers the metal to the central vacuole of the shoot cells, leading to a high level of tolerance to this element (Krämer *et al*., 2000). It is clear from cellular Ni distribution studies (Krämer *et al*., 2000), that a substantial amount of cellular Ni also accumulates outside the vacuole, suggesting the need for a cytoplasmic-based tolerance mechanism. Nicotianamine may also play an important role in the detoxification of extravacuolar Ni in hyperaccumulating plants (Vacchina *et al*., 2003). Due to the constitutively enhanced activity of serine acetyltransferase, glutathione concentrations in Thlaspi hyperaccumulators are also constitutively elevated, leading to enhanced tolerance to Ni-induced oxidative stress (Freeman *et al*., 2004). In a later experiment, it was also proved that the glutathione-mediated Ni tolerance mechanism observed in Ni-hyperaccumulating Thlaspi species is signalled by the constitutively elevated levels of SA. It was also observed that both biochemical and genetic manipulations that increase SA in *Arabidopsis thaliana* (L.) Heynh plants mimic the glutathione-related phenotypes of the hyperaccumulating *Thlaspi*, and that these biochemical changes in the non-accumulator are associated with increased glutathione-mediated Ni resistance. Such observations suggest that SA may be one of the regulators involved in coordinating certain key biochemical differences between Ni/Zn hyperaccumulators and non-accumulator plant species.

2.2 Drought

Plants are exposed to drought stress when there is not sufficient water available, or when, for some other reason, the water present cannot be taken up by the plants, e.g. if the ground is dry, if there is intense evaporation or severe frost, or if the soil has a high salt content, leading to strong osmotic water-binding. The first, most sensitive sign of water deficiency is a reduction in turgor, leading to the retardation of growth processes, especially lengthwise growth. Drought stress also reduces photosynthesis, for a number of reasons: i) hydroactive stomatal closure reduces the CO_2 supply to the leaves, ii) water deficiency damages the cytoplasm ultrastructure and enzyme activity, iii) dehydrated cuticles, cell walls and plasma membranes are less permeable to CO_2. An analysis of the correlation between drought and the carbohydrate metabolism reveals that one characteristic symptom of water deficiency is the mobilisation of the starch stored in the chloroplasts. As there is also a reduction in the translocation of carbohydrates during drought stress, this leads to a change in source-sink relationships.

Although ion uptake and water uptake are not closely related, water deficiency has also been found to jeopardise nutrient uptake. This can be attributed to the fact that when the soil moisture content is low, there is a

reduction in water migration and thus in the quantity of ions transported by the water. A further difficulty arises due to the retardation of root growth in dry soil, with a consequent reduction in ion uptake. The negative effect of drought stress is first felt in the uptake of phosphorus from the upper soil layers. Water deficiency also has a major effect on the nitrogen metabolism. There is a considerable decline in protein synthesis in water-deficient plants, due to the reduced number of polysome complexes in tissues with a lower water content. Another noticeable change is the decline in nitrate reductase activity. Parallel with the drop in water potential, there is a reduction in the intensity of transpiration and in the quantity of nitrate transported by water flow, in the xylem, resulting in lower nitrate content and nitrate reductase activity in the leaves. Another characteristic symptom of drought stress is the formation and accumulation of ABA. The biosynthesis of this stress hormone is induced by the turgor reduction, arising due to water loss. ABA-dependent and ABA-independent signal transduction chains have been shown to function between the primary signal, induced by drought or cold stress and the expression of specific genes. One pathway of ABA-dependent signal transduction systems requires protein synthesis, while the other does not. The regulation of one ABA-independent pathway, on the other hand, involves a dehydration responsive element in the case of both drought and salt- or cold-induced stress, while the other pathway is only initiated by drought or salt stress. The genes induced by dehydration also control the genes responsible for the signal transduction pathway of the response to drought stress. One group contains proteins involved in stress tolerance (e.g. water channel proteins, proteins protecting macromolecules and membranes, such as LEA proteins or chaperones), while the other contains proteins, participating in signal transduction and gene expression (e.g. protein kinases, transcription factors, phospholipase C).

When wheat seeds were soaked in acetyl SA (which may be degraded into SA in aqueous solution) the plants had better resistance to drought stress (Hamada, 1998; Hamada and Al-Hakimi, 2001). Soaking in 100 ppm acetyl SA for 6 h, before sowing not only allevated the inhibitory effects of drought but also had a stimulatory effect, as both the dry matter gain in the shoots and roots and the transpiration rate showed a marked increase. Treatment with ascorbic acid or thiamine had a similar protective effect, which was attributed to the protection of the photosynthetic apparatus from oxidation and the retardation of dark respiration (Hamada, 1998). In another experiment, irrespective of the SA concentration (1-3 mM) and the level of water stress, plants treated with SA generally exhibited higher moisture content, dry mass, carboxylase activity of Rubisco, superoxide dismutase (SOD) activity and total chlorophyll, compared to untreated seedlings (Singh and Usha, 2003). In the case of water stress, SA treatment protected nitrate

reductase activity and maintained the protein and nitrogen contents of the leaves, compared to water-sufficient seedlings. The results signify the role of SA in regulating the drought response of plants and suggest that SA could be used as a potential growth regulator to improve plant growth, under water stress.

Both SA and acetyl SA proved effective in protecting tomato and bean plants against drought stress at concentrations of 0.1 mM and 0.5 mM. Above and below this concentration range, however, no positive results were recorded (Senaratna *et al*., 2000). It is interesting to note that protection was afforded by SA or aspirin against not only drought, but also against low and high temperature stress, irrespective of whether the seeds were soaked for 1 day, or whether two-week-old plants were treated, either through the soil or in the form of spraying. Among several other plant growth substances, for example brassinolide, methyl jasmonic acid (methyl JA), ABA, 1-aminocyclopropane-1-carboxylic acid (ACC), 2-chloro-ethylphosphonic acid (ethephon), gibberellic acid and kinetin (but not indoleacetic acid or zeatin), SA also improved the protoplasmic drought tolerance of free-cell suspensions prepared from fully turgid leaves of *Sporobolus stapfianus* (Ghasempour *et al.,* 2001). In maize, however, although a 1-day preliminary treatment with 0.5 mM SA increased the polyamine content of the plants, drought tolerance was not improved; in fact, plants treated in this way became more susceptible to drought (Németh *et al*., 2002). A negative effect was also recorded when wheat plants were treated in this way, suggesting that the effect of SA is influenced by the method of treatment and by the developmental stage of the plant.

The SA level in leaves of *Phillyrea angustifolia* L. plants exposed to drought showed a strong negative correlation with the relative water content, and increased progressively to as much as 5-fold during drought (Munne-Bosch and Penuelas, 2003). During recovery, SA levels decreased, but remained slightly higher than those observed before drought. SA levels were positively correlated with those of □-tocopherol during drought, but not during recovery. This result also indicates the possible role of endogenous SA in the induction of a protective mechanism during water stress.

In another experiment the effect of moderate or severe water deficit on the SA content in the leaves and roots, and the effect of pre-treatment with SA on the response to water stress were evaluated in barley plants (Bandurska and Stroinski, 2005). Water deficit increased the SA content in the roots, whereas the SA content in the leaves did not change. Plant treatment with SA before stress reduced the damaging effect of water deficit on the cell membrane in the leaves. SA treatment increased the ABA content in the leaves of the studied genotypes. An increase in the proline level was observed only in the wild species *Hordeum spontaneum*. The results suggest

that ABA and proline may contribute to the development of the antistress reactions, induced by SA.

2.3 Heat tolerance

Plants growing in cold regions of the world are much more sensitive to heat than those from the temperate zone, which again are less resistant to heat than those indigenous in the tropics. Heat-sensitive species suffer damage at temperatures of 30–40°C, while heat-tolerant plants are capable of hardening and can tolerate 50°C for a considerable length of time. One common defence against overheating is transpirational cooling, which is only possible if sufficient water is available. The primary effect of high temperature is to alter protein conformations and membrane status. In plants, as in other living organisms, the synthesis of most proteins slows down at temperatures well above optimum, and may cease altogether if the temperature continues to rise. At the same time, the transcription and translocation of heat shock proteins (HSPs) is stimulated. Heat shock proteins belong to multigene families, most of which are regulated by high temperature, while a small number are induced by other abiotic stress factors. Proteins known as chaperones are responsible for the secondary and tertiary structure of the polypeptides synthesised in the cell, for linking up the subunits and for transporting them to the required cell component. If this complex process is disturbed, for example by high temperature, newly synthesised proteins form insoluble aggregates. It is currently thought that the majority of stress proteins are molecular chaperones, most of which have been identified as HSPs. The thylakoid membranes of the chloroplasts are the most important for heat sensitivity, so one critical aspect of heat tolerance in plants is the continual maintenance of photosynthesis. Consequently, one of the first signs of high temperature stress is a reduction in photosynthetic activity. Recent research has proved that many HSPs are transported into the chloroplasts, where they promote the heat tolerance of the photosynthetic system.

The capacity to survive heat shock varies with the plant species and genotype, and also with the developmental stage. Plants may have basal thermotolerance in the absence of pre-adaptation. In addition, plants subjected to mild heat stress may transiently acquire tolerance to previously lethal high temperatures: this phenomenon is known as acquired thermotolerance or heat acclimatisation, and is probably an adaptation to the gradual increases in temperature in the natural environment (Clarke *et al.,* 2004).

The first paper to demonstrate the effect of SA on heat tolerance showed that spraying with SA improved the heat tolerance of mustard plants and that

this effect was concentration-dependent: SA only exhibited a protective effect at low concentrations (0.01–0.1 mM). Both, treatment with 0.01 mM SA and hardening at 45°C for 1 h led to an increase in the H_2O_2 level and a reduction in catalase activity (Dat *et al*., 1998a). The role of SA in the signal transduction process of heat tolerance development is also confirmed by the increase in the level of endogenous bound and free SA during the heat acclimatisation of mustard plants (Dat *et al*., 1998b). When tobacco was grown *in vitro* for 4 weeks on medium containing SA, low concentrations (0.01 mM) were found to increase heat tolerance, while a concentration of 0.1 mM no longer had any protective effect (Dat *et al*., 2000). The endogenous glucosylated SA content was enhanced in the shoots of plants grown on 0.01 or 0.1 mM SA, while free SA was also enhanced in those grown on 0.1 mM SA. The shoot H_2O_2 content increased, while the catalase activity declined as the SA concentration in the medium increased. In a similar manner, 0.01 mM acetyl SA was able to improve the heat tolerance of potato in tissue culture, while there was an increase in the endogenous H_2O_2 level in the plants (Lopez-Delgado *et al.,* 1998, 2004). It was also shown that potato microplants grown from explants incubated for 1 h in 0.1-50 mM H_2O_2 exhibited a concentration-dependent decrease in stem height, but were significantly more thermotolerant than the controls, even more than a month after the H_2O_2 treatment, showing that not only SA but also H_2O_2 is able to increase the heat tolerance of potato plants. SA treatment was also found to reduce the oxidative damage caused by heat stress in *Arabidopsis* plants. In addition to SA, ethylene, ABA and calcium have also been shown to play a role in the development of tolerance to high temperatures (Larkindale and Knight, 2002).

The efficacy of heat acclimation and SA treatment applied as a 0.1 mM foliar spray in inducing thermotolerance was also tested in *Cicer arietinum* L. plants (Chakraborty and Tongden, 2005). A substantial reduction in the relative level of membrane injury was observed in plants pre-treated with SA in comparison to heat-acclimatized and untreated control seedlings subjected to lethal temperature treatment. Both treatments resulted in an increase in the protein and proline contents over the control seedlings, and led to the induction of peroxidase and ascorbate peroxidase (Apx), while there was a reduction in catalase activity.

Turf quality often declines during summer when temperatures exceed the optimum range. Physiological measurements, including turf quality, leaf photosynthetic rate and levels of oxidative damage demonstrated that among several other signaling compounds, such as ABA, $CaCl_2$, H_2O_2 or ACC, the foliar application of SA increased the heat tolerance of creeping bent-grass (*Agrostis stolonifera* var. palustris). The better heat tolerance of pre-treated plants as compared to the control was related to the protection from

oxidative damage under heat stress: although SA pre-treatment had no effect on peroxidase activity and the catalase activity was lower than in the control plants, it increased the APx activity (Larkindale and Huang, 2004). In another experiment involving SA concentrations from 0 to 1.5 mM, 0.25 mM most effectively enhanced heat tolerance in Kentucky bluegrass (*Poa pratensis* L.), which was manifested by improved re-growth potential following heat stress at 46°C for 72 h and the maintenance of leaf water content. Contrary to the results obtained in mustard, potato or creeping bent-grass species, increased SOD and catalase activities were observed under heat stress after SA application in Kentucky bluegrass (He *et al.,* 2005). A primary economic concern of sod producers is the loss of sod quality during the transportation and storage phases of a sale. Unfortunately, even when proper cultural guidelines are followed, excessive sod heating and tissue damage often occurs. The foliar application of 0.5 kg ha^{-1} SA enhanced the photochemical efficiency of the pre-harvest canopy in both Kentucky bluegrass and tall fescue (*Festuca arandinacea* Schreb.) sod (Ervin *et al.,* 2005). SA also reduced visual injury and enhanced post-harvest root strength, suggesting that pre-harvest foliar SA application may improve the shelf life and transplant success of supraoptimally heated cool-season sod.

Another type of study investigates the effect of a lack of SA in transgenic plants or in plants with a mutation affecting SA biosynthesis. *NahG* transgenic plants carry a salicylate-hydroxylase gene of bacterial (*Pseudomonas putida*) origin, which prevents them from accumulating SA, as the salicylate-hydroxylase enzyme converts SA into catechol (Gaffney *et al*., 1993). It was found that after heat stress *NahG Arabidopsis* plants became more sensitive to oxidative damage caused by high temperature than non-transformed plants with a normal level of SA. Furthermore, exogenous SA pre-treatment for 1 h enhanced survival and reduced the level of thiobarbituric acid-reactive substances (TBARS), the indicator of oxidative damage to membranes, in *Arabidopsis* plants after a 40°C heat treatment (Larkindale and Knight 2002). However, it has recently been shown that catechol, the product of SA degradation in *NahG* plants, induces susceptibility to pathogens in wild-type *Arabidopsis* plants, most probably due to catechol-mediated H_2O_2 production (van Wees and Glazebrook 2003). It is thus important to check, when evaluating the experiments, that the observed changes were not caused by the catechol produced during the decomposition of SA.

To investigate the importance of different processes in the development of heat stress tolerance, *Arabidopsis* mutants and one transgenic line were tested for basal and acquired thermotolerance at different stages of growth (Larkindale *et al.,* 2005). The plants tested either had defective signalling pathways (ABA, SA, ethylene, and oxidative burst signalling) or reactive

oxygen metabolism (ascorbic acid or glutathione production, catalase), or had previously been found to have temperature-related phenotypes (e.g. fatty acid desaturase mutants, the UV-sensitive mutant, *uvh6*). The mutants were assessed for thermotolerance defects in seed germination, hypocotyl elongation, root growth and seedling survival. To assess oxidative damage and alterations in the heat shock response, TBARS, HSP 101, and small HSP levels were determined. ABA signalling mutants and *uvh6* showed the strongest defects in acquired thermotolerance of root growth and seedling survival. Mutations in NADP oxidase homologue genes, ABA biosynthesis mutants and *NahG* transgenic lines showed weaker defects. Ethylene signalling mutants and reactive oxygen metabolism mutants were more defective in basal than acquired thermotolerance, especially under high light. All the mutants accumulated wild-type levels of HSP 101 and small HSP. These data indicate that, apart from HSP induction, ABA, active oxygen species and SA pathways are involved in acquired thermotolerance and that UVH6 plays a significant role in temperature responses, in addition to its role in UV stress.

However, it was shown, using *Arabidopsis* genotypes with modified SA signalling that SA-dependent signalling plays a role in the maintainance of basal thermotolerance: 0.5 or 1 mM SA pre-treatment promotes basal thermotolerance in 3-week-old *Arabidopsis* plants, together with the induction of PR proteins, demonstrated by the less pronounced electrolyte leakage from the leaves after heat stress (Clarke *et al.,* 2004). The level of endogenous SA correlated with basal thermotolerance. Recovery from heat shock apparently involved an NPR1-dependent pathway but thermotolerance during heat shock did not. It was also shown that SA is not essential for acquired thermotolerance: all the genotypes could be heat acclimated, irrespective of their endogenous SA content. It may be that SA is dispensable because heat acclimation is always initiated by some other key factor(s), or because SA is only one of multiple alternative acclimation signals. If SA were a potential mediator of a heat-induced acclimation response, SA levels should be heat-inducible. A moderate, transient, but statistically significant increase in glucosylated SA during heat treatment was also shown in wild-type *Arabidopsis* plants. Given the significant basal levels of SA in *Arabidopsis* and mustard, the extent to which such changes in the SA metabolism provide additional thermotolerance is uncertain, but they suggest that despite the metabolic stress of heat treatment, the plant actively maintains biosynthesis of this hormone. In plants, SA is subject to glucosylation, which might be involved in transport (Seo *et al*., 1995a) or vacuolar localization (Dean *et al*., 2003). Heat-induced SA increases were not apparent in the other *Arabidopsis* genotypes, although this might be because of their altered SA metabolism or signalling (Clarke *et al.,* 2004).

2.4 Cold stress

Temperature is one of the major determinants of the occurrence and spread of natural plant associations. For crops, too, low temperature is one of the most important factors restricting cultivation in a given area. Plants are said to be cold-sensitive if they die or suffer severe damage at temperatures between 0 and 15°C. Cold-tolerant plants, on the other hand, are still able to grow near freezing point and are capable of surviving temperatures as low as 10–15°C below zero. Apart from genetic factors, cold sensitivity also depends on the stage of development and the level of metabolic activity. Plants are more sensitive to cold in the early phases of development, during the day, in bright light, under dry conditions and in the case of potassium deficiency, for example. Results achieved so far suggest that differences in membrane composition make a substantial contribution to sensitivity to low temperature stress. The quantity of unsaturated fatty acids has been shown to be much lower in cold-sensitive plants than in those tolerant of low temperatures. The duration and intensity of the cold effect is also an important factor. If the plant is only exposed to cold for a short period, the process is reversible and no serious damage is incurred. If, however, the cold is severe or continues for a long period, cell damage is accelerated and the process becomes irreversible. The rate at which cooling takes place also has a considerable influence. If cooling is slow, the hardening process prepares the plant to survive frost and winter weather. This is of great importance in winter cereals, for example. Sudden frost stress is a more severe form of low temperature stress, which causes the membranes to lose their semipermeability and thus their active ion transporting ability. The phospholipids begin to decompose, a phase transition occurs and the distribution of the membrane proteins changes. The greatest danger is the formation of ice crystals within the cells. Plants growing in areas exposed to long-term cold survive either by avoiding temperatures below the freezing point or by tolerating freezing. The most important way of avoiding freezing is the accumulation of osmolytes induced by low temperature (sugars, polyalcohols, amino acids, polyamines, quaternary ammonium compounds, etc.), which cause the freezing point within the cell to drop. This process prevents the dehydration of the cytoplasm as the result of frost. Plants such as perennials and conifers tolerate frost through extracellular ice formation in the apoplasts, where it does not have the lethal consequences it would have within the cell. Recent research has proved that the antifreeze proteins demonstrated in many plant species are capable of protecting the cell by preventing the formation of large crystals.

SA and other phenol derivatives are known to improve the cold tolerance of plants. It was shown that the addition of 0.5 mM SA to the hydroponic

growth solution of young maize plants under normal growth conditions provided protection against subsequent low-temperature stress. Besides the obvious visual symptoms this observation was confirmed by chlorophyll fluorescence parameters and electrolyte leakage measurements from the leaves (Janda *et al.,* 1997, 1999). Among the antioxidant enzymes, the activity of catalase decreased, while that of glutathione reductase and guaiacol peroxidase increased as a result of the preliminary treatment with SA. These changes might explain the increased cold tolerance. Since it was earlier shown that the chilling-induced level of ACC, the precursor of ethylene, is negatively correlated with the cold tolerance of maize plants (Janowiak and Dörffling, 1996), an investigation was also made on changes in ACC and malonyl ACC (MACC) after chilling stress. Chilling at 5°C caused an increase in ACC content; however, this increase was less pronounced in cold-acclimated plants, and in those which were pre-treated with SA for 1 d, before cold stress. Changes in MACC at low temperature showed no correlation with chilling tolerance in maize (Szalai *et al.,* 2000). Further studies proved that not only SA, but also related compounds, such as BA, aspirin or coumaric acid, may have a protective role against chilling stress in young maize plants (Janda *et al.,* 1998, 2000; Horváth *et al.,* 2002). It should be mentioned, however, that these compounds may cause severe damage to plants at normal growth temperature. There is a decrease in net photosynthesis, stomatal conductivity and transpiration after 1 day of SA, BA or aspirin treatment under normal growth conditions (Janda *et al.,* 1998, 2000). These compounds may cause a decrease in the growth rate in young, developing maize seedlings. In contrast to this, the foliar application of SA or aspirin enhanced photosynthetic rates in both soybean and maize (Khan *et al.,* 2003). Stomatal conductance and transpiration were also increased. Other stimulating effects of SA were reported in embryogenic cell suspension cultures of *Coffea arabica* treated with picomolar concentrations of SA (Quiroz-Figueroa *et al.,* 2001).

In a later work, the chilling tolerance of leaves or hypocotyls was significantly increased by the application of 0.5 m*M* SA not only in maize, but also in cucumber and rice (Kang and Saltveit 2002). This was manifested as a reduction in the chilling-induced electrolyte leakage from excised maize and rice leaf discs, and from excised cucumber hypocotyl segments in plants pre-treated with SA. However, SA treatment did not significantly alter the electrolyte leakage from radicles excised from any of the chilled seedlings. Obviously, exposure to SA was a severe shock to the seedlings even at concentrations as low as 0.5 mM, as indicated by the significant reduction in subsequent radicle growth. Other studies confirmed that the addition of exogenous SA to the hydroponic solution may cause severe damage to the roots (Pál *et al.,* 2002). This severe inhibitory effect on subsequent radicle

growth was observed when the seedlings were exposed to SA solutions after being inhibited in water and growth on capillary cloth, moistened with water. By contrast, exposure to aerated aqueous SA solutions during imbibition and growth did not significantly affect the rate of radicle growth at 25°C in cucumber seedlings. It is assumed that seedlings exposed to SA from germination may become acclimated to SA and did not perceive it as a stress or stress signal (Kang and Saltveit, 2002).

Pre-treatment of the leaves of chilling-sensitive banana seedlings with 0.5 mM SA solution by spraying the foliage or irrigating the roots for 1 day, induced an increase in chilling tolerance during subsequent 5°C chilling stress (Kang *et al.,* 2003a). These treatments did not result in any great change in the SOD activity, but reduced the catalase and APx activity, and increased the peroxidase activity. However, SA pre-treatment caused an activation of SOD, catalase and APx activities during a period of 5°C chilling stress, while it did not change the peroxidase activity. These effects of SA on activities of various protective enzyme could be associated with H_2O_2 metabolism. Measurement of H_2O_2 levels indicated that SA pre-treatment at 30/22°C resulted in H_2O_2 accumulation; however, it reduced H_2O_2 overproduction during the subsequent 5°C chilling stress,. These results suggested that the H_2O_2 metabolism might also be involved in the enhanced chilling tolerance, induced by SA in banana plants (Kang *et al.,* 2003b).

Pre-soaking seeds before sowing could also be an effective way of improving cold tolerance. In tomato and bean plants, 0.1 mM and 0.5 mM concentrations of both SA and acetyl SA proved effective not only against heat and drought stress, but also against low temperature stress (Senaratna *et al*., 2000). Certain salicylates, such as 2,6-dihydroxy BA, aspirin and SA hastened the germination of carrot seeds at 5°C (Rajasekaran *et al.,* 2002). Priming pepper (*Capsicum annuum* L.) seeds imbibed in KNO_3 solution containing aspirin at a concentration range of 0.05-0.5 mM resulted in an increase in the germination percentage, and the germination at low temperature became faster and better synchronated (Korkmaz, 2005). Other hormones, such as methyl JA in the 0.001-0.005 mM range and spermine in the 1-5 mM range, had a similar effect, as shown in both pepper and watermelon seeds (Korkmaz *et al.,* 2004, Korkmaz, 2005). Higher concentrations may have an inhibitory effect on germination.

Another related compound, methyl SA, used at a final vapour concentration of 10^{-4} M for 1 day at room temperature, increased resistance against chilling injury in green bell pepper (*Capsicum annuum* L. cv. Century) freshly harvested on a warm day. Methyl JA application led to similar results (Fung *et al.,* 2004). Increases in respiration, particularly via the alternative pathway, have been observed in response to chilling. An

increase in the steady-state mRNA level of the genes for alternative oxidase has also been reported in rice at low temperature (Ito *et al.,* 1997). These increases may result in increased heat evolution, especially in chilling tolerant species. The increased capacity to produce respiratory heat after exposure to chilling temperatures may contribute to the cold-acclimation process (Moynihan *et al.,* 1995). The alternative respiratory pathway may moderate chilling injury by keeping the production of ROS in balance with the levels of antioxidants and active oxygen-scavenging enzyme systems. The expression patterns of alternative oxidase and of several other genes involved in the defence against oxidative stress before and during the early chilling period suggested that the pre-treatment of pepper fruit with methyl SA or methyl JA vapours increased preferentially the transcript levels of alternative oxidase. The increase in alternative oxidase transcript levels caused by methyl JA or methyl SA before cold treatment was correlated with a reduced incidence of chilling injury.

The positive effect of SA on cold tolerance was shown not only under chilling but also under freezing conditions in winter wheat (Tasgin *et al.,* 2003). Not only cold acclimation, but also exogenous SA used at 0.01, 0.1 and 1 mM concentrations decreased freezing injury in the leaves of plants grown under cold and control conditions. Cold conditions caused an increase in ice nucleation activity by apoplastic proteins, which were isolated from the leaves. Exogenous SA caused an increase in ice nucleation activity under cold and control conditions. These results show that SA can increase freezing tolerance in winter wheat leaves by affecting apoplastic proteins. However, this effect cannot be generalised as experiments with winter rye showed that the apoplastic proteins accumulated after SA treatment had no antifreeze activity (Yu *et al.,* 2001).

The *in vitro* conservation of germplasm is often achieved by reducing the rate of tissue growth to a minimum either by reducing the temperature or by withdrawing a nutrient from the medium or by adding a growth retardant. For the long-term conservation of plant germplasm, cryopreservation is actually a more valuable technique since, when frozen in liquid nitrogen, the metabolism ceases to function, tissues are maintained without growth and genetic alterations that do not take place even during a very long period of storage. Embryonic axes of Persian lilac (*Melia azedarach* L.) encapsulated in calcium alginate beads with sucrose and SA were subjected to the cryopreservation technique, involving dehydration and freezing in liquid nitrogen, or to cold preservation by storing the alginate beads in empty petri dishes for 4 months at 4°C. The 0.2 mM SA significantly enhanced the viability percentage of encapsulated embryonic axes (Bernard *et al.,* 2002). The SA used in these experiments did not interfere with the development of the axes at all; in fact, significantly better growth recovery was monitored on

axes which benefited from protection by SA during cryopreservation, probably due to better conservation of the integrity of the tissues. This suggests that the addition of SA to the encapsulation medium may help to reinforce the tolerance of the tissues, particularly for those that are sensitive to the dehydration caused by conservation procedures.

The growth of *Arabidopsis* plants under chilling conditions could be related to their SA levels (Scott *et al.,* 2004). *NahG* plants and Col-0 wild types grew at similar rates at 23°C, and the growth of both genotypes was slowed by transfer to 5°C. However, at 5°C, *NahG* plants displayed relative growth rates about one-third greater than Col-0, so that by 2 months *NahG* plants were typically 2.7-fold larger. This resulted primarily from greater cell expansion in *NahG* rosette leaves. Specific leaf areas and leaf area ratios remained similar in both genotypes. Net assimilation rates were similar in the two genotypes at 23°C, but higher in *NahG* at 5°C. Chlorophyll fluorescence measurements revealed no PSII photodamage in chilled leaves of either genotype. At 5°C Col-0 shoots accumulated SA, particularly in glucosylated form. A similar tendency was observed in *NahG* shoots at 5°C, but at greatly depleted levels. Growth and SA levels were also examined in SA-signalling and metabolism mutants at 5°C. The partially SA-insensitive *npr1* mutant displayed growth intermediate between *NahG* and Col-0, while the SA-deficient *eds5* mutant behaved like *NahG*. In contrast, the *cpr1* mutant accumulated very high levels of SA at 5°C and its growth was much more inhibited than that of the wild type. At both temperatures, cpr1 was the only SA-responsive genotype in which oxidative damage was significantly different from that of the wild type.

2.5 Salinity

Soils with high salt contents already existed before the appearance of Man, but problems have only arisen since the spread of agriculture, and particularly irrigation. Salt stress affects around 20% of the world's cultivated areas and nearly half of the irrigated land. The ionic equilibrium is disturbed by a high salt concentration, leading to hyperosmotic stress in the plants. The regulation of ion homeostasis is a fundamental criterion for physiological activity in plants. Salt stress destroys the homeostasis in the water potential and ionic distribution at both cell and whole plant level. This drastic change in the equilibrium state causes damage at the molecular level, the cessation of growth and, eventually, to the death of the plant. In addition to these primary effects, oxidative damage is also frequently observed. High salt concentration primarily damages cell membrane integrity, the activity of various enzymes and the functioning of the photosynthetic apparatus.

Plants give a complex molecular response to salt stress. In salt-tolerant (halophytic) plants several mechanisms are available to counteract salt stress, the most important of which are: i) the transportation of sodium and chloride ions from the cytoplasm to the vacuoles with the aid of Na^+/K^+-ATPases, leading to a higher concentration of K^+ ions in the cytoplasm. Recent observations draw attention to the role of non-selective cation channels in regulating the entry of sodium ions into the cytoplasm, and that of certain ion transporters in maintaining the ionic equilibrium, or restoring it after salt stress. ii) The synthesis of the compatible osmolytes mentioned above and the conversion of certain ions into less soluble forms. iii) An increase in the enzyme concentration to overcome the enzyme inhibition caused by higher salt concentrations. iv) Modifications in photosynthesis, respiration and the hormone metabolism. The latter involves increases in ABA and ethylene and decreases in cytokinins.

It has been shown that SA could provide multiple stress tolerance (Senaratna, 2000). Similarly, soaking wheat seeds in SA solution provided protection against not only drought, but also salinity stress (Hamada and Al-Hakimi, 2001). By contrast, SA may promote the formation of ROS in the photosynthetic tissues of *Arabidopsis* plants during salt stress and osmotic stress. The widespread necrotic lesions observed on the shoots of wild-type plants after NaCl or mannitol treatment were not exhibited by *NahG* plants, incapable of SA accumulation (Borsani *et al*., 2001).

SA pre-treatment also provided protection against salinity in tomato plants, probably due to the increased activation of aldose reductase and APx enzymes and to the accumulation of osmolytes, such as sugars, sugar alcohol or proline (Tari *et al.,* 2002, 2004; Szepesi *et al.,* 2005).

The soaking of wheat (*Triticum aestivum* L.) seeds in 0.05 mM SA also reduced the damaging effects of salinity on seedling growth and accelerated the growth processes (Shakirova *et al.,* 2003). The treatment of wheat plants with SA increased the level of cell division within the apical meristem of seedling roots, causing an increase in plant growth and elevated wheat productivity. It was found that SA treatment caused the accumulation of both ABA and indoleacetic acid, but did not influence cytokinin content, while diminishing the changes in phytohormone levels in wheat seedlings, under salinity. A high ABA level was also maintained in wheat seedlings, treated with SA. The SA-induced increase in ABA might contribute to the pre-adaptation of plants to stress, since ABA is known to have a key role in inducting the synthesis of a range of stress proteins ensuring the development of antistress reactions, for example, the maintenance of proline accumulation. The stress-induced accumulation of active oxygen species and, therefore, the level of SOD and peroxidase activity in the roots of young wheat seedlings, pre-treated with SA, were significantly lower than in

untreated plants, indicating that these enzymes contribute to the protective effect of SA on plants under conditions of salination (Sakhabutdinova, 2004).

Certain abiotic stresses, such as salt, cold and drought greatly stimulated the expression of the AcPMP3-1 and AcPMP3-2 genes coding for plasma membrane protein in the monocotyledonous halophyte *Aneurol-epidium chinense*. ABA, H_2O_2 and SA also triggered the expression of AcPMP3 genes. The expression of the AcPMP3-1 gene was able to restore yeast mutants, lacking the Na^+/H^+ antiporter and Na^+-ATPase, Na^+ efflux systems and the transformants accumulated lower amounts of Na^+ than the mutant cells under saline conditions (Inada *et al.,* 2005). These results suggested that AcPMP3-1 acted as a regulator of both Na^+ and K^+ accumulation in the cells. *In situ* hybridization showed that the AcPMP3-1 transcript was localized in the cells of the root cap and root epidermis, making it highly probable that AcPMP3-1 is essential for regulating Na^+/K^+ transportation between plant roots and the outer environment, under salt stress.

An increase in the NaCl level often reduces the germination percentage, the growth parameters and the contents of potassium, calcium, phosphorus and insoluble sugars both in shoots and roots. It may also reduce photosynthetic pigment contents and increase the electrolyte leakage from the cells. SA pre-treatment (grain soaking in 1 mM SA solution prior to sowing) increased the relative water content, the fresh and dry mass, the contents of water, photosynthetic pigments, insoluble saccharides and phosphorus, and peroxidase activity in salt-stressed barley seedlings. By contrast, the Na^+ and soluble protein contents, the lipid peroxidation level and the electrolyte leakage were markedly lower under salt stress with SA than without it. Under stress conditions, plants pre-treated with SA exhibited less Ca^{2+} and more K^+ accumulation and soluble sugars in roots at the expense of these contents in the plant shoots. The application of exogenous SA appeared to induce a pre-adaptive response to salt stress, leading to the promotion of protective reactions to the photosynthetic pigments and the maintenance of membrane integrity in barley plants, which was reflected in an improvement in plant growth (El Tayeb, 2005).

It was also recently shown that salt-induced protein (SALT) was present in the xylem parenchyma cells of vascular bundles in the major and minor leaf veins. The expression of the gene encoding SALT was up-regulated following the treatment with a fungal elicitor, JA, ABA, or NaCl. However, SA alone or in combination with one of the other elicitors not only strongly inhibited SALT gene expression but also exhibited an antagonistic effect in suspension cells and leaves (Kim *et al.,* 2004).

2.6 Ozone stress

Ozone is responsible for more crop losses than any other air pollutant. It is formed through photochemical reactions between nitrogen oxides, carbon monoxide and hydrocarbons released, primarily, through the burning of fossil fuels in urban areas (Mauzerall and Wang, 2001). Ozone production is particularly favoured in summer months by strong sunlight, high temperature and stagnant high-pressure systems and concentrations, therefore, tend to be at their highest during the growing season of most of the world's crop plants. Long-term chronic exposure to ozone can lead to a reduction in growth and crop yield, resulting from the inhibition of photosynthesis, premature senescence, altered biomass partitioning and changes to reproductive processes (Black *et al.*, 2000; Pell *et al.*, 1997; Saitanis and Karandinos, 2002; Sandermann, 1996). Ozone is also able to act as an abiotic elicitor of plant defence reactions and acute exposure can result in the appearance of small necrotic hypersensitive response (HR)-like lesions on foliage (Rao and Davis, 2001). Ozone is toxic to plants and animals because it is a powerful oxidizing agent, which is able to react directly with lipids and proteins. Such reactions and the decomposition of ozone in aqueous environments such as the plant apoplast can lead to the production of other ROS such as the hydroxyl radical, singlet oxygen and H_2O_2 (Kanofsky and Sima, 1991; Mehlhorn *et al.*, 1990; Evans *et al.,* 2005).

The primary site of ozone interaction with plant cells is the extracellular matrix where ozone challenges the antioxidant protection of the cells (Baier *et al.,* 2005). Accordingly, ozone sensitivity is generally correlated with the ascorbate status of the leaf tissue (Conklin & Barth 2004), which can accumulate to millimolar concentrations in leaf apoplasts, where it may scavenge significant amounts of ozone. Ozone sensing takes place through the covalent modification of redox-sensitive components of the plasma membrane, for example ion channels such as the plasma membrane Ca^{2+}-channels. This modification has been characterized as the earliest response to ozone, resulting in an elevation in cytosolic-free calcium, which takes place within seconds of exposure (Evans *et al.,* 2005). Ozone was found to elicit distinct calcium responses in the aerial tissue and roots of seedlings. The calcium response in the cotyledons and leaves was biphasic and sensitive to the rate at which the ozone concentration increased. The response in the root was monophasic and insensitive to the rate of increase in ozone concentration. Experiments utilizing inhibitors of the antioxidant metabolism demonstrated that the magnitude of the first peak in Ca^{2+} in the aerial tissues was dependent upon the redox status of the plant. Seedlings were shown to be able to distinguish between ozone and H_2O_2, producing a Ca^{2+} signal in response to one of these ROS when they had become

refractory to the other. Pre-treatment with ozone altered the Ca^{2+} response to H_2O_2 and vice versa, indicating that the Ca^{2+} response to a given ROS may reflect the stress history of the plant. Subsequent intracellular signal transduction is an intriguing network of hormone, Ca^{2+} and MAPK signalling pathways, significantly overlapping with oxidative burst-induced pathogen signalling. A change in the expression of several genes in response to ozone has been documented (Pell *et al.,* 1997; Sandermann *et al.,* 1998), including the genes of antioxidant enzymes, which may offer some protection against further oxidative stress (Conklin and Last, 1995).

Ozone treatment led to the accumulation of SA, the synthesis of PR protein and the development of virus resistance in tobacco (Yalpani *et al*., 1994). The role of SA in counteracting ozone stress was also demonstrated in *Arabidopsis* plants, where *NahG* plants were more sensitive to the damaging effect of ozone. The synthesis of some of the ozone-induced mRNAs was SA-dependent, so only a few were found in transgenic plants. Plants exposed to ozone exhibited enhanced resistance to virulent *Pseudomonas syringae* strains. The results indicate that there is overlapping between the development of ozone- and pathogen-induced resistance and that both are SA-dependent (Sharma *et al*., 1996). Other authors reported that both a deficiency and an excess of SA caused greater ozone sensitivity (Rao and Davis, 1999). The Cvi-0 *Arabidopsis* genotype, which accumulates SA, is ozone-sensitive, since the large quantity of SA induces oxidative processes during ozone stress, leading to cell death similar to that caused by the hypersensitive reaction. In *NahG* plants, however, which are incapable of accumulating SA, the lack of a satisfactory antioxidant response led to increased ozone sensitivity (Rao and Davis, 1999). In the Cvi-0:*NahG* genotype the lack of SA reduced the level of ozone-induced cell death.

Recent studies demonstrate that ethylene, JA and SA signalling pathways do not act independently, but interact in a complex manner to regulate plant defence responses (Feys and Parker, 2000; Rao and Davis, 2001). Ethylene is a plant hormone that promotes leaf damage in ozone-exposed plants. Ethylene-responsive factors (ERFs) are important in regulating plant pathogen resistance, abiotic stress tolerance and plant development. A tomato ERF protein, TSRF1, was also recently shown to be transcriptionally up-regulated by ethylene, SA or pathogen infection (Zhang *et al.,* 2004). SA inhibits JA biosynthesis and/or JA-dependent gene expression (Gupta *et al.*, 2000). Conversely, JA inhibits the SA pathway in response to wounding (Niki *et al.*, 1998). Pre-treatment with methyl JA hindered the accumulation of SA or H_2O_2, thus preventing ozone-induced necrosis (Rao *et al.,* 2000). The interactions between ethylene, JA and SA that are induced by ozone, and the inhibition of the ethylene or SA pathways by JA have also been investigated. It was shown that active oxygen species mediated SA

biosynthesis and that the SA signalling pathway in the ozone-sensitive ecotype was influenced by the JA pathway (Rao *et al.*, 2000). In addition, plants with deficient SA signalling failed to produce ethylene in response to ozone, indicating that the SA pathway is required for ethylene signalling (Rao *et al.*, 2002). The results of a cDNA macroarray assay indicate that ozone-induced defence gene expression was mainly regulated by ethylene and JA at this stage, and that the SA pathway acts as a strong antagonist to gene expression, induced by ethylene and JA signalling (Tamaoki *et al.,* 2003).

SA biosynthesis has also been investigated during ozone fumigation. When C^{14}-labelled BA was applied to ozone-exposed tobacco leaves, it was efficiently metabolized to SA (Ogawa *et al.,* 2005). However, there was no increase in the activity or mRNA level of isochorismate synthase. In contrast, the activity of this enzyme was increased in ozone-exposed *Arabidopsis*. These results suggest that SA is synthesized via BA from phenylalanine in ozone-exposed tobacco leaves but via isochorismate in *Arabidopsis*. During ozone exposure, transgenic plants with reduced ozone-induced ethylene production accumulated less SA than did wild-type plants. Ozone increased the activity of phenylalanine ammonia-lyase and the transcript levels of the chorismate mutase and phenylalanine ammonialyase genes in wild-type tobacco, but their induction was suppressed in the transgenic plants. These results indicate that ethylene promotes SA accumulation by regulating the expression of the chorismate mutase and phenylalanine ammonialyase genes in ozone-exposed tobacco plants. A comparison of recent transcriptome analysis revealed that in addition to genes generally induced by all kinds of oxidative stress, for example, transcripts for PR-proteins and most antioxidant enzymes, approximately one-third of the responsive transcripts are ozone-specific, indicating JA, SA and ethylene-independent redox signalling triggered by extracellular redox sensing. These data suggest ROS signalling is more sophisticated than previously realized and raise questions about current models of ozone perception (Evans *et al.,* 2005).

2.7 Ultraviolet radiation

Depletion of the stratospheric ozone layer may result in an increase in the level of potentially harmful ultraviolet (UV) radiation reaching the surface of the earth. UV radiation is traditionally divided into UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) wavelength ranges, which have increasing level of energy and harmful effects. Plants, which use direct sunlight for photosynthesis are unable to avoid UV radiation, therefore,

mechanisms which may protect them from the harmful effects of UV radiation are of particular interest (Hollósy, 2002)

Like ozone, UV radiation was shown to induce the accumulation of SA, while also stimulating PR-protein synthesis and inducing virus resistance in tobacco plants (Yalpani *et al.,* 1994). Increased SA levels were accompanied by the accumulation of an SA conjugate and by an increase in the activity of BA 2-hydroxylase, which catalyses SA biosynthesis. This was paralleled by the appearance of induced resistance to a subsequent attack by tobacco mosaic virus. The results suggest that UV light, ozone fumigation and tobacco mosaic virus can activate a common signal transduction pathway that leads to SA and PR-protein accumulation and increased disease resistance (Yalpani *et al.,* 1994).

Besides heat stress, UV-B radiation may also significantly contribute to the quality decline and death of Kentucky bluegrass (*Poa pratensis* L.) during summer transplanting. UV-B irradiation stress substantially reduced the turf quality and photochemical efficiency when measured 10 days after the initiation of UV-B. Antioxidants and protective pigments may be involved in plant defence against oxidative stress caused by UV-B. It was recently shown that exogenous SA at 150 mg m^{-2} may alleviate UV-B damage by upregulating plant defence systems (Ervin *et al.,* 2004). Endogenous α-tocopherol, SOD and catalase were reduced by UV-B stress. The anthocyanin content increased from day 1 to day 5 and then decreased from day 5 to day 10 of continuous UV-B irradiation. The application of SA enhanced the photochemical efficiency by 86% when measured 10 days after UV-B initiation. In addition, the application of SA increased the α-tocopherol concentration, the SOD and catalase activity and the anthocyanin content compared to the control 10 days after UV-B initiation, leading to better visual quality under UV-B stress. These results suggest that the foliar application of SA may alleviate the decline in photochemical efficiency and turf quality associated with increased UV-B light levels during the summer.

3. POSSIBLE ACTION MECHANISMS

3.1 SA-induced gene expression

As indicated above, the abiotic stress tolerance, induced by SA, may have various causes. Resistance to many stress factors, however, can be attributed to common properties. As already illustrated in previous sections, there are several genes which can be induced by both biotic and abiotic

stress factors, while several "stress-related" compounds, such as ABA, H_2O_2, etc. as well as SA may also induce their expression (Salzman *et al.,* 2005).

Although abiotic stresses affect plant growth and development, its direct effect on the regulation of the components of the DNA replication machinery is still largely unknown. It was shown that the expression of TOP2 which encodes topoisomerase II, is up-regulated by various abiotic stresses including salinity and low temperature, and by phytohormones such as ABA and SA (Hettiarachchi *et al.,* 2005). Transgenic studies on various deletion versions of the TOP2 promoter in tobacco define several promoter determinants responsible for specific abiotic stress responsiveness. These results demonstrate the direct involvement of stress in the transcriptional regulation of TOP2. The differential expression of the gene coding for a chloroplast translation elongation factor (EF-Tu) in response to various abiotic stresses in pea showed that it is down-regulated in response to salinity and ABA and up-regulated in response to low temperature and SA treatment. These results indicate that the regulation of this gene in pea may have an important role in plant adaptation to environmental stresses (Singh *et al.,* 2004).

Mobile genetic elements play a crucial role in the genome restructuring, induced by environmental challenges (McClintock, 1984). According to this theory, the transcription and transposition of these elements should be greatly influenced by external factors such as biotic and abiotic stress conditions. The TLC1 family is one of the four families of long terminal repeat retrotransposons identified in the genome of *Lycopersicon chilense*. It was shown that this family of retroelements is transcriptionally active and its expression is induced in response to diverse stress conditions such as wounding, protoplast preparation and high salt concentrations, but not drought stress (Tapia *et al.,* 2005). Several stress-associated signalling molecules, including ethylene, methyl JA, SA, and 2,4-dichlorophenoxy-acetic acid, but not ABA or H_2O_2, are capable of inducing the expression of the TLC1 family *in vivo*.

Cyclophilins (Cyp) are ubiquitous proteins with intrinsic peptidyl-prolyl cis-trans isomerase activity that catalyses the rotation of X-Pro peptide bonds and facilitates the *in vivo* folding of proteins. Usually, higher amounts of Cyp mRNAs are found in developing tissues (Marivet *et al.,* 1994). Cyp mRNA accumulates in leaves infected with alfalfa mosaic virus and after ethephon and SA treatments in bean. In response to localized chemical treatment, Cyp mRNA accumulation was observed in the untreated parts of the plants. A comparative study of Cyp mRNA accumulation in bean and maize in response to various external stimuli showed striking differences in profiles between the two plants. For instance, in response to heat shock, maize Cyp mRNA accumulated to a significant extent, whereas no mRNA

was found to remain in bean a few hours after the beginning of the heat stress. Two putative heat shock elements were identified in the promoter region of a maize Cyp genomic clone; a metal regulatory element and a third heat shock element were localized in the 5' untranslated leader (Marivet *et al.,* 1995). Differences in mRNA accumulation profiles are also observed after salt stress, which induces a response earlier in maize than in bean, whereas the opposite situation is observed when plants are cold-stressed. All these findings suggested that cyclophilin might be a stress-related protein and might play a role in signal transduction processes (Marivet *et al.,* 1994). The StCyP clone encoding a cytosolic form of Cyp was isolated from a cDNA library prepared from potato (*Solanum tuberosum*) tubers infected with the fungus *Solani f.* sp. *eumartii* (Godoy *et al.,* 2000). StCyP is expressed at high levels in all the tissues of healthy potato plants, except in the tubers. Northern blot analysis revealed that both wounding and fungal infection increased the level of StCyP mRNA in tubers. StCyP mRNA accumulation is also stimulated by heat-shock or by the application of ABA or methyl JA in tubers, but not by fungal elicitor or, in contrast to bean plants, by SA.

Studies were also made on the effect of SA on the synthesis of HSPs in tomato. It was found that, although a low concentration of SA alone was unable to induce HSP synthesis, it promoted heat-induced synthesis. At higher, cytotoxic concentrations (1 mM), SA alone was able to induce Hsp70/Hsc70 expression (Cronje and Bornman, 1999).

Some genes can be induced by both drought and SA. For example, the expression of a water stress-induced gene from *Brassica oleracea* (BoWS), encoding a 95-amino-acid protein, was upregulated by ABA, mannitol, NaCl, drought, SA and H_2O_2, indicating that this gene is closely related to water-deficit stress in this species (Li *et al.,* 2004). Transcripts of the BcDh2 dehydrin-like gene isolated from *Boea crassifolia* accumulated to a great extent when the plants were exposed to drought, salinity, exogenous ABA and moderate heat shock, while accumulation was low in response to low temperature stress. BcDh2 also accumulated slightly in response to wounding signals such as MeJA and a low concentration of SA (Shen *et al.,* 2004). By contrast, transcripts of another drought-inducible gene BcMYBI, which was strongly induced by drought stress and also responded to polyethylene-glycol (PEG), high salinity and low temperature, did not accumulate to any great extent after treatment with exogenous ABA, methyl JA or SA. These results indicate that BcMYBI might be involved in the regulation of gene expression in response to dehydration stress through an ABA-independent pathway, but does not seem to be a regulatory component in wounding signalling (Chen *et al.,* 2005). Differential responses to drought and SA were also exhibited by other genes, such as the BnBDC1 gene,

which encodes a protein containing the BURP domain, and which was isolated from oilseed rape (*Brassica napus*) following drought treatment (Shunwu *et al.,* 2004). The transcript of this gene was found to be specifically expressed in the shoots, but not in the roots. The expression level of the BnBDC1 transcript is upregulated by mannitol, NaCl and ABA, and downregulated by UV irradiation and SA, while it is unresponsive to H_2O_2 and cold treatment. The expression level of BnBDC1 was significantly reduced after SA treatment and the effect was prolonged beyond 2 days, indicating that BnBDC1 is one of the target genes in the SA signalling pathway. Although there is insufficient information to assess the precise function of BnBDC1, it is probably involved in multiple cell signalling pathways, and may play an important role in the response to osmotic stress and plant pathogen infection in *Brassica napus*.

A submergence-induced gene, OsGGT, was recently cloned from submerged plants of a submergence-tolerant cultivar of rice (*Oryza sativa* L.) (Qi *et al.,* 2005). Its deduced amino acid sequence is homologous with glycogenin glucosyltransferase. The expression of this gene increased during submergence in the submergence-tolerant cultivar, but decreased in a submergence-intolerant cultivar. The expression of the OsGGT gene in the tolerant cultivar was induced by SA and benzyladenine. The accumulation of OsGGT mRNA also increased in response to ethylene, gibberellin, ABA, drought and salt treatment, but methyl JA treatment and cold stress had no effect. These results suggest that the OsGGT gene could be related to submergence stress and associated with a general defensive response to various environmental stresses.

The SAR8.2 genes of pepper (*Capsicum annuum* L.), designated CASAR82A, B and C, which are induced by all biotic and abiotic stresses, are not constitutively expressed in any of the organs of healthy pepper plants. Besides induction by pathogens, a strong induction of the CASAR82A gene was found in pepper leaves treated with ethylene, methyl JA, indole-3-acetic acid, ABA, SA, benzothiadiazole, DL-beta-n-amino butyric acid or H_2O_2. Interestingly, the transcription of the CASAR82A gene was rapidly triggered by high salinity, drought or low-temperature stresses, but not by mechanical wounding. *In situ* hybridization results revealed that the CASAR82A mRNAs were localized in the phloem and epidermal cells of pepper leaf and stem tissues infected by *Colletotrichum coccodes* and *Phytophthora capsici*, or treated with SA. These results suggest that pepper SAR8.2 genes may be valuable as molecular markers for the detection of various pathogen infections, abiotic elicitors and environmental stresses (Lee and Hwang, 2003).

In *Arabidopsis*, the cytosolic, patatin-related phospholipase A enzymes (PLA) comprise a family of 10 genes, designated AtPLAs and thought to be

involved in auxin and pathogen signalling (Holk *et al.,* 2002). The first indication that plant PLA had a function in signal transduction was the rapid activation of PLA activity by auxin (Scherer and André, 1989). Later, the activation of PLA by pathogens and elicitors was shown (Lee *et al.,* 1992). Its synthesis is up-regulated after treatment by SA, benzo[1,2,3]thiadiazole-7-carbothionic acid-S-methylester (Bion), wounding, ACC and JA. The properties of this member of the patatin-related phospholipase A gene family suggest that it is a defence, iron-stress and phosphate-stress gene, being transcriptionally up-regulated within hours or days (Rietz *et al.,* 2004).

Chloroplastic lipoxygenase, CPRD46, a single copy gene isolated from dehydrated cowpea (*Vigna unguiculata*) plants, was shown to be induced by high-salinity stress and exogenous ABA, but not by cold stress. The CPRD46 gene is also responsive to heat stress, methyl JA and SA (Iuchi *et al.,* 1996).

Glycine-betaine is an osmoprotectant accumulated by barley (*Hordeum vulgare*) plants in response to high levels of NaCl, drought, cold stress or ABA treatment (Jagendorf and Takabe, 2001). Additional inducers of glycine-betaine accumulation have been detected in barley seedlings, including other inorganic salts, oxidants and organic compounds. The same concentrations of aspirin or SA (added to the hydroponic solution as sodium salicylate) that induced glycine-betaine accumulation increased TBARS level. Since H_2O_2 also increased the glycine-betaine level, the glycine-betaine-inducing effect of salicylates can be explained by their ability to increase the H_2O_2 level. Although illumination is needed for optimal induction, a significant increase in the leaf glycine-betaine level is also found in complete darkness.

Among the growing list of promising genes for plant improvement, some of the most versatile appear to be those involved in the sugar alcohol metabolism. Mannitol, one of the best known sugar alcohols, is a significant photosynthetic product in many higher plants. In addition to its osmoprotectant properties, mannitol is an antioxidant and may have a significant role in plant-pathogen interactions. The catabolic enzyme mannitol dehydrogenase is a prime modulator of mannitol accumulation in plants. An up-regulation by SA and down-regulation by salt, osmotic stress and ABA was recently shown for its *mtd* gene in *Arabidopsis* plants (Zamski *et al.,* 2001). In contrast, the massive up-regulation of the expression of this gene in the vascular tissues of salt-stressed *Arabidopsis* roots suggests a possible role for mannitol dehydrogenase in mannitol translocation and unloading and its interrelation with the sugar metabolism.

Osmotin was detected in tobacco cells, exposed to gradually increasing concentrations of NaCl, which led to phenotypic adaptation and increased tolerance to NaCl (Singh *et al.,* 1987). A cDNA clone encoding osmotin,

PhOSM, was isolated from a cDNA library constructed from petal protoplast cultures of *Petunia hybrida* (Kim *et al.,* 2002). This gene was expressed primarily in roots and slightly in the pistil, 3 days after flowering. Its expression was strongly induced in leaves that were exposed to certain pathogens. Upon wounding, PhOSM transcripts were induced in the damaged leaf, but not systemically. Moreover, PhOSM transcript levels increased in response to octadecanoid pathway intermediates and treatment with aspirin or SA. These results indicate that PhOSM is developmentally regulated as well as being involved in stress signal transduction.

Investigations on the expression of SbPRP gene, encoding a soybean proline-rich protein showed that it accumulates in the leaves and epicotyls of soybean seedlings, but not in the cotyledons, hypocotyls or roots (He *et al.,* 2002). SbPRP mRNA was also expressed in response to SA and virus infection. In addition, SbPRP gene transcription was regulated by circadian rhythm, salt stress, drought stress and plant hormones. These results indicate that the SbPRP gene might play a role in plant responses to multiple internal and external factors. The expression of another proline-rich protein, encoded by the PvSR1 (*Phaseolus vulgaris* stress-related protein) gene, was greatly enhanced in the leaf tissue not only by alfalfa mosaic virus infection, wounding, heat shock, UV, drought and salt stress, but also by exogenous factors such as SA and H_2O_2. The precise biological role of PvSR1 is still unknown, but the expression of PvSR1 genes, in various forms of stress, suggests that PvSR1 may play an important role in maintaining cellular integrity during the stress, by forming strong linkages with the cell wall (Chai and Zhang, 1999).

Using the microarray technique several cytochrome P450 genes were detected in *Arabidopsis*, of which the expression was induced not only by biotic and abiotic stresses, but also by hormone treatments, including SA, suggesting cross-talk between different types of stress factors (Narusaka *et al.,* 2004). Most cytochrome P450 genes induced by both abiotic and biotic stresses, contained the recognition sites of MYB and MYC, ACGT-core sequence, TGA-box and W-box for WRKY transcription factors in their promoters. These cis-acting elements are known to participate in the regulation of plant defence. The response of each gene to multiple stress is strictly regulated. Multiple forms of cytochrome P450-dependent monooxygenases catalyse the in-chain hydroxylation, end-terminal hydroxylation, and epoxidation of medium- and long-chain fatty acids. In plants, fatty acid hydroxylases are particularly important in the synthesis of plant cuticles and signalling molecules derived from fatty acids. Some members of the *Arabidopsis thaliana* CYP86A and CYP94B cytochrome P450 monooxygenase subfamilies, which share some sequence homology with animal and fungal fatty acid hydroxylases, have been functionally

defined as fatty acid omega-hydroxylases. Due to these activities, these and other fatty acid hydroxylases have a potential role in the synthesis of cutin, the production of signalling molecules, and the prevention of the accumulation of toxic levels of free fatty acids. The constitutive and stress-inducible patterns of the five *Arabidopsis* CYP86A subfamily members have now been defined (Duan and Schuler, 2005). Very distinct expression patterns exist for each of these fatty acid hydroxylases under normal growth conditions and in response to environmental and chemical stresses. CYP86A1 transcripts were transiently induced by ABA and ACC treatments, more continuously induced by mannitol and clofibrate treatments, and more slowly induced by cold treatment and brassinosteroid treatments, while they were repressed by IAA and SA treatments. CYP86A2 transcripts were transiently induced by wounding, ABA, mannitol, IAA and clofibrate treatments, more continuously induced by drought treatment, and induced more intensively in etiolated and dark-adapted seedlings compared to light-grown seedlings of the same age. CYP86A4 transcripts were transiently induced by ABA and IAA treatments and more continuously induced by cold treatment. In contrast to the transient responses of other CYP86A transcripts, CYP86A4 transcripts were repressed by short-term ACC treatment and induced by long-term ACC treatment. These transcripts were also repressed by wounding in etiolated seedlings. CYP86A7 transcripts were transiently induced by clofibrate treatment, more continuously induced by MeJA treatment, and slowly induced by ABA treatment, while being significantly repressed by drought, SA, wounding, ACC and mannitol treatments. CYP86A8 transcripts were induced only by IAA and ABA treatments and repressed by wounding. An analysis of the promoter sequences for each of these genes together with their expression patterns has highlighted a number of elements in current databases that potentially correlate with the responses of individual genes. The observed patterns of inducible expression suggest that, in addition to their roles in normal growth and development, each of these P450s has a particular role in stress responses, although these results also suggest that the activation of these CYP86A genes does not involve SA- or MeJA-dependent pathways (Duan and Schuler, 2005).

The detection and development of gene promoters specific for the pericarp epidermis (epicarp) of barley (*Hordeum vulgare* L.) is a critical step in the targeting of transgene-mediated disease resistance, since this tissue constitutes an early point of entry and proliferation of *Fusarium graminearum*, the main causal agent of disease. Plant lipid transfer proteins (LTPs) were defined by their ability to facilitate the transfer of phospholipids between membranes *in vitro*. Suggested roles *in planta* include the formation and reinforcement of plant surface layers,

embryogenesis, defence against pathogens, symbiosis, and adaptation to various abiotic stress conditions (Kader, 1996). Transcripts of the TaLTP1 gene were increased by water stress, such as treatment with various PEG concentrations or NaCl, hormone treatment (SA, ethephon), H_2O_2, or wounding (Jang *et al.,* 2004). The search for a cereal promoter capable of driving preferential transgene expression in the pericarp epidermis of developing barley resulted in the cloning of a novel gene (Federico *et al.,* 2005). This encoded a polypeptide of 124 amino acids showing 87% identity with WBP1A, a wheat lipid transfer protein, but much lower homology to other barley LTPs. In addition to the epicarp, this Ltp-like gene, *Ltp6*, is highly expressed in coleoptiles and embryos under normal growth conditions. In contrast to other Ltps, such as *Ltp3* or *Ltp4*, the expression of which was increased by salt and ABA but not by drought, cold, or SA in leaves, mRNA levels of Ltp6 increased in seedling tissues during salt and cold treatments and after the application of ABA and SA. An ATMYB2A-binding site was found in the Ltp6 promoter. The *Arabidopsis* AtMyb2A gene encodes an MYB-related protein, which is strongly induced by ABA, drought and high salt (Urao *et al.,* 1993). If an MYB transcription factor related to ATMYB2A exists in barley, this MYB-binding site could account for the ABA and high salinity response observed in the Ltp6 gene. In *Arabidopsis*, low temperature responsive elements, containing the CCGAC core-motif, have been found in many cold- and drought-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 1996). Ltp6 consists of a sequence which could represent a binding site for both the HvCBF1 and HvCBF2 barley transcription factors, which have been shown to activate cold-inducible genes through a CCGAC core-motif (Xue, 2002; 2003), and which might account for the responsiveness of Ltp6 to cold stress. The presence of putative ABA- and gibberellin-responsive elements in the promoter region suggests that *Ltp6* may respond to either gibberellin or ABA under certain developmental or stress conditions (Federico *et al.,* 2005).

The expression of the *Arabidopsis* phospholipase A IIA (AtPLA IIA) gene, which is a member of the patatin-related PLA gene family in plants and is homologous to the Ca^{2+}-independent PLA_2 gene family in animals, was induced by various treatments such as pathogen inoculation, cold, high salinity, ABA, SA (sprayed at a concentration of 5 mM), methyl JA, ethephon, paraquat, rose bengal, UV-C and $CuSO_4$ (Narusaka *et al.,* 2003). The sequences of putative cis-acting elements were found in the promoter region of the AtPLA IIA gene. This gene has two ACGT-sequences and a TGA-box in the promoter, which are known to be the core sequences of activation sequence-1 (Lam *et al.,* 1989), which acts as an oxidative stress-responsive element. Therefore, these sequences may function as cis-acting elements in the ROS-responsive promoters. The AtPLA IIA gene was also

induced by both osmotic stress and ABA treatment. Although the signalling factors involved in the induction of AtPLA IIA are unclear, it may play an important role in plant defence responses.

3.2 Relationship with oxidative stress

It is a well-known fact that most stress factors, whether biotic or abiotic, are usually associated with oxidative stress. This relationship may be indirect or secondary, as in the case of flooding stress, which may activate the antioxidant system in spite of the fact that it tends to cause oxygen deficiency (Yordanova *et al*., 2004). In many cases the increased tolerance can be attributed to the efficiency with which the plant is capable of neutralising ROS.

Exposure to sublethal biotic and abiotic stresses renders plants more tolerant to a subsequent, normally lethal, dose of the same stress, a phenomenon referred to as acclimation or acquired resistance (Vierling, 1991; Sticher *et al.,* 1997). In fact, this induced stress resistance is not restricted to the same type of stress, cross-tolerance having been reported between different stresses (Strobel and Kuc, 1995; Bowler and Fluhr, 2000; Chini *et al.,* 2004). Antioxidant defence responses have long been associated mainly with enhanced antioxidant enzyme activity and increased levels of antioxidant metabolites, such as ascorbic acid, glutathione, α-tocopherol and carotenoids. More recently, the induction of small HSPs, the cellular protection gene glutathione-S-transferase, and the PR gene *PR2* have also been associated with the acquisition of oxidative stress tolerance (Vranová *et al.,* 2002).

When applied at suitable concentrations, SA causes transient oxidative stress in plants, which acts as a hardening process, increasing the antioxidative capacity of the plants (Knörzer *et al*., 1999) or inducing the synthesis of stabilising substances such as polyamines (Németh *et al*., 2002). In tobacco and cucumber plants SA moderated the oxidative stress caused by paraquat (Strobel and Kuc, 1995). When young barley plants were treated with 0.5 mM SA for 1 day in the dark, the inhibitory effect of paraquat on photosynthesis could be averted. There was also a reduction in the H_2O_2 production, lipid peroxidation and membrane damage, induced by paraquat (Ananieva *et al*., 2002). At higher concentrations, however, SA itself may cause a high level of stress.

One frequent result of both SA treatment and the acclimatisation process is a temporary reduction in catalase activity and a rise in the H_2O_2 level (Janda *et al*., 2003). The increased H_2O_2 level is mainly the result of the effect of SA on antioxidant enzymes (Klessig *et al*., 2000; Ganesan and Thomas, 2001). In *Arabidopsis* a long course of SA treatment reduced the

activity of APx as well as that of catalase, leading to cell death similar to the hypersensitive reaction (Rao *et al*., 1997). SA also led to a reduction in catalase and APx activity in *Astragalus adsurgens* Pall. callus culture, resulting in an increase in the H_2O_2 level (Luo *et al*., 2001). SA has proved capable of binding directly to catalase enzyme, isolated from tobacco, inhibiting its activity (Chen *et al*., 1993b; Conrath *et al*., 1995). The *in vitro* catalase-inhibiting effect of SA has also been demonstrated in many other plant species (e.g. *Arabidopsis*, tomato, cucumber) (Sánchez-Casas and Klessig, 1994; Horváth *et al*., 2002). It is thought that the increased H_2O_2 level produced by the catalase-inhibiting effect of SA may play a role in the development of SAR (Chen *et al*., 1993a) and in the defence against abiotic stress effects involving oxidative damage (Gechev *et al*., 2002). Nevertheless, the significance of catalase inhibition in the induction of resistance is still dubious, partly because the binding of SA to catalase is not specific (it also binds to other iron-containing proteins such as aconitase; Rüffer *et al*., 1995), and partly because inhibition has not been observed unambiguously for all plant species. In tobacco all the catalase isoenzymes are inhibited by SA (Durner and Klessig, 1996), but in maize and rice a significant level of inhibition could not be detected (Sánchez-Casas and Klessig, 1994). Mild inhibition was caused by 1 mM SA in the activity of the CAT2 isoenzyme isolated from maize scutellum (Guan and Scandalios 1995). This contradiction was partially solved when later studies found differences between the catalase isoenzymes in their sensitivity to SA, both in maize and rice. A substantial level of non-competitive inhibition was caused by 2 mM SA in the activity of the CAT1 isoenzyme of maize, while in the case of CAT2 the inhibition was competitive and weak (Horváth *et al*., 2002). In rice SA inhibited the activity of the CATb isoenzyme, but not that of CATa (Chen *et al*., 1997). The CAT1 isoenzyme of maize and the CATb isoenzyme of rice, both of which are sensitive to SA, exhibited considerable sequence homology with tobacco catalase, which is also inhibited to a great extent by SA. The tissue-specific expression of various catalase isoenzymes may lead to differences in the effect of SA on the given tissue if catalase does indeed play a role in transmitting the effect of SA.

As regards the mechanism of catalase inhibition, it is thought that SA might act as an electron donor, diverting catalase to the slower peroxidative pathway. At low levels of H_2O_2 this is manifested as inhibition, while at damaging levels of H_2O_2 it protects the enzyme (Durner and Klessig 1996). In the course of catalase inhibition, however, SA is converted into a free radical, which may then cause lipid peroxidation. Both the higher H_2O_2 level caused by catalase inhibition and the lipid peroxidation arising in the course of inhibition are thought to be involved in the signal transduction process leading to SA-dependent resistance (Anderson *et al.,* 1998). There is

evidence that not only does SA cause a rise in the quantity of ROS in the cell, but ROS also lead to the accumulation of SA (León *et al*., 1995, Enyedi 1999). This observation suggested the existence of a self-induced SA-H_2O_2 cycle resulting in the accumulation of ROS and the death of the cell (Van Camp *et al*., 1998). SA and its biologically active analogues may cause lipid peroxidation (Anderson *et al.*, 1998) and may also lead to oxidative damage to proteins and to the formation of chlorophyll and carotene isomers. H_2O_2 treatment alone did not cause such a great extent of damage to membranes or proteins. Dimethyl-thiourea, on the other hand, which reduces the level of H_2O_2, moderated the damaging effect of SA treatment (Rao *et al*., 1997). A similar observation was made when the induction of somatic embryogenesis by SA was examined in callus culture of *Astragalus adsurgens* Pall. SA (0.2 mM) increased the level of endogenous H_2O_2, but exogenous H_2O_2 was unable to substitute fully for the effect of SA, while dimethyl-thiourea moderated the effect of SA by reducing the H_2O_2 level (Luo *et al*., 2001). This suggests that the effect of SA is only mediated in part by H_2O_2. In addition to H_2O_2, a role may also be played by the SA free radical arising in the course of catalase inhibition and the consequent lipid peroxidation (Klessig *et al*., 2000). Recent results revealed that the SA-induced H_2O_2 accumulation in germinating wheat seedlings was not associated with the inhibition of catalase or APx. It is suggested that the abiotic stress signal is transduced via ABA, Ca^{2+} and H_2O_2, which might be responsible for the activation of a common transcription factor associated with certain antioxidant enzymes (Agarwal *et al.,* 2005).

As previously mentioned, several studies have supported a major role of SA in modulating plant responses to abiotic and biotic stresses, by the induction of antioxidant capacity. In some cases SA stimulates the activity of the Cu- and Zn-SOD enzymes, which again may contribute to a rise in the H_2O_2 level (Rao *et al.,* 1997; Azevedo *et al.,* 2004). There are several enzyme systems capable of removing excess H_2O_2, including the ascorbate-glutathione cycle in the chloroplasts. The glutathione metabolism in plants may play a key role in determining the degree of expression of defence genes controlled by various signalling pathways both before and during stress. This control may reflect the physiological state of the plant at the time of the onset of an environmental challenge and suggests that changes in the glutathione metabolism may be one means of integrating the functions of different signalling pathways (Kocsy et al. 1997, 2001, 2004; Ball *et al.,* 2004). The activity of glutathione reductase is also stimulated by SA *in vivo*, as shown in mustard and maize plants after exogenous SA treatments (Dat *et al.,* 1998a, Janda *et al.,* 1999). Tobacco plants growing *in vitro* in the presence of 0.01 or 0.1 mM SA also showed increased glutathione reductase and dehydroascorbate reductase activity in the shoots, although there was no

significant effect on APx. SA at 0.1 mM also increased the monodehydroascorbate reductase (MDHAR) activity. The ascorbate redox-ratio was maintained in the shoots of plants grown on 0.01 mM SA, despite increases in the ascorbate and dehydroascorbate levels. However, on 0.1 mM SA, the ascorbate redox-ratio decreased by more than 40% due to a 300% increase in dehydroascorbate. The glutathione redox-ratio was maintained in the shoots of plants grown on either SA concentration, despite increase in the glutathione level (Dat *et al.,* 2000). In cucumber hypocotyls, SA significantly increased the activity of these enzymes in control and chilled tissue, while having no effect on the activities in radicle tissues (Kang and Saltveit, 2002). The treatment of *Ficus carica* leaves with SA by submerging the leaves into 5 mM SA solution did not cause any significant increase in the mRNA level of peroxidase (Kim *et al.,* 2003a). However, it should be mentioned that although the total activity did not increase substantially in maize treated with SA, a new peroxidase isoform was detected (Janda *et al.,* 1999).

MDHAR maintains reduced pools of ascorbate by recycling the oxidized form of ascorbate. The screening of a *Brassica campestris* cDNA library led to the identification of an MDHAR cDNA (BcMdhar) which encodes a polypeptide containing 434 amino acids. This polypeptide possesses domains characteristic of FAD- and NAD(P)H-binding proteins (Yoon *et al.,* 2004), and shows a high level of identity to the cytosolic MDHAR of rice, pea and tomato, but does not possess an N-terminal leader sequence, suggesting that it encodes a cytosolic form of MDHAR. The level of BcMdhar mRNA increased in response to oxidative stress invoked by H_2O_2, SA, paraquat and ozone.

Glutathione peroxidases are also enzymes which protect cells against the oxidative damage generated by ROS. Up to now seven related proteins have been identified in *Arabidopsis thaliana* plants, and the expression of the one showing the strongest response to abiotic stress was also affected by several plant hormones, including SA (Milla *et al.,* 2003). Along with plasmalemmal redox systems, cell-wall peroxidase is involved in the production of superoxide and H_2O_2. Under stress conditions, some soluble peroxidase isoforms are easily secreted into the apoplast. Various membranotropic compounds, SA in particular, can also induce this process. Mobile peroxidase forms are thought to induce the plant defence response (Minibaeva *et al.,* 2003).

Glutathione S-transferases (GST) form a large family of non-photosynthetic enzymes known to function in the detoxification of xenobiotics. The effect of SA is ambiguous in the case of the GST enzyme. The *in vitro* activity of the enzyme is inhibited non-competitively by SA (Watahiki *et al*., 1995), which, however, stimulates its expression. PEG and

heavy metals rapidly induced the osgstu4 and osgstu3 genes in rice seedling roots (Moons, 2003). Osgstu4 and osgstu3 were also induced in roots by hypoxic stress but not by cold nor heat shock. Salt stress and ABA also induced osgstu3 in rice roots, whereas osgstu4 exhibited late salt stress and no ABA response. SA, JA and the auxin naphthalene acetic acid triggered osgstu4 and osgstu3 expression. Osgstu4 and osgstu3 were rapidly and markedly induced by the antioxidant dithiothreitol and by the strong oxidant H_2O_2, suggesting that redox perturbations and ROS are involved in their stress response regulation. Similarly, SA caused an increase in the expression of the Gnt35 gene coding for GST in tobacco cells. Recently a novel, low temperature-regulated, *Solanum commersonii* GST gene (Scgst1) was cloned and characterized from a cold acclimated wild potato species and the level of its transcription was studied in freezing-tolerant and sensitive *Solanum* genotypes (Seppanen *et al.,* 2000). Increased GST enzyme activity was observed in *S. commersonii* and SH9A after 2 days of cold acclimation, whereas the activity declined in *S. tuberosum* during the same period. ROS were associated with the early steps of Scgst1 regulation since a strong mRNA signal was detected in plants treated with H_2O_2 and SA. Under experimental conditions where the formation of ROS is known to accelerate, such as excessive light at low temperature, a significant accumulation of the transcript was observed in *S. commersonii*. Under similar experimental conditions, the Scgst1 transcript did not accumulate in freezing-sensitive *S. tuberosum* though a single copy of the Scgst1 sequence was present in both species. The abundance of Scgst1 transcript correlated well with the freezing tolerance of the parental lines and the somatic hybrid SH9A. However, further studies of potato lines derived by selfing the somatic hybrid revealed a more complex relationship between freezing tolerance and Scgst1 expression level. A SA-responsive component (as-1) was found in the promoter region of some GST genes, which is activated not only by SA but also by auxin and methyl JA via reactive oxygen forms (Garretón *et al.,* 2002).

3.3 Role of alternative oxidases

One of the best known roles of endogenous SA is its role in heat production, which is due to the enhanced activity of the cyanide-resistant or alternative respiration chain (Raskin *et al.,* 1987). An increase in the alternative pathway can also be observed during chilling stress (Moynihan *et al.,* 1995). It was assumed that the increased capacity to produce respiratory heat after exposure to chilling temperatures might contribute to the cold-acclimation process. A correlation was also found between the activity of the alternative respiratory pathway and the chilling tolerance of maize lines

(Vandeventer, 1985; Luxova and Gasparikova, 1999). On the other hand, studies of the electron partitioning between the cytochrome and the alternative respiratory pathways during chilling recovery, using the oxygen isotope fractionation technique, revealed that electron partitioning to the alternative pathway was greater in the chilling-sensitive maize line, which suffered greater stress, so it was suggested that the increased activity of the alternative pathway is not related to the tolerance of the plant to chilling (Ribas-Carbo *et al.,* 2000). The existence of multiple pathways to regulate the expression of the alternative oxidase genes encoding the alternative oxidase was recently demonstrated in soybean (Djajanegara *et al.,* 2002). The Aox1 gene is specifically induced by a variety of stress and metabolic conditions, including SA, via at least two independent signal transduction pathways. Similar results were found when using the mRNA differential display technique; seven cDNAs were isolated that were rapidly induced when cultured tobacco (*Nicotiana tabacum*) cells were treated with antimycin A (Maxwell *et al.,* 2002). All the cDNAs, as well as Aox1, were found to be strongly induced by H_2O_2 and SA. The antimycin, H_2O_2 or SA treatment of tobacco cells caused a rapid rise in intracellular ROS accumulation but if it was prevented by antioxidant treatment, gene induction was inhibited. Besides antimycin, both H_2O_2 and SA were found to disrupt normal mitochondrial function, resulting in decreased rates of electron transport and a lowering of cellular ATP levels. These findings suggest that the mitochondrion may play an important role in conveying intracellular stress signals to the nucleus, leading to alterations in gene expression.

3.4 Protein kinases

Reversible protein phosphorylation/dephosphorylation plays an important role in signalling adaptive responses to several types of stress (Bassett, 2001). The first step in signal relay is the perception of a chemical or physical signal, such as a change in temperature or light. One type of sensor commonly used to initiate a response to the signal is a receptor protein kinase (RPK). Plants utilize two types of RPKs: histidine-phosphorylating types, such as the ethylene receptor, and serine/threonine-phosphorylating types, such as the brassinosteroid receptor. A receptor-like protein kinase gene (Ppsrkl1) was isolated from a peach (*Prunus persica* L. Batsch.) bark cDNA library prepared with RNAs isolated from bark collected in December under cold acclimation conditions (Bassett *et al.,* 2005). Because peach is a self-compatible species and the gene was originally identified in December bark tissue, it can be assumed that the expression of this gene might be responsive to environmental stresses related to winter, i.e. low temperatures,

short-day photoperiod or water limitation. This gene is related to the S-locus family of receptor protein kinases (SRKs), which belongs to a group of serine/threonine-phosphorylating type RPKs. In bark tissues, Ppsrkl1 was induced by water deficit treatment, and repressed by short-day photoperiods, while it showed no response to cold treatment. There was also an increase in Ppsrkl1 mRNA in the roots in response to water deficit. The addition of 25mM SA prevented the decline in Ppsrkl1, 12 h after wounding in fruit, but did not further induce the mRNA in samples taken earlier. The quantity of Ppsrkl1 mRNA rapidly decreased in fruit after 10-min exposure to UV-C radiation, followed by a return to normal levels within 1.5 h. These experiments indicate that Ppsrkl1 is negatively regulated by light and positively influenced by SA treatment in fruit and by water stress in bark and roots.

In plants, two classes of stress-activated protein kinases, mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) have so far been reported to integrate multiple environmental stresses and undergo rapid biochemical activation upon exposure to biotic and abiotic stimulation. The MAPK signal transduction cascades are routes through which eukaryotic cells deliver extracellular messages to the cytosol and nucleus (Morris, 2001). These signalling pathways direct cell division, cellular differentiation, metabolism, and both biotic and abiotic stress responses. In plants, MAPKs and the upstream components of the cascades are represented by multigene families, organized into different pathways which are stimulated and interact in complex ways. In particular, it was proposed that two MAPKs, tobacco SA-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK), and their respective orthologue in other plant species functioned as central convergence points in stress signalling (Zhang and Klessig, 1997; 1998; Jonak *et al.,* 2002). Plants normally respond to wounding with enhanced levels of JA, which in turn is involved in the induction of wound-induced genes (Farmer and Ryan, 1992). However, SA also increased in wounded leaves (Seo *et al.,* 1995b). It was assumed that WIPK may regulate JA synthesis by the phosphorylation of cytoplasmic phospholipase A2, and that JA may suppress SA synthesis. The opposite effects of SA and JA have also been reported for the regulation of a wound-inducible ipomoelin gene isolated from sweet potato (*Ipomoea batatas*). Besides mechanical wounding, ethylene and methyl JA were identified as signal transducers leading to the accumulation of ipomoelin mRNA. However, treatment with SA reduced the production of mRNA, further supporting the involvement of the octadecanoid pathway in the signal transduction of wounding in sweet potato. The application of the protein phosphatase inhibitor okadaic acid, a calcium ion chelator or channel blockers also blocked the methyl JA- or ethylene-induced accumulation of

ipomoelin mRNA, indicating that the activation of this gene by both MeJA and ethylene proceeded via dephosphorylated proteins and that the calcium ion was also involved in the activation process (Chen *et al.,* 2003). Another example of the opposite effect of SA and JA has been reported in sweet potato: SA, as an inhibitor of the octadecanoid pathway, strongly suppressed the promoter function of sporamin, a tuberous storage protein stimulated by wounding and methyl JA treatments (Wang *et al.,* 2002). Nevertheless, there are also genes where the effect of SA and JA is the same, as it was reported for the expression of resveratrol synthase genes, which can be induced by biotic and abiotic factors, such as UV light, wounding or paraquat, and by stress hormones, such as ethylene, JA and SA, but not by ABA (Chung *et al.,* 2001; 2003).

Two protein kinases with molecular masses of 48 and 40 kD are activated in tobacco cells exposed to NaCl (Hoyos and Zhang, 2000). The 48-kD protein kinase was identified as SIPK. The activation of the 40-kD protein kinase is rapid and dose-dependent. Other osmolytes such as Pro and sorbitol activate these two kinases with similar kinetics. The activation of 40-kD protein kinase is specific for hyperosmotic stress, as hypotonic stress does not activate it. Therefore, this 40-kD kinase was named HOSAK (high osmotic stress-activated kinase). HOSAK is a Ca^{2+}-independent kinase and uses myelin basic protein and histone equally well as substrates. The kinase inhibitor rapidly activates HOSAK in tobacco cells, implicating a dephosphorylation mechanism for HOSAK activation. The activation of both SIPK and HOSAK by high osmotic stress is Ca^{2+}- and ABA-independent. Furthermore, a mutation in the Ca^{2+} sensor-encoding SOS3 locus, which leads to a salt overly sensitive phenotype does not affect the activation of either kinase in *Arabidopsis* seedlings. These results suggest that SIPK and 40-kD HOSAK are two components in a Ca^{2+}- and ABA-independent pathway that may lead to plant adaptation to hyperosmotic stress. It was also shown that a rice gene encoding an MAPK kinase, OsEDR1, is constitutively expressed in seedling leaves and is up-regulated within a few minutes upon wounding, or treatment with JA, SA, ethylene, ABA or H_2O_2 (Kim *et al.,* 2003b). Protein phosphatase inhibitors, the fungal elicitor chitosan, drought, high salt and sugar, and heavy metals also dramatically induce its expression. OsEDR1 expression was altered by the co-application of JA, SA and ethylene, and required *de novo* synthesized protein factor(s) in its transient regulation. Using an *in vivo* system it was shown that OsEDR1 responds to changes in temperature and environmental pollutants. The expression of OsEDR1 varied significantly in vegetative and reproductive tissues, suggesting a role for OsEDR1 in defense/stress signalling pathways and development.

Two novel rice (*Oryza sativa* L.) MAPKs, OsMSRMK3 (multiple stress responsive) and OsWJUMK1 (wound- and JA-uninducible), which most likely exist as single copy genes in the genome, were recently isolated (Agrawal *et al.,* 2003). The steady state mRNA analysis of these MAPKs, which are constitutively expressed in the leaves of two-week-old seedlings, revealed that OsMSRMK3 was up-regulated upon wounding, JA, SA, ethylene, ABA, H_2O_2, protein phosphatase inhibitors, chitosan, high salt/sugar, and heavy metals, whereas OsWJUMK1 was not induced by either wounding, JA or SA, and showed up-regulation only as the result of H_2O_2, heavy metals and cold stress. Moreover, these MAPKs were developmentally regulated. These results strongly suggest a role for OsMSRMK3 and OsWJUMK1 in both stress-signalling pathways and development in rice.

Calcium may control the activity of plant protein kinases through indirect or direct interaction with the enzymes. Indirect interactions involve calmodulin, a calcium-binding protein. Six *Arabidopsis* genes designated as AtSR1-6, related to a tobacco early ethylene-responsive gene, encoding a calmodulin-binding protein, were also shown to be rapidly and differentially induced by environmental signals such as temperature extremes, UV-B, salt and wounding, by hormones such as ethylene and ABA and by signal molecules such as methyl JA, H_2O_2 and SA (Yang and Poovaiah, 2002). AtSR1 targets the nucleus and specifically recognizes a novel 6-bp CGCG box (A/C/G)CGCG(G/T/C). Multiple CGCG cis-elements are found in promoters of genes such as those involved in ethylene signalling, ABA signalling and light signal perception. These results suggest that the AtSR gene family encodes a family of calmodulin-binding/DNA-binding proteins involved in multiple signal transduction pathways in plants. The direct interaction of calcium with enzymes implicates a multi-family protein referred to as calcium-dependent protein kinases (CDPKs), which fall into the serine/threonine class of protein kinases found mainly in plants and in some protozoans (Harmon *et al.*, 2001). CDPKs from many species have been shown to be involved in stress responses. Cold treatment has previously been shown to enhance the activity of a rice CDPK (Martin and Busconi, 2001), while the over-expression of the rice CDPK, OsCDPK7, confers cold and salt tolerance in the transgenic tissues (Saijo *et al.*, 2000). Transcripts of a previously identified tomato CDPK, LeCDPK1, increase transiently in plants subjected to mechanical wounding, both at the wound site and in non-wounded leaves (Chico *et al.*, 2002). The increase observed in LeCDPK1 mRNA upon wounding correlates with an increase in the activity of a soluble CDPK detected in extracts of tomato leaves. CDPK mRNA accumulation has been shown previously to be induced by GA, ABA, cytokinin (Yoon *et al.*, 1999), indole-3-acetic acid (Davletova *et al.*, 2001), and brassinolide

(Yang and Komatsu, 2000). The *Capsicum annuum* calcium-dependent protein kinase 3 (CaCDPK3), localized in the cytosol in chili pepper protoplasts, was rapidly induced in response to various osmotic stress factors and exogenous ABA application in pepper leaves. CaCDPK3 RNA expression was also induced by an incompatible pathogen and by plant defence-related chemicals such as ethephon, SA and JA. It is assumed that CaCDPK3 is implicated in biotic and abiotic stresses in pepper plants (Chung *et al.,* 2004).

A cDNA clone (LeCRK1), encoding a novel isoform of calcium-dependent protein kinase (CDPK), was isolated by screening a tomato (*Lycopersicon esculentum*) cDNA library (Leclercq *et al.,* 2005). The protein derived from the full-length sequence indicated that it belongs to the family of CDPK-related kinases (CRKs) and the predicted amino acid sequence shows a modular organization of the protein, consisting of various characteristic domains. The kinase domain of LeCRK1 shows a high degree of similarity with the catalytic domain of CDPKs. In contrast to canonical members of the family, LeCRK1 has a degenerate sequence in the C-terminal calmodulin-like domain. LeCRK1 protein was shown to be a functional kinase, but, consistent with the lack of calcium-binding activity, its autophosphorylation activity did not require calcium. LeCRK1 harbours an amphiphilic amino acid region, revealed by *in vitro* assay to be a functional calmodulin-binding site. The native protein is anchored to the plasma membrane by acylated residues. Expression studies revealed a significant accumulation of LeCRK1 transcripts during fruit ripening, although transcripts were also detected in stem, leaf and flower. LeCRK1 transcript levels are low in unstressed leaves, but increase in response to wounding and cold treatment. Gene expression was slightly induced by ethylene and by spraying the leaves with a 4 mM solution of SA, and by mechanical wounding or cold treatment.

ROS accumulating due to unfavourable changes in environmental conditions may also trigger the activation of signalling cascades such as the mitogen-activated protein kinase cascade and the accumulation of plant hormones, such as JA, SA and ethylene. It was shown that in *Arabidopsis* plants ozone treatment caused a transient activation of 43 and 45 kDa MAPKs, identified as AtMPK3 and AtMPK6, via the generation of ROS in the apoplast (Ahlfors *et al.,* 2004). The initial AtMPK3 and AtMPK6 activation in response to ozone was not dependent on ethylene signalling, though ethylene is likely to have secondary effects on AtMPK3 and AtMPK6 function, whereas functional SA signalling was needed for full-level AtMPK3 activation by ozone. It was also shown that AtMPK3, but not AtMPK6, responded transcriptionally and translationally during ozone exposure. The activated AtMPK3 and AtMPK6 are translocated to the

nucleus during the early stages of ozone treatment. The use of ozone to induce apoplastic ROS formation offers a non-invasive *in planta* system amenable to reverse genetics that can be used for the study of stress-responsive MAPK signalling in plants (Ahlfors *et al.,* 2004). In another study, the regulation of the MAP kinase gene (OsMAPK2) expression was investigated in rice under metal stress conditions (Hung *et al.,* 2005). The accumulation of OsMAPK2 transcripts was enhanced by copper and H_2O_2 in rice root-tip cells. Glutathione, calcium chelator, plasma membrane calcium channel blocker and the protein phosphatase inhibitor, cantharidin inhibited copper-induced OsMAPK2 gene activation. These results support the idea that the stimulation of OsMAPK2 transcript accumulation by copper in rice roots may operate through an intracellular signalling cascade mediated by ROS, extracellular calcium ions and cantharidin-sensitive protein phosphatase.

As shown in tobacco plants, ozone may also induce rapid activation of SIPK. Transgenic manipulation has previously shown that the overexpression of SIPK leads to enhanced ozone-induced lesion formation with the concomitant accumulation of ROS. Ozone treatment strongly induced ethylene formation in sensitive SIPK-overexpressing plants at ozone concentrations that failed to elicit stress ethylene release in wild-type plants. By contrast, SIPK-overexpressing plants displayed no ozone-induced SA accumulation, whereas wild-type plants accumulated SA upon ozone exposure. The epistatic analysis of SIPK-overexpressing function suggests that the ozone-induced cell death observed in SIPK-overexpressing plants is either independent, or upstream, of SA accumulation (Samuel *et al.,* 2005).

4. RELATIONSHIP BETWEEN BIOTIC AND ABIOTIC STRESS FACTORS

It was established by Pastori and Foyer (2002) that the mechanisms of abiotic and biotic stress resistance have many points in common. The enhanced resistance of barley (*Hordeum vulgare* L.) against barley powdery mildew was induced by abiotic stresses, such as osmotic or proton stresses (Wiese *et al.,* 2004). Another interesting example of common pathways for the regulation of protective mechanisms against biotic and abiotic stresses was recently shown using the *edr1* (enhanced disease resistance 1) *Arabidopsis* mutant, which displays enhanced stress responses and spontaneous necrotic lesions under drought conditions in the absence of pathogens, suggesting that EDR1, which encodes a CTR-1 like kinase, is also involved in stress response signalling and cell death regulation. Double

mutant analyses showed that the *edr1*-mediated growth inhibition and cell death phenotypes are also SA-dependent (Tang *et al.,* 2005).

Despite these similarities, however, there are also differences, raising the question of how the two types of responses are integrated if plants are exposed simultaneously to both biotic and abiotic stress (Mittler, 2002). SA and ROS play a key role in both processes.

An antifungal protein (GtAFP1) showing antimicrobial activity against phytopathogenic fungi was recently purified from leaves of *Gentiana triflora* (Kiba *et al.,* 2005). The deduced amino acid sequence of the cDNA of the corresponding gene, GtAFP1, showed 94, 75, 72 and 63% amino acid identity with peroxyredoxin Q from *Populus balsamifera* x P. deltoides subsp. trichocarpa, *Sedum lineare*, *Suaeda maritima* and *Arabidopsis thaliana*, respectively. It is suggested the GtAFP1 gene is present in the genome in one to two copies and was expressed in leaves, roots and stems. The expression of GtAFR1 was induced by treatment with SA, but not by methyl JA. Recombinant GtAFP1 protein showed not only antifungal activity but also thioredoxin-dependent peroxidase activity. The overexpression of GtAFP1 in tobacco plants improved tolerance not only against fungal diseases but also against oxidative stress. These results indicate that GtAFP1 might act as a disease and oxidative stress defensive gene in plants and could be useful for engineering stress-resistant plants.

The recent characterization of a number of genes suggested that ubiquitin-mediated protein degradation has a role in plant defence responses. The 26S proteasome involved in the degradation of proteins covalently modified with polyubiquitin consists of the 20S proteasome and the 19S regulatory complex. The NbPAF gene encoding the alpha6 subunit of the 20S proteasome was identified from *Nicotiana benthamiana* (Kim *et al.,* 2003c). NbPAF mRNA was detected abundantly in flowers and weakly in roots and stems, but it was almost undetectable in mature leaves. In response to stresses, the accumulation of NbPAF mRNA was stimulated by methyl JA, NaCl and SA, but not by ABA or cold treatment in leaves. Recently two cDNAs (NtUBA1 and NtUBA2) encoding ubiquitin-activating enzyme from *Nicotiana tabacum* cv. BY-2 were isolated (Takizawa *et al.,* 2005). These enzymes and the corresponding transcripts were up-regulated by infection with tobacco mosaic virus and tomato mosaic virus, and to a lesser extent by cucumber mosaic virus. Furthermore, they were also up-regulated by wounding stress, and by the plant hormones SA, JA and the ethylene precursor, ACC. These findings support the idea that the ubiquitin-proteasome system plays a role in plant disease defences.

Ten peroxidase genes (designated TmPRX1 to TmPRX10) were recently isolated and characterized from a cDNA library constructed from the leaf epidermis of diploid wheat (*Triticum monococcum*) infected with the

powdery mildew fungus (*Blumeria graminis* f. sp. *tritici*) (Liu *et al.,* 2005). Consistent with its abundance in the EST collection, TmPRX1 expression showed the highest induction during pathogen attack and fluctuated in response to the fungal parasitic stages. TmPRX1 to TmPRX6 were expressed predominantly in mesophyll cells, whereas TmPRX7 to TmPRX10, which feature a putative C-terminal propeptide, were detectable mainly in epidermal cells. The differential expression profiles of the TmPRXs after abiotic stresses and signal molecule treatments were used to dissect the potential role of these peroxidases in multiple stress and defence pathways.

Transcription of the pepper defensin CADEF1 gene isolated from a cDNA library constructed from pepper leaves infected with an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* was induced earlier and more strongly by *X. campestris* pv. *vesicatoria* infection in the incompatible than in the compatible interaction. CADEF1 mRNA was constitutively expressed in the stem, roots and green fruit of pepper. Transcripts of CADEF1 gene accumulated to a great extent in pepper leaf tissues treated with SA, methyl JA, ABA, H_2O_2, benzothiadiazole and D,L-β-amino-n-butyric acid. *In situ* hybridization results revealed that CADEF1 mRNA was localized in the phloem areas of vascular bundles in leaf tissues treated with exogenous SA, methyl JA and ABA. A strong accumulation of CADEF1 mRNA occurred in pepper leaves in response to wounding, high salinity and drought stress. These results suggest that bacterial pathogen infection, abiotic elicitors and certain environmental stresses may play a significant role in the signal transduction pathway for CADEF1 gene expression (Do *et al.,* 2004).

An *Arabidopsis* mutant, designated *adr1* (activated disease resistance1), constitutively expressed SA-dependent defence genes and was resistant to a broad spectrum of virulent pathogens (Grant *et al.,* 2003). ADR1 was found to encode a coiled-coil/nucleotide-binding site/leucine-rich repeat protein, which possessed domains of homologous with serine/threonine protein kinases. It was also shown that either the constitutive or conditional enhanced expression of ADR1 conferred significant drought tolerance. This was not a general feature of defence-related mutants because *cir* (constitutive induced resistance)*1*, *cir2* and *cpr* (constitutive expressor of PR genes)*1*, which constitutively express systemic acquired resistance, failed to exhibit this phenotype. The increased drought tolerance of *adr1* was not indicative of cross-tolerance, because it did not show an increase in tolerance of other stress factors, they showed increased sensitivity to heat and salinity stress. Furthermore, neither hemizygous nor homozygous *adr1* plants exhibited either increased tolerance or sensitivity to heavy metal stress. In a similar fashion, *adr1* plants failed to exhibit any significant tolerance to

freezing (Chini *et al.,* 2004). Similar observations were made when a P4-chitinase genomic sequence was isolated from a bean genomic library using a P4-ch cDNA. Various stress conditions, such as wounding, SA and NaCl treatments, heat and cold stress were applied to bean (*Phaseolus vulgaris*) plants. Whereas wounding, NaCl treatment and cold stress were ineffective, the transcription of P4-chitinase mRNA was induced by SA treatment and, surprisingly, in response to heat stress (Margispinheiro *et al.,* 1994). Hence, *adr1*-activated signalling may antagonise some stress responses. The Northern analysis of abiotic marker genes revealed that the dehydration-responsive element DREB2A was expressed in adr1 plant lines, but not DREB1A, RD(response to dehydration)29A or RD22. Furthermore, DREB2A expression was SA-dependent but NPR (non-expressor of PR genes)1-independent. In the double mutants adr1/ADR1 *NahG*, *adr1*/ADR1 *eds* (enhanced disease susceptibility)1 and *adr1*/ADR1 abi1 (ABA insensitive), drought tolerance was significantly reduced. The microarray analysis of plants containing a conditional adr1 allele demonstrated that a significant number of the up-regulated genes were previously implicated in responses to dehydration (Chini *et al.,* 2004).

5. CONCLUSIONS

It is clear from the above results, that SA could be a very promising compound for the reduction of the abiotic stress sensitivity of crops, since under certain conditions it has been found to mitigate the damaging effects of various stress factors in numerous plant species (Figure 1).

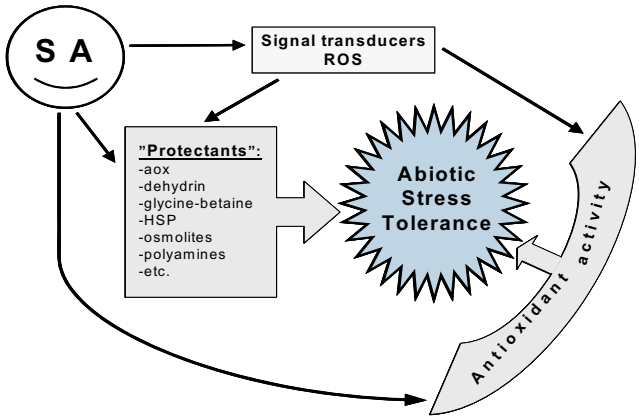

Figure 1. Schematic model of the action of SA on the induction of abiotic stress tolerance. For details see the text.

However, a number of questions remain unanswered at both the theoretical and the practical level. A similar effect is exerted by many related compounds involved in the synthesis of SA, whether artificial or naturally occurring in plants. This raises the question of whether SA is the only, or the most important key molecule. In certain cases contradictory results are obtained in investigations on the effect of exogenous SA application and that of endogenous SA levels. It is not yet clear whether the effect of exogenous SA is direct or whether it is connected with that of endogenous SA. SA is part of an extremely complex signal transduction network. The clarification of these questions could bring us closer to an understanding of the control mechanism.

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