SZENT ISTVÁN UNIVERSITY

BIOCHEMICAL AND MOLECULAR STUDIES ON THE ROLE OF REACTIVE OXYGEN SPECIES AND ANTIOXIDANTS IN PLANT DISEASE RESISTANCE

PhD THESIS

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NTRODUCTION

Reactive oxygen species (free radicals)

Recently, it turned out that there is a certain balance between the action of reactive oxygen species (ROS) and antioxidants in microorganisms, plant as well as in animal cells. As a result of stress or infection, this balance is abnormal or does not exist. Thus, the role of ROS in different forms of disease resistance or in symptom expression of susceptible plants seems to be pivotal.

Reactive oxygen species (ROS) are involved in many important processes in plants (Elstner et al., 1994). ROS are believed to have important roles in plants in general and in plant-pathogen interactions in particular. They are involved in signal transduction, cell wall reinforcement, hypersensitive response (HR) and phytoalexin production, and have direct antimicrobial effects (Abdu et al., 1993; Galal et al., 1993; Király et al., 1993). The main toxic ROS are the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) in the biological systems (Elstner, 1982, 1987; Tzeng and DeVay 1993). As is seen, some ROS are free radicals and some are reactive molecules.

Oxidative stress

Oxidative stress is caused by herbicides, infections (biotic stress) and abiotic stresses, such as air pollution, high light intensity, heat shock etc. Oxidative stress in the cells results in damaging the membranes, the lipids, amino acids, nucleic acids, pigments and proteins. The end result could be senescence or cell death.

As a result of senescence, superoxide is produced in a high level in the membranes and, at the same time, some antioxidants such as SOD are reduced in the activity (Droillard et al., 1987, 1989).

Role of antioxidants

Antioxidants are substances that delay or inhibit oxidative damage to target molecules such as lipids, proteins, nucleic acids and carbohydrates. Antioxidants might protect a target by scavenging oxygen-derived species or minimizing the formation of oxygen-derived species.

Induced resistance

A common response to necrogenic pathogen infection is the development of systemic acquired resistance (SAR) to a subsequent pathogen attack. This induced resistance or SAR results in broad-spectrum, non-specific immunity in non-infected parts of the plant and provides protection against several subsequent pathogens and non-pathogens (Ryals et al., 1994, 1995).

The accumulation of salicylic acid (SA) is an important component in the signal transduction pathway leading to SAR (Métraux et al., 1990; Malamy et al., 1990). It was suggested that the role of SA in SAR signal transduction can inhibit catalase activity, leading to elevated levels of H_2O_2 which could in turn function as a second messanger of SA in SAR signal transduction (Chen and Klessig, 1991; Chen et al., 1993).

The use of chemicals to activate SAR-type reaction provides novel alternatives for disease control in agronomic systems. 2,6-dichloroisonicotinic acid

and its methyl ester (both referred to as INA) were the first synthetic chemical compounds shown to activate SAR-type reaction, thus providing broad spectrum disease resistance. Thus, INA seems to be a proper compound for practical agronomic use.

The aim of my research

- I tried to have a deeper insight into the role of ROS (H_2O_2) in inducing symptom expression in powdery mildew infected barley.
- Induction of HR type necroses in virus-infected local lesion tobacco even at 30°C with the application of ROS.
- Chemically induced ROS and resistance caused by 2,6-dichloroisonicotinic acid (INA) treatment in susceptible barley plants.
- Immunization of tobacco plants with very low concentrations of H_2O_2.

MATERIALS AND METHODS

Plant materials

Tobacco (*Nicotiana tabacum*) cultivar Xanthi-nc, which is a local lesion (HR) host of TMV, was used in this study. Susceptible barley (*Hordeum vulgare*) expressing the gene *Mlo* and near-isogenic lines of cultivar Ingrid carrying the genes *mlo5*, *Mla12* and *Mlg* for resistance against the powdery mildew were also used.

Pathogens

Tobacco mosaic virus (TMV) U1 strain and *Pseudomonas savastanoi (syringae)* pv. *phaseolicola* GSPB 1205 were used in this study. The following fungal pathogens were involved in this study: barley powdery mildew (*Blumeria graminis* f. sp. *hordei* race A6) and *Botrytis cinerea* strain Bc-1.

Artificial production of necroses at 30°C

The riboflavin/methionine photochemical system

Inoculated tobacco leaves were detached three days after infection with TMV and put on the riboflavin/methionine solutions in Petri dishes. Six ml of mixture containing 266 or 532 µM riboflavin as well as 10 or 20 mM L-methionine, respectively was poured on filter paper in each Petri dish. The Petri dishes were illuminated (100 µE m^{-2} s^{-1}) in an incubator at 30°C for three days. The same treatments were conducted with inoculated intact leaves by infiltration with a syringe. TMV-infected leaves (without riboflavin treatment) and riboflavin treated healthy leaves (without infection) were used as controls.

ROS-producing glucose-glucose oxidase system and direct application of H_2O_2

Six ml of solutions containing 50, 100, 150, 200, 250 and 300 units of glucose oxidase/ml and 2 mM glucose were poured on filter paper in each Petri dish or injected into intact leaves. TMV inoculated leaves were detached three days after inoculation and put into these Petri dishes for three days. TMV-infected

leaves (without glucose-glucose oxidase) and glucose–glucose oxidase treated healthy leaves (without virus infection) were used as controls.

Direct application of 10, 25, 50, 100, 150 and 200 mM H_2O_2 was carried out by injecting the intact leaves or treating the detached leaves in Petri dishes as described above.

The reversible action of antioxidant enzymes

In tobacco infected with TMV, the action of ROS at 30°C was reversed by treatment with 4000 U/ml of superoxide dismutase (SOD) and 5000 U/ml of catalase (CAT). SOD and CAT were applied to leaves treated with riboflavin/methionine three days after inoculation with TMV. CAT was applied on leaves treated with glucose-glucose oxidase or directly treated with H_2O_2 three days after inoculation with TMV.

Barley leaves were injected with a water solution contained 2500 units superoxide dismutase (SOD) and 5000 units catalase (CAT)/ ml and leaves were sprayed with H_2O_2 after water evaporation. Leaves were infected with the powdery mildew fungus after H_2O_2 treatment.

Tobacco leaves inoculated with TMV, *P. syringae* pv. *phaseolicola* and *Botrytis cinerea* were injected immediately after inoculation with a solution which contained 5000 units CAT and 2500 units SOD/ ml.

Determination of concentration of TMV

Xanthi-nc tobacco plants infected with TMV and treated with riboflavin, glucose-glucose oxidase or H_2O_2 were kept at 30°C. TMV-infected tobacco pants held at 20°C served as controls. Four days after inoculation necroses (HR) appeared. ELISA test performed for determining concentration of TMV according to Clark and Adams (1977) and Tobiás et al. (1982).

Xanthi-nc tobacco plants pre-treated with low concentration of H_2O_2 were inoculated with TMV one day after the treatment. Plants infected with TMV were kept in the greenhouse for three days. TMV-infected untreated plants served as controls.

ELISA test performed for determining concentration of TMV as mentioned above.

Determination of concentration of bacteria

Infection with *P. syringae* pv. *phaseolicola* was carried out one day after treatment with low concentration of H_2O_2 and kept in the greenhouse for one day. Plants infected with the bacterium served as controls. To determine the bacterial concentration, we used the plate-count technique 24, 48 and 72 hours after inoculation.

Application of H_2O_2 to barley leaves

Leaves of 8-10-day-old barley seedlings were inoculated with *Blumeria graminis* f. sp. *hordei*. Some intact inoculated leaves were detached at different time periods after inoculation. Either intact or detached leaves were sprayed with 25-50 mM H_2O_2 one, two and three days after inoculation. The water solution of H_2O_2 contained 0.5% Tween. Leaves, which were treated with H_2O_2 only or infected only with the pathogen, served as controls.

Application of low concentration of H_2O_2 in tobacco

The fourth and fifth true leaves of 8-10-week-old Xanthi-nc plants were treated by spraying the plant leaves with an aqueous solution of H_2O_2. Plants were sprayed with 5, 7, 10, and 12.5 mM H_2O_2 solution. The control plants were treated with water alone. After one day of H_2O_2 treatments, the leaves infected with TMV, *Pseudomonas syringae* pv. *phaseolicola* or with *Botrytis cinerea*.

Chemical induction of resistance by INA

Susceptible barley seedlings (*Hordeum vulgare* cultivar Ingrid) were treated with 2,6-dichloroisonicotinic acid (INA) applied as a soil-drench (6-mg/litre soil) 3-4 days after sowing. Inoculation with the powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*) was carried out 4 days after INA treatment (7-8 days after sowing). Leaf samples were harvested 12, 18, 24, 36, 48, 60, 72, 84 and 96 hours after inoculation with the powdery mildew fungus.

Histochemical analysis of ROS

Histochemical staining for superoxide production in leaf tissue was based on the ability of $O_2^{\cdot-}$ to reduce nitro blue tetrazolium (NBT). Superoxide was visualised as a purple discoloration of NBT. Discoloration of leaf discs was quantified using a ChemiImager 4000 digital imaging system .

H_2O_2 was visualised as a reddish-brown discoloration of 3,3-diaminobenzidine (DAB). Detection of H_2O_2 was performed using 0.1% DAB, as described by Thordal-Christensen et al. (1997) and Hückelhoven et al. (1999).

To detect H_2O_2 spectrophotometrically with a peroxidase independent reaction, a xylenol orange based method was used according to Gay et al. (1999).

To detect H_2O_2 by the spectrofluorometer, 2', 7'-dichlorofluorescein diacetate (DCFH-DA) dye was used. This dye reacts with H_2O_2 in the presence of peroxidase yielding the fluorescent dichlorofluorescein (DCF). We used this method described by Lu and Higgins (1998).

Biochemical and gene expression assays of the antioxidant enzymes

Activities of antioxidant enzymes, such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), guaiacol peroxidase (POX) as well as activity of NADPH oxidase were determined by biochemical (spectrophotometric) assays. The gene expression of antioxidant enzymes, alternative oxidase and BAX inhibitor gene were determined using the RT-PCR technique.

3. RESULTS

Role of hydrogen peroxide in symptom expression of barley susceptible and resistant to powdery mildew

Under natural conditions barley leaves, carrying the gene *Mlo*, exhibited susceptible response to infection, the *mlo* and *Mlg* barley leaves were resistant but did not develop HR necrotic symptoms. The *Mla12* barley was resistant and developed HR-type symptoms. Under the influence of treatment with H_2O_2 (25-50

mM), leaves of the susceptible *Mlo* and the resistant *mlo5* or *Mlg* plants exhibited HR-type symptoms with tissue necroses. The *Mla12*-resistant genotype produced HR earlier and the number of necrotic lesions increased, as compared to untreated but infected control leaves. H_2O_2 alone without infection did not induce symptoms. These experiments show that ROS may have role in symptom expression and disease resistance.

Treatment of barley genotypes with H_2O_2 before establishment of infection (one day after inoculation) resulted in inhibition of the pathogen and symptomless response in all of the four genotypes, because the pathogen was probably killed before the establishment.

It was possible to reverse the inhibitory effect as well as the HR-producing actions of H_2O_2 by injection of barley leaves with a combination of superoxide dismutase (SOD) and catalase (CAT) before treatment with H_2O_2.

Immunization of tobacco with low concentration of hydrogen peroxide against oxidative stress caused by viral, bacterial and fungal infections

In tobacco leaves pre-treated with low concentration (5-7 mM) of hydrogen peroxide (H_2O_2) the number and size of necroses caused by tobacco mosaic virus (TMV) infection was suppressed and the antioxidant capacity augmented, as compared to the untreated but infected control. Particularly the activity of CAT, ascorbate peroxidase (APX) and guaiacol peroxidase (POX) was stimulated.

Suppression of necroses and increased activity of these antioxidant enzymes were also demonstrated in pre-treated tobacco leaves infected with *Pseudomonas syringae* pv. *phaseolicola*. Pre-treatment of tobacco with H_2O_2 similarly suppressed necrotization caused by the fungal pathogen *Botrytis cinerea*. Injection of a mixture of SOD and CAT into leaves infected with TMV, *P. syringae* pv. *phaseolicola* and *B. cinerea* significantly diminished tissue necrotization caused by these pathogens.

Concentration of TMV and the number of bacteria did not change significantly in infected leaves, which were pre-treated with H_2O_2. Viral and bacterial concentrations were also not diminished when exogenously applied SOD and CAT suppressed tissue necrotization. Low concentration of H_2O_2 exerted no action on the growth of *B. cinerea* in an artificial medium.

It is concluded that spraying leaves with low concentration of H_2O_2 can immunize tobacco leaves to necrotic symptoms caused by viral, bacterial and fungal pathogens by increasing activity of several antioxidant enzymes, but multiplication of pathogens is not enhanced.

Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization associated with resistance of an N gene encoding tobacco

It is known that local lesion hosts of TMV cannot develop HR-type necroses at high temperature, such as 30°C and the concentration of TMV increases at this high temperature. Chemical compounds which generate reactive oxygen species (ROS), such as riboflavin/methionine and glucose/glucose oxidase

systems, or the directly applied H_2O_2 are able to induce HR-type necroses in Xanthi-nc tobacco infected with TMV even at 30°C. It was possible to suppress the chemically induced HR-type necrotization at 30°C by the application of antioxidants, such as SOD and CAT. In this case TMV content, as determined by the ELISA-test, did not change.

The amount of superoxide ($O_2^{\cdot-}$) decreased and that of the H_2O_2 slightly increased in leaves of infected and healthy Xanthi-nc tobacco at 30°C, as compared to 20°C. Activity of NADPH-oxidase and the mRNA levels of the NADPH-oxidase and an alternative oxidase were also significantly lower at 30°C, as compared to 20°C. Activity of a dehydroacorbate reductase (DHAR) significantly increased at 30°C, as compared to 20°C. However, the mRNA level of SOD and CAT did not change. Interestingly, expression of the gene Bax inhibitor (*NtBI-1*) was stimulated in TMV-infected tobacco kept at 30°C. The development of HR-type necroses caused by TMV infection depends on a certain level of superoxide and other ROS.

Accordingly, suppression of virus multiplication in resistant tobacco is independent of the appearance of necroses but depends on high temperature.

Changes in prooxidants (ROS) and antioxidants in barley leaves in which resistance was induced chemically by INA

2,6-dichloroisonicotinic acid (INA) is an analogue of salicylic acid (SA) which acts as a chemical inducer of resistance. Treatment of barley leaves with INA four days before inoculation induces resistance against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). The frequency of epidermal cell death (HR) significantly increased in INA-treated barley leaves upon powdery mildew infection and accompanied by elevated levels of ROS, including H_2O_2. Activities and expression of the genes SOD and DHAR were significantly inhibited as a result of INA treatment.

NEW SCIENTIFIC RESULTS

- It was demonstrated that spraying barley leaves with hydrogen peroxide (H_2O_2) could alter the type of symptoms and induces HR-type necrosis in the *Mlo*-susceptible genotype. This effect could be reversed with the application of antioxidant enzymes (SOD and CAT).
- As a result of H_2O_2-treatment, the *Mla12*-resistant genotype produced HR earlier and the number of necrotic lesions increased, as compared to untreated but infected control leaves. Leaves of the resistant non-necrotic *mlo5* or *Mlg* plants exhibited HR-type symptoms. It was also possible to reverse this action of H_2O_2 with the antioxidants.

- Pre-treatment of tobacco plants with low concentration of H_2O_2 suppressed tissue necrotization caused by viral, bacterial and fungal infections. Suppression of tissue necrotization is in correlation with the high activities of several antioxidants (CAT, APX and POX). H_2O_2-treatment did not alter the multiplication (replication) of the pathogens.

- HR-type necrosis was induced in Xanthi-nc tobacco infected with TMV even at 30°C by chemical compounds which produce ROS or with direct application of H_2O_2. This action can be reversed with antioxidants. At 30°C

the level of superoxide was significantly reduced and activity of enzymes associated with the production of superoxide as well as their gene expression were also suppressed.

- The expression of the gene BAX inhibitor was stimulated at 30°C, which corresponds to the suppression of necrotization.

- Resistance can be induced chemically with 2,6-dichloroisonicotinic acid (INA) in barley susceptible to powdery mildew. As a result of INA-treatment, level of ROS increased and the activity as well as gene expressions of antioxidants were reduced. Correspondingly, HR-type necrosis developed in the originally susceptible barley.

CONCLUSIONS

Role of hydrogen peroxide in symptom expression of barley

1. Spraying four genotypes of barley (cultivar Ingrid) expressing the genes *Mlo*, *mlo5, Mla12* and *Mlg* with H_2O_2 and infected with *Blumeria graminis* f. sp. *hordei* resulted in HR-type necrosis and produced necroses earlier in the *Mla12* barley than in the control.

2. Treatment of barley genotypes with hydrogen peroxide (H_2O_2) before establishment of infection (one day after inoculation) resulted in inhibition or killing the pathogen and symptomless response in all of the four genotypes.

3. It was possible to reverse these actions of H_2O_2 with injection of barley leaves with a combination of superoxide dismutase (SOD) and catalase (CAT) before treatment with H_2O_2.

Imunization of tobacco with low concentration of H_2O_2

1. In tobacco leaves pre-treated with low concentration of H_2O_2 the number and size of necroses caused by tobacco mosaic virus (TMV) infection was suppressed and the antioxidant capacity augmented, as compared to the untreated but infected control. Particularly the activity of CAT, ascorbate peroxidase (APX) and guaiacol peroxidase (POX) was stimulated. Suppression of necroses and increased activity of these antioxidant enzymes were also demonstrated in pre-treated tobacco leaves infected with *Pseudomonas syringae* pv. *phaseolicola*.

Pre-treatment of tobacco with H_2O_2 similarly suppressed necrotization caused by the fungal pathogen *Botrytis cinerea*.

2. Injection of a mixture of SOD and CAT into leaves infected with TMV, *P. syringae* pv. *phaseolicola* and *B. cinerea* significantly diminished tissue necrotization caused by these pathogens, showing that the increased activities of antioxidants could be responsible for immunization.

3. Concentration of TMV and the number of bacteria did not change significantly in infected leaves, which were pre-treated with H_2O_2. Also, viral and bacterial concentrations were not diminished when exogenously applied SOD and CAT suppressed tissue necrotization. Low concentration of H_2O_2 exerted no action on the growth of *B. cinerea* in an artificial medium.

Role of reactive oxygen species (ROS) and antioxidants in TMV-induced necrotization at high temperature

1. Chemical compounds which generate reactive oxygen species (ROS), such as riboflavin/methionine and glucose/glucose oxidase systems, or the directly applied H_2O_2 are able to induce HR-type necroses in Xanthi-nc tobacco infected with TMV even at 30°C.
2. Chemically induced HR-type necrotization at 30°C was suppressed by the application of antioxidants such as SOD and CAT. In this case TMV content, as determined by the ELISA-test, did not change.
3. The amount of superoxide ($O_2^{\cdot-}$) decreased and that of the H_2O_2 slightly increased in leaves of infected and healthy Xanthi-nc tobacco at 30°C, as compared to 20°C.
4. Activity of NADPH-oxidase and the mRNA levels of NADPH-oxidase as well as an alternative oxidase were also significantly lower at 30°C, as compared to 20°C. Activity of a dehydroacorbate reductase (DHAR) significantly increased at 30°C, as compared to 20°C. However, gene expressions of SOD and CAT on the mRNA level did not change. Interestingly, expression of the gene Bax inhibitor (*NtBI-1*) was stimulated in TMV-infected tobacco kept at 30°C.

Induced resistance caused by 2,6-dichloroisonicotinic acid (INA) in barley

Treatment of barley leaves with 2,6-dichloroisonicotinic acid (INA) four days before inoculation induces resistance against barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). The frequency of epidermal cell death (HR) significantly increased in INA-treated barley leaves upon powdery mildew infection and resistance was accompanied by elevated level of ROS (H_2O_2) and reduced activities of SOD and DHAR

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