

Communication

Plastoquinone: possible involvement in plant disease resistance[✉]

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The plant *Solanum nigrum* treated with the pathogen *Phytophthora infestans*-derived elicitor responded by elevated reactive oxygen species (ROS) production, lipid peroxidation and lipoxygenase (EC 1.13.11.12) activity in comparison with control plants indicating that oxidative stress took place. We demonstrate that these events are accompanied by a significant increase in plastoquinone (PQ) level. It is postulated that PQ may be associated with mechanisms maintaining a tightly controlled balance between the accumulation of ROS and antioxidant activity that determines the full expression of effective defence.

Oxidative stress, caused by reactive oxygen species (ROS), such as singlet oxygen, superoxide radicals, hydroxyl radicals and hydrogen peroxide which are inevitable by-products of physiological redox reactions, is now considered as the main etiological agent involved in the progressive deterioration of cell structures. All biological molecules may be modi-

fied by ROS. Lipid peroxidation, protein oxidation and DNA damage are the main consequences of oxidative stress.

It has been established that contact between plant and a pathogen or a pathogen-derived elicitor results in a rapid production of ROS (oxidative burst) at the site of infection (Ebel & Mithöfer, 1998). The genera-

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Abbreviations: CF, culture filtrate; LOX, lipoxygenase; NBT, nitroblue tetrazolium; PQ, plastoquinone; ROS, reactive oxygen species; TBARS, 2-thiobarbituric acid-reactive substances; UQ, ubiquinone.

tion of ROS plays several important roles in prevention of pathogen spread but also has important consequences for the host plant. A role for hydrogen peroxide as antimicrobial agent (Wu *et al.*, 1995) and signal molecule for the hypersensitive cell death response (Levine *et al.*, 1994) and for salicylic acid biosynthesis (Wu *et al.*, 1997) has already been demonstrated. However, the level of ROS must be controlled to avoid damage to surrounding noninvaded tissue whose viability is required for coordinated defence response. In plants, an oxidative burst is usually accompanied by enhanced biosynthesis of antioxidants and increased activity of ROS-scavenging enzymes (Vanacker *et al.*, 1998). Most such enzymes are water soluble and have limited access to hydrophobic membranes. In recent years experimental evidence has accumulated from studies with mammalian tissues which strongly suggest that ubiquinone (UQ) in its reduced form may play an important role as endogenous cellular antioxidant (Beal, 2002; Albano *et al.*, 2002). It has also been found that, together with α -tocopherol and β -carotene, plastoquinone (PQ) in its reduced form can function as an antioxidant in chloroplast thylakoid membranes (Hundal *et al.*, 1995). In contrast to tocopherol and carotene, associated with specific pigment-protein complexes (Cogdell & Frank, 1987), the abundant plastoquinone with its high mobility within the hydrophobic membrane might constitute a very suitable scavenger of toxic oxygen species. The localisation of PQ synthesis in the Golgi apparatus and the endoplasmic reticulum as well as in chloroplasts (Swiezewska *et al.*, 1993) suggests that it could exert its antioxidative function in different cellular compartments.

The goal of the present study was to test whether the oxidative reactions: ROS production, peroxidation of lipids and lipoxygenase (LOX) activity exhibited by the resistant plant *Solanum nigrum* in response to the pathogen *Phytophthora infestans*-derived

elicitor include changes in the level of plastoquinone.

MATERIALS AND METHODS

Plant and pathogen. Axenic shoots of *Solanum nigrum* were propagated in vitro on hormone-free MS medium (Murashige & Skoog, 1962) as previously described (Maciejewska *et al.*, 1999). Growth of the pathogen *P. infestans* and preparation of culture filtrate (CF) that served as elicitor were described by Awan *et al.* (1997).

Elicitor treatment of leaves. Leaves harvested from 4 week-old plants were placed on moist filter paper in Petri dishes. The culture filtrate was applied on the surface of each leaf 100 μ l per 150 mg fresh mass. In parallel, dishes with control leaves with an equal amount of distilled water were prepared. Leaves were incubated for 18 h at 25°C under continuous light (40 μ m m⁻² s⁻¹) from fluorescent tubes (Pila, Poland) or in darkness where indicated.

Assay for ROS generation. The determination of O₂⁻ generated by leaves based on the reduction of nitroblue tetrazolium (NBT) was according to Doke (1983). After treatment with culture filtrate or H₂O the leaves were incubated for 1 h in NBT mixture. The reduction of NBT is expressed as ΔA_{580} /h per g fresh mass.

Peroxidation of lipids. Lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances (TBARS) according to the modified method of Oteiza & Bechara (1993). The tissues were homogenised in 5% trichloroacetic acid and centrifuged at 10 000 $\times g$ at 4°C for 10 min. The 1 ml reaction mixture contained the cell extract, 0.3% (w/v) SDS, 0.25% (w/v) TBA in 50 mM NaOH and 6% HCl. TBARS were extracted with 1 ml of 1-butanol. The specific absorbance of the organic phase was measured at 532 nm and corrected for absorbance measured at 600 nm. Measurements are expressed as A₅₃₂/g fresh mass.

Lipoxygenase activity. Lipoxygenase (EC 1.13.11.12) activity in leaf extracts was determined using a Clark oxygen electrode with linoleic acid as substrate (Fournier *et al.*, 1993). The results are expressed as nmole of O₂/g fresh mass per s.

Lipid extraction. Leaves of *S. nigrum* were homogenised using a Waring Blender at high speed for 2 × 1 min in 0.25 M sucrose. The resulting slurry was filtered through four layers of nylon net and the residue homogenised and filtered once more. Lipids from the homogenate were extracted with chloroform/methanol/water (CMW) 1:1:0.3 (by vol.) for 1 h at 20°C and the extract adjusted to a final CMW ratio of 3:2:1 (by vol.). The organic phase was evaporated to dryness. The extracted lipids were dissolved in hexane and applied to a silica gel 60 (230–400) mesh, ASTM column equilibrated with hexane. A step gradient of 3, 6, 10 and 20% diethyl ether in hexane yielded a fraction of partially purified quinones.

HPLC analysis. Lipids were analyzed by reversed-phase HPLC using a Hewlett-Packard Hypersil ODS 3- μ m column at a flow rate of 1.5 ml/min in a linear gradient program from A: methanol/water (9:1, v/v) to B: methanol/2-propanol (8:2, v/v). The external quantitative plastoquinone standard prepared as described earlier (Swiezewska *et al.*, 1993) was used.

Statistical evaluation. Statistical analysis was performed using Student's *t*-test; differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Our objective was to define if the oxidative processes activated in leaves by elicitor treatment induced changes in the level of plastoquinone. Leaves of a resistant *S. nigrum* plant were treated with culture filtrate (CF) from the pathogen *P. infestans*-derived elicitor time chosen to assess that after ROS forma-

tion had taken place (it proceeds up to 24 h, not shown) subsequent events were established.

Formation of ROS is one of the earliest consequences of pathogen attack. Superoxide anions, among others, are generated in that process. NBT reduction is a good indicator of their presence. The results presented in Table 1 show a 2.33-fold increase in NBT reduction in CF treated leaves in comparison with control leaves.

Another consequence of elicitation is accumulation of the products of lipid peroxidation. This is a secondary damage occurring during oxidative stress in plants. The quantification of the accumulation of lipid peroxidation products in elicitor-treated leaves shows a 33% increase in the level of TBARS as compared to control leaves (Table 1). The induction of transcription of LOX genes and the corresponding increase of lipoxygenase activity following pathogen attack have been described (Kolomiets *et al.*, 2000). This may imply the release of specific lipid peroxide molecules such as fatty acid hydroxyperoxides which are subsequently metabolized into molecules with known or hypothesized regulatory activities. The results presented in Table 1 show a 2.6-fold increase in lipoxygenase activity upon treatment with elicitor.

Having established the concerted events that occur after elicitor treatment, we investigated whether they are accompanied by changes in the level of plastoquinone in infected leaves.

Lipids from CF-treated and untreated control leaves were extracted as described in Materials and Methods and subjected to HPLC chromatography. Figure 1 shows one of the runs. To make sure that in both samples the peak identified based on the retention time of a plastoquinone standard (30.6 min) really represents PQ, partially purified quinones were subjected to reduction with sodium borohydride. The disappearance of the peak at 30.6 min is the confirmation of its identity. The external plastoquinone standard made it

Table 1. Oxidative reactions in *Solanum nigrum* leaves treated with culture filtrate (CF) of *Phytophthora infestans*.

Data represent the mean \pm S.D. of four independent experiments ($P < 0.02$).

Treatment	Reaction		
	NBT reduction (ΔA_{580} /g fresh mas per h)	TBARS (A_{532} /g fresh mass)	LOX activity (nmol of O_2 /g fresh mass per s)
H ₂ O	0.18 \pm 0.07	1.35 \pm 0.20	3.66 \pm 0.71
CF	0.42 \pm 0.11	1.81 \pm 0.24	9.83 \pm 1.35

possible to determine the amount of PQ in treated and untreated leaves. Bearing in mind the influence of light on the pool of plasto-

quinones (Gaunt & Stowe, 1967) we compared the possible changes in the level of plastoquinone in CF-treated leaves under light and

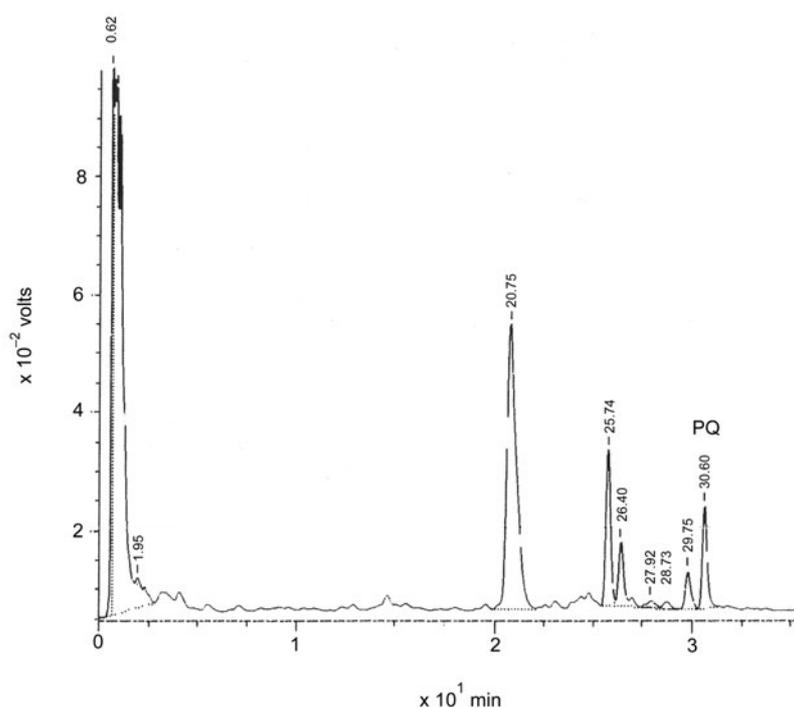


Figure 1. HPLC analysis of partially purified quinones synthesized after elicitor treatment.

Lipid extraction and purification are as described in Materials and Methods. PQ indicates the plastoquinone peak.

dark condition. Table 2 demonstrates that, independently of light, elicitor treatment of leaves results in a statistically significant in-

crease ($P < 0.01$) in the amount of PQ in comparison with control leaves (34% and 96%, light and darkness, respectively). However,

Table 2. The effect of elicitor treatment on the level of plastoquinone.

Data represent the mean \pm S.D. of three independent experiments ($P < 0.01$)

Treatment	Plastoquinone content $\mu\text{g/g}$ fresh mass	
	Light	Dark
H ₂ O	1.12 \pm 0.10	0.54 \pm 0.06
CF	1.50 \pm 0.12	1.06 \pm 0.11

the amount of plastoquinone in light-exposed leaves was higher than in those kept in the dark. It may be explained considering that the amount of PQ in illuminated leaves is determined by two processes, light-stimulated synthesis and decay of plastoquinone (Wanke *et al.*, 2000) while in the darkness there is no such stimulation (Gaunt & Stowe, 1967). So the elicitor-induced synthesis of PQ appears to be more evident in the darkness since it is not masked by the influence of light. One could expect that elicitor treatment might cause similar changes in the level of UQ. However, the ratio of UQ to PQ in *S. nigrum* is so low that HPLC measurement of ubiquinone content is statistically insignificant (not shown).

Antioxidant activity of reduced plastoquinone (PQH₂) has been demonstrated. However, the experiments were performed on isolated spinach chloroplast thylakoid membranes subjected to strong illumination and the defence mechanism against oxidative damage concerned only the photosynthetic apparatus (Hundal *et al.*, 1995).

As documented here, in elicitor-treated leaves the total amount of plastoquinone significantly increased. This phenomenon may be associated with mechanisms involved in maintaining a tightly controlled balance between the accumulation of ROS and antioxidant activity that determines the full expression of effective defence. This may suggest that plastoquinone, similarly to ubiquinone in animal systems, may function as a universal plant antioxidant. The control of lipid peroxidation processes in membranes, not necessarily at the site of infection but also in surrounding cells, might be the possible mechanism of the antioxidant PQ activity. The reduced form of PQ needed for exerting the antioxidant activity could be the product of reductases described in the literature, i.e. ferredoxin-PQ reductase (Bendall & Manasse, 1995) or NAD(P)H-PQ oxidoreductase (Cornelle *et al.* 1998).

The use of leaves instead of cell suspensions or isolated membranes allowed us to investigate more complex events that reflect naturally occurring reactions which take place after elicitor treatment. For the first time we demonstrate that the level of plastoquinone significantly increases after pathogen attack. However, there is a disadvantage in this model. Extraction, purification and HPLC analysis of plastoquinone from the tissue are processes causing reoxidation of PQH₂ to PQ, thus allowing only total quantification. Clearly, methods for *in vivo* determination of the PQ/PQH₂ ratio are necessary. Such work is in progress.

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