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The gene encoding glutathione-dependent formaldehyde dehydrogenase/GSNO reductase is responsive to wounding, jasmonic acid and salicylic acid

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Abstract It has recently been discovered that glutathione-dependent formaldehyde dehydrogenase (FALDH) exhibits a strong *S*-nitrosoglutathione reductase activity. Plants use NO and *S*-nitrosothiols as signaling molecules to activate defense mechanisms. Therefore, it is interesting to investigate the regulation of FALDH by mechanical wounding and plant hormones involved in signal transduction. Our results show that the gene encoding FALDH in *Arabidopsis* (*ADH2*) is down-regulated by wounding and activated by salicylic acid (SA). In tobacco, FALDH levels and enzymatic activity decreased after jasmonate treatment, and increased in response to SA. This is the first time that regulation of FALDH in response to signals associated with plant defense has been demonstrated.

Key words: Glutathione-dependent formaldehyde dehydrogenase; GSNO reductase; Wound response; Jasmonic acid; Salicylic acid

1. Introduction

Glutathione-dependent formaldehyde dehydrogenase (FALDH; EC 1.2.1.1) is a ubiquitous enzyme also known as class III alcohol dehydrogenase. The enzyme catalyzes the NAD^+-dependent formation of *S*-formylglutathione from *S*-hydroxymethylglutathione that forms spontaneously by condensation between formaldehyde and glutathione. It has been long proposed that the main function of this enzyme in biological organisms is to detoxify endogenous and exogenous formaldehyde. This toxic compound forms intracellularly from demethylation of several amino acids [1] and, in plants, also from the catalase-mediated oxidation of methanol, derived from pectin hydrolysis during leaf expansion [2]. Alternative substrates for FALDH are aldehydic fatty acid derivatives [3] that can be generated intracellularly from lipid membranes in a situation of oxidative stress [4]. Recently, it has been demonstrated that FALDH is very active in the reduction of *S*-nitrosoglutathione (GSNO), the condensation product of glutathione and NO, that is a naturally occurring NO reservoir and also a reactive nitrogen intermediate. This GSNO reductase activity has been demonstrated for the enzyme purified from different organisms, including *Arabidopsis thaliana* [5–7].

The nature of the FALDH substrates strongly suggests that this enzyme might play an important role in cell protection against different types of stress and, in particular, in signal transduction processes that rely on NO. Biotic stress in plants activates an oxidative burst with production of O_2^- and H_2O_2, accompanied by an increase in salicylic acid (SA) and perturbations of cytosolic Ca^{2+} to trigger defense mechanisms [8]. Recent data indicate that NO and GSNO are signaling molecules in plant defense [9]. NO cooperates with reactive oxygen intermediates (ROIs) in the activation of hypersensitive cell death [10], which requires a delicate balance between ROIs and NO production [11]. In addition, many data support the linkage of oxidative stress to other abiotic stresses, such as cold, UV, air pollutants and drought. On the other hand, phytohormones are also involved in the signaling events triggered in response to different types of stresses to activate different sets of protectant genes. For instance, ethylene, abcisic acid (ABA), jasmonic acid (JA) and SA have been implicated in stress responses such as wounding, anaerobiosis, drought, cold and salinity [12].

In this work we have explored the response of the *ADH2* gene (*At*5g43940), coding for the FALDH/GSNO reductase, to wounding and to several hormones that act as signals for environmental stress. Our results demonstrate that plant FALDH, although highly expressed in all plant tissues [13], is transcriptionally regulated in response to signals associated with plant defense.

2. Materials and methods

2.1. Biological material and treatments

BY-2 cells were cultivated as described in [14]. *A. thaliana* ecotype Columbia was grown in soil under a 16-h light/8-h dark regime, at 22°C. For the different treatments, *Arabidopsis* seeds were surface-sterilized and grown as in [15]. Stocks were prepared as follows: 100 mM ABA in dimethyl sulfoxide; 100 mM JA in *N*,*N*-dimethyl formamide; 50 mM SA in water (pH 6). Control plants were treated with an equivalent amount of the corresponding solvent. Wounding experiments were performed with plants grown in soil, as in [15]. BY-2

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Abbreviations: ABA, abcisic acid; APAD, 3-acetylpyridine adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; FALDH, glutathione-dependent formaldehyde dehydrogenase; GSNO, S-nitrosoglutathione; JA, jasmonic acid; ROI, reactive oxygen intermediate; SA, salicylic acid; SAR, systemic acquired resistance; SDS, sodium dodecyl sulfate

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cell viability was calculated with fluorescein diacetate (0.1 mg/ml) and subsequent observation under a fluorescence microscope (Leica DRMB), using an I3 filter (Leica).

2.2. Immunochemical procedures, protein extracts and enzymatic activity

Antibody against *Arabidopsis* FALDH was produced in rabbits by conventional methods (unpublished). For Western blotting, 20 μg of proteins was electrophoresed on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore) and incubated with a FALDH antiserum (dilution 1:3000). Membranes were revealed with the Immun-Star detection kit system (Bio-Rad). Protein content was assessed by Bradford analysis [16], and equal loading confirmed by membrane staining (0.1% Coomassie blue in 7% acetic acid and 50% methanol). Protein extracts were obtained in 0.1 M sodium phosphate buffer, pH 8, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. FALDH activity was determined as in [13], using *S*-hydroxymethylglutathione as substrate and 3-acetylpyridine adenine dinucleotide (APAD) as cofactor. The assay mixture contained 1 mM formaldehyde, 1 mM glutathione, 0.6 mM APAD, in 0.1 M sodium phosphate buffer, pH 8.

2.3. Northern blots

RNA preparation and Northern blot analysis were performed as in [13]. The complete cDNA for FALDH (*ADH2*) was used as a ^{32}P-radiolabeled probe and hybridized in 0.2 M sodium phosphate, pH 7.2, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% bovine serum albumin, 7% SDS, at 65°C. The membrane was washed twice in 40 mM sodium phosphate, pH 7.2, 1 mM EDTA, and 1% SDS, at 65°C.

3. Results

3.1. Wound-induced transcriptional repression of ADH2 gene

Mechanical damage of plant tissue, or wounding, occurs upon insect attack and initiates a series of biochemical and molecular events that are identical to those involved in the defense response to insects. To assess whether FALDH is involved in host defense, we examined the *ADH2* mRNA levels at different times after wounding. Fig. 1A shows a transient decrease in the amount of the transcripts, both in the locally injured leaves and in the systemic or unwounded leaves, indicating a transcriptional repression of the gene. The repression was higher in damaged leaves 2 h after wounding than in unwounded leaves (rosette and cauline). The mRNA levels were recovered 48 h after wounding. As a control, the same membrane was subsequently hybridized with the wound-inducible gene *WR3*, showing a transient accumulation of mRNA levels, as previously described [15]. The decrease in FALDH mRNA correlated with a decrease in the protein levels, both in wounded and systemic leaves (Fig. 1B). This effect was observed 2 h after wounding and persisted up to 48 h after wounding.

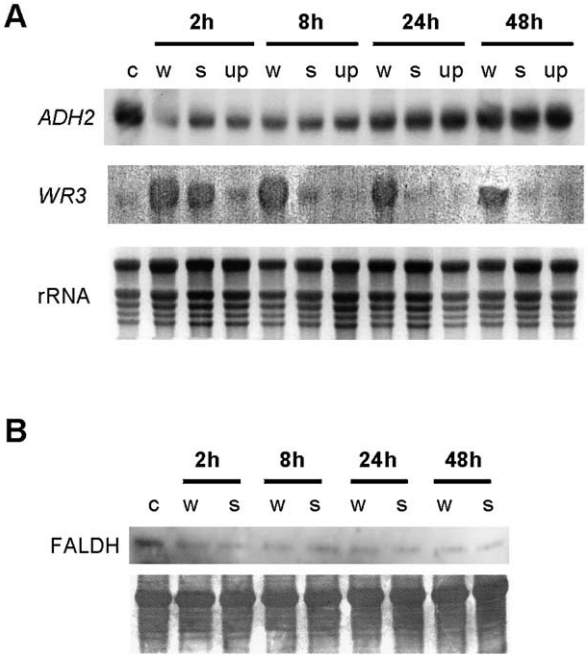

Fig. 1. Wound-induced repression of *ADH2* gene in *A. thaliana*. Plants were sampled at the indicated times after wounding. A: 5 μg of total RNA was loaded per lane and blots were hybridized with ^{32}P-labeled probes derived from *ADH2* or from *WR3*. Ethidium bromide-stained rRNAs are shown as loading control. B: Western blot analysis. The Coomassie blue-stained membrane is shown as loading control. c, control, rosette leaves from unwounded plants; w, wounded rosette leaves; s, systemic, unwounded rosette leaves from wounded plants; up, cauline leaves from wounded plants.

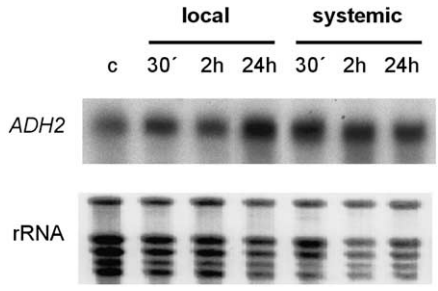

Fig. 2. Wound response of *ADH2* gene in *Arabidopsis coi* mutant. Plants were sampled at the indicated times after wounding and Northern blots were performed as in Fig. 1. c, control, rosette leaves from unwounded plants; local, locally wounded rosette leaves; systemic, unwounded rosette leaves from wounded plants.

3.2. Hormonal regulation of FALDH

The expression of some wound-inducible genes, such as *Jr1*, *Jr2*, or *VPS* is also strongly induced by application of exogenous JA. On the other hand, in potato and tomato, ABA is required for the wound-induced activation of *Pin2* [17]. To ascertain whether JA is a mediator in the wound-induced regulation of *ADH2* gene in *Arabidopsis*, we used the *coi1 Arabidopsis* mutant, which is jasmonic-insensitive [18]. No repression of *ADH2* gene was observed after wounding, but, to the contrary, mRNA levels were slightly higher both in the wounded and the systemic leaves (Fig. 2). This result supports the idea that JA mediates the wound-induced repression of *ADH2* gene in *Arabidopsis*. To get more insight into the molecular signals involved in the wound-induced transcriptional repression of *ADH2* gene, ABA and JA were added exogenously and FALDH expression was measured during a time course. The experiments were performed using tobacco BY-2 suspension cells and *Arabidopsis* plantlets grown in liquid medium. After treatment with 50 μM JA, a dramatic decrease in the amount of FALDH from tobacco protein extracts was observed (Fig. 3A) that correlated with a similar decrease in the specific activity of the enzyme (Fig. 3B). The response was first observed at 24 h after treatment and persisted after 72 h. Treatment with 100 μM ABA resulted in no effect on FALDH levels (not shown). Addition of 50 μM JA to *Arabidopsis* plantlets resulted in no significant change in both FALDH and *ADH2* mRNA levels. Increasing the concentration up to 500 μM did not have any effect, nor did the use of

methyl jasmonate at different concentrations from 200 μM to 1 mM (Figs. 3C,D). We do not have an explanation for the lack of response of the *ADH2* gene to exogenous JA in *Arabidopsis* but one possible speculation is that a synergism with other factors is necessary.

3.3. *Regulation of* ADH2 *by SA*

SA is an important signal in plant defense responses. An increase in the intracellular SA levels is necessary for transcriptional activation of defense genes [19,20] and for the establishment of the systemic acquired resistance (SAR) [21]. Biosynthesis of intracellular SA is also very tightly linked to both oxidative and nitrosative stress, as has been demonstrated by the increase in SA levels provoked by UV and ozone treatments [22,23], and in NO-treated tobacco plants [9].

The exogenous application of 150 μM SA to BY-2 tobacco cells provoked a marked increase in both protein accumulation (Fig. 4A) and specific activity of FALDH (Fig. 4B). The response was maximal at 48 h after treatment. Exogenous application of 150 μM SA to *Arabidopsis* plantlets had very little effect on FALDH levels (data not shown). However, treatment with 500 μM SA and 1 mM SA provoked a clear increase in FALDH levels (Fig. 4C) that correlated with an increase in *ADH2* mRNA levels (Fig. 4D). The higher accumulation of the protein was observed at 48 h after treatment, whereas the mRNA levels were higher at 5 h and persisted up to 48 h. These results strongly suggest a transcriptional up-regulation of *ADH2* gene by SA.

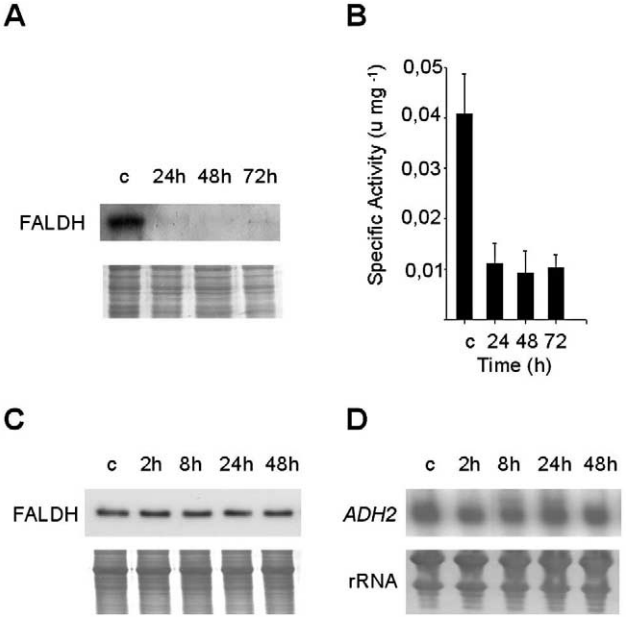

Fig. 3. Effect of JA treatments on FALDH expression in tobacco BY-2 cells and in *Arabidopsis* plants. A,B: BY-2 cells (day 2 after subculturing) were treated with 50 μM JA or the corresponding amount of *N*,*N*-dimethyl formamide (c) for the indicated times. Samples were analyzed by Western blot (panel A) and FALDH-specific activities (panel B). Error bars indicate standard deviations of three independent experiments. C,D: *Arabidopsis* plantlets grown in liquid medium were treated with 200 μM methyl jasmonate for the indicated times. Samples were analyzed by Western blot (panel C) and Northern blot (panel D). Membranes stained with Coomassie blue (panels A and C) or methylene blue (panel D) are shown as loading controls.

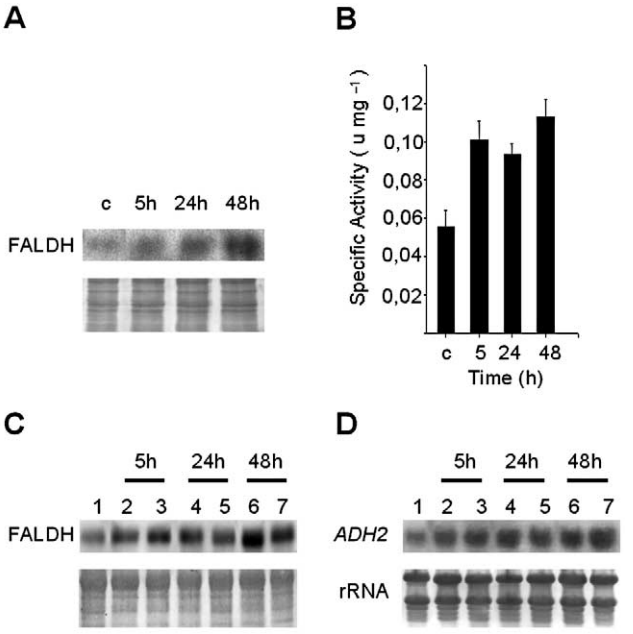

Fig. 4. Effect of SA treatments on FALDH expression in tobacco BY-2 cells and in *Arabidopsis* plants. A,B: BY-2 cells (day 2 after subculturing) were treated with 150 μM SA or the corresponding amount of distilled water (c), for the indicated times. Samples were analyzed by Western blot (panel A) and FALDH-specific activities (panel B). Error bars indicate standard deviations of three independent experiments. C,D: *Arabidopsis* plantlets were treated with 0.5 mM SA (lines 2, 4 and 6) or 1 mM SA (lines 3, 5, and 7) for the indicated times. C: Western blot analysis. D: Northern blot analysis, with 15 μg of total RNA and hybridized with ^{32}P-labeled *ADH2*. Membranes stained with Coomassie blue (panels A and C) or methylene blue (panel D) are shown as loading controls.

4. Discussion

Our results demonstrate that *ADH2* gene expression is regulated by molecular signals related to plant defense. *ADH2* gene is down-regulated by wounding, and the response is transient and systemic. A stronger repression was observed in locally injured leaves than in systemic leaves and, in both cases, the expression returned to basal levels by 48 h after wounding. Western blot analysis demonstrated a concomitant decrease in FALDH levels that was first observed 2 h after wounding and persisted up to 48 h. Two distinct signaling pathways involved in the wound response have been proposed [15]. One of them does not require JA and is mainly responsible for the activation of genes at the site of the local damage. The other is JA-dependent and activates genes both locally and systemically to the wound site. On the other hand, some genes require ABA to be activated in response to wounding [17]. Our results strongly suggest that *ADH2* is a JA-responsive and ABA-unresponsive gene, both in tobacco and in *Arabidopsis*.

ADH2 gene is also regulated by SA, an endogenous signal that induces certain defense-related genes and that is necessary for the establishment of SAR. SA also promotes a type of cell death associated with the hypersensitive response in some plant–pathogen-incompatible interactions [24,25], whereas in other cases it suppresses it [26,27]. It has been demonstrated that SA binds to an endogenous catalase and inhibits its activity [28], leading to an increase in the H_2O_2 concentration that can exacerbate the oxidative stress at the local site. More-

over, inhibition of catalase and of ascorbate peroxidase might generate the damaging SA free radical [29] that could initiate lipid peroxidation. We observed that addition of exogenous SA to tobacco BY-2 cells resulted in cell death in a dose-dependent manner. At 1 mM SA, only 1% of the cells was alive after 24 h treatment. For this reason, treatments of BY-2 cells were performed with 150 μM SA (95% viability, same as control), and a clear increase in both specific activity and FALDH protein levels was observed that was maximal at 48 h. In *Arabidopsis* plantlets, however, 0.5 mM SA was required to observe an increase in FALDH and the corresponding *ADH2* mRNA levels. Neither cell necrosis nor cell death was observed after 48 h of treatment, revealing a different sensitivity from the tobacco suspension cells. The up-regulation of the *ADH2* gene in response to SA suggests a role of FALDH in protection against oxidative stress and/or nitrosative stress. Although the interrelationship between the NO and ROS signaling pathways in plants is currently unclear, both compounds stimulate the accumulation of SA [9,30]. One of the consequences of the oxidative stress is lipid peroxidation that might generate formaldehyde and other reactive lipid peroxidation products that can be eliminated by FALDH [3]. On the other hand, the importance of FALDH in nitrosative stress has been demonstrated in mice and yeast, where deletion of the gene encoding FALDH increases the susceptibility of the cells to nitrosative challenge and produces an accumulation of nitrosylated proteins [6].

It is difficult to speculate about the functional significance of FALDH repression caused by wounding and JA. It has been reported that jasmonate reduces synthesis of Rubisco and other proteins related to basal cell functions [31]. One of the cell's responses to a herbivore attack might be the transient repression of housekeeping genes and the concomitant synthesis of specific defense proteins. Alternatively, JA down-regulation of *ADH2* could play a role in pathogenesis. For instance, a transient decrease of FALDH might be necessary to avoid its GSNO-consuming activity during the first moments following a pathogen attack. Though there is no evidence of a NO-mediated signal in wound defense, it cannot be ruled out. It has been reported that NO negatively modulates the expression of wound-inducible defense genes in tomato by a SA-independent signaling pathway [32], but the experiments were performed in excised leaves and, thus, the effects observed only locally. On the other hand, the substrate of FALDH is GSNO and it is not known how the NO/GSNO pool is regulated. NO also mediates *S*-glutathiolation and *S*-nitrosation of proteins that modulate intracellular signaling [33,34]. NO and *S*-nitrosothiols might thus operate at different levels in the NO-mediated signaling pathways, and understanding the regulation of the enzymes involved in NO metabolism is of crucial importance. FALDH might play a dual role in turning off/on NO or GSNO signaling, and in modulating the concentration of intracellular thiol compounds that can generate nitrosative stress. Our results, showing a down-regulation of FALDH/GSNO reductase by wounding, strongly suggest a modulation of NO metabolism as part of the wound defense response. The generation of transgenic plants with modified levels of FALDH would provide a good tool to investigate the importance of this enzyme in NO signaling in plants.

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References

[1] Uotila, L. and Koivusalo, M. (1989) in: Coenzymes and Cofactors. Glutathione: Chemical, Biochemical and Medical Aspects (Dolphin, D., Poulson, R., Avramovic, O., Eds.), Vol III, part A, pp. 517–55, Wiley, New York.
[2] Fall, R. and Benson, A.A. (1996) Trends Plant Sci. 1, 296–301.
[3] Boleda, M.D., Saubi, N., Farrés, J. and Parés, X. (1993) Arch. Biochem. Biophys. 307, 85–90.
[4] Mano, J. (2002) In: Oxidative Stress in Plants (Inzé, D. and Van Montagu, M., Eds.), pp. 217–245, Taylor and Francis, London.
[5] Jensen, D.E., Belka, G.K. and Du Bois, G.C. (1998) Biochem. J. 331, 659–668.
[6] Liu, L., Hausladen, A., Zeng, M., Que, L., Heitman, J. and Stamler, J.S. (2001) Nature 410, 490–494.
[7] Sakamoto, A., Ueda, M. and Morikawa, H. (2002) FEBS Lett. 515, 20–24.
[8] Lamb, C. and Dixon, R.A. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251–275.
[9] Durner, J., Wendehenne, D. and Klessing, D.F. (1998) Proc. Natl. Acad. Sci. USA 95, 10328–10333.
[10] Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nature 394, 585–588.
[11] Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Proc. Natl. Acad. Sci. USA 98, 13454–13459.
[12] Leshem, Y.Y. and Kuiper, P.J.C. (1996) Biol. Plant 38, 1–18.
[13] Martínez, M.C., Achkor, H., Persson, B., Fernández, M.R., Shafqat, J., Farré, J., Jörnvall, H. and Parés, X. (1996) Eur. J. Biochem. 241, 849–857.
[14] Nagata, T., Nemoto, Y. and Hasezawa, S. (1992) Int. Rev. Cytol. 132, 1–30.
[15] Titarenko, E., Rojo, E., León, J. and Sánchez-Serrano, J. (1997) Plant Physiol. 115, 817–826.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Peña-Cortés, H., Fisahn, J. and Willmitzer, I. (1995) Proc. Natl. Acad. Sci. USA 92, 4106–4113.
[18] Feys, B.J.F., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) Plant Cell 6, 751–759.
[19] Klessig, D.F. and Malamy, J. (1994) Plant Mol. Biol. 26, 1439–1458.
[20] Vernooij, B., Uknes, S.J., Ward, E. and Ryals, J. (1994) Curr. Opin. Cell Biol. 6, 275–279.
[21] Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y. and Hunt, M.D. (1996) Plant Cell 8, 1809–1819.
[22] Yalpani, N., Enyedi, A.J., León, J. and Raskin, I. (1994) Planta 193, 372–376.
[23] León, J., Lawton, M.A. and Raskin, I. (1995) Plant Physiol. 108, 1673–1678.
[24] Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T. (1999) Plant Cell 11, 1695–1708.
[25] Brading, P.A., Hammond-Kosack, K.E., Parr, A. and Jones, J.D. (2000) Plant J. 23, 305–318.
[26] Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Science 261, 754–756.
[27] Rate, D.N. and Greenberg, J.T. (2001) Plant J. 27, 203–211.
[28] Conrath, U., Chen, Z., Ricigliano, J. and Klessig, D.F. (1995) Proc. Natl. Acad. Sci. USA 92, 7143–7147.
[29] Durner, J. and Klessing, D.F. (1995) Proc. Natl. Acad. Sci. USA 92, 11312–11316.
[30] Kumar, D. and Klessig, D.F. (2000) Mol. Plant Microbe Int. 13, 347–351.
[31] Reinbothe, S., Reinbothe, C. and Parthier, B. (1993) Plant J. 4, 459–467.
[32] Orozco-Cárdenas, M.L. and Ryan, C.A. (2002) Plant Physiol. 130, 487–493.
[33] Klatt, P. and Lamas, S. (2000) Eur. J. Biochem. 267, 4928–4944.
[34] Stamler, J.S., Lamas, S. and Fang, F.C. (2001) Cell 106, 675–683.