Reconstitution in vitro of the components and conditions required for the oxidative cross-linking of extracellular proteins in French bean (*Phaseolus vulgaris* L.)

Przemyslaw Wojtaszek[1], Jonathan Trethowan, G. Paul Bolwell*

Division of Biochemistry, School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK

Received 30 January 1997

Abstract Oxidative cross linking of three (hydroxy)proline-rich cell wall proteins, known to be immobilised during the elicitor-induced oxidative burst in French bean cells, was modelled using peroxidases with cysteine or H_2O_2. Further reconstitution of the homologous system was achieved with a 46 kDa bean cell-wall peroxidase using conditions known to appertain in cell walls prior to the immobilisation. Thus, cell wall alkalinisation and secretion of a reductant have been reconstituted by addition of apoplastic fluid to the wall proteins and the 46 kDa peroxidase with resultant cross-linking. This is the first demonstration of the oxidative cross-linking of cell wall glycoproteins without the addition of external H_2O_2.

Key words: Oxidative cross-linking; In vitro reconstitution; (Hydroxy)proline-rich glycoprotein; Extracellular matrix; Defense response; *Phaseolus vulgaris*

1. Introduction

Pathogen infection of a plant leads to the activation of a wide range of defence mechanisms. In true incompatible interactions this results in the hypersensitive response (HR) [1]. The rapid and transient production of active oxygen species (AOS), termed 'the oxidative burst' is one of the earliest reactions observed during this response [2,3], and is thought to drive the cross-linking of extracellular components to form a barrier 'papilla' preventing further progression of the pathogen. The papilla is thought to consist of callose, wall proteins and phenolics [4]. However, it is not possible to investigate its formation biochemically due to the amount of tissue available. Therefore, the changes in the cell wall are modelled using elicitor-treated suspension-cultured cells.

The plant cell wall is altered in response to either internal, e.g. developmental, or external stimuli, e.g. wounding, infection or in the presence of elicitors of either microbial and/or host origin. This response leads to lignification in tissues and immobilisation of specific (hydroxy)proline-rich glycoproteins (HRGPs or PRPs), and other antimicrobial wall proteins [5–12]. Differential changes in protein extractability have also been noted for some proteins in suspension-cultured cells of French bean in response to elicitor treatment [8–10,12]. Until recently, no direct evidence has been provided demonstrating the mechanism by which these proteins become immobilised. Now studies involving animals (sea urchin [13]), algae (*Chlamydomonas* [14]), and plants (soybean [9,11], and French bean [9,12]) indicate the possible involvement of a transient rapid production of H_2O_2 (the oxidative burst) as a prerequisite for the oxidative cross-linking of the extracellular proteins in response to elicitation, wounding and during developmental processes.

The present work demonstrates in vitro the oxidative cross-linking of three (hydroxy)proline-rich proteins purified from cell walls of suspension-cultured French bean cells. The components of the model system utilising horse radish peroxidase (HRP), and H_2O_2 or cysteine are replaced sequentially to demonstrate the homologous reconstitution of the cross-linking reaction. Firstly, the reaction is catalysed by a 46 kDa cell wall peroxidase (FBP1) isolated from French bean [15], capable of generating H_2O_2 at alkaline pH. Secondly, the cross-linking reaction of a 42 kDa proline-rich, chitin-binding protein (PRP), isolated from French bean [16] is reconstituted in vitro using only components isolated from French bean suspension-cultured cells. An attempt to determine the nature of cross-links in such high molecular weight complexes of cross-linked proteins is also presented.

2. Materials and methods

2.1. Plant material

Suspension cultures of French bean (*Phaseolus vulgaris* L.) were derived and maintained as described previously [17].

2.2. Preparation of proteins

Horse radish peroxidase (HRP) was from Sigma, UK, and for the experiments a stock solution of 1 unit/μl in 50 mM phosphate buffer, pH 7.0 was prepared. A 46 kDa cationic cell-wall peroxidase (FBP1) was isolated from French bean cell cultures as described previously [15]. For the isolation of 230 and 136 kDa HRGP-type glycoproteins [12], and a 42 kDa chitin-binding protein [16], cell wall proteins were isolated from intact French bean cells with 0.2 M $CaCl_2$ [18]. Following precipitation of the bulk of the extracted proteins with 10% trichloroacetic acid, the resultant supernatant was extensively dialysed against water and freeze-dried. Proteins were dissolved in 0.1% (v/v) TFA (trifluoroacetic acid) at a concentration of 1 mg/ml, applied to an Aquapore RP 300 C8 column (220×2.1 mm – Brownlee, obtained from Applied Biosystems, UK), and eluted with a 0–70% gradient of 0.1% TFA (v/v) in 90% acetonitrile (v/v). Fractions containing proteins of interest were collected, dialysed against water, and freeze-dried.

2.3. Preparation of apoplastic fluid

For all experiments, bean cells were used 4–5 days after subculture. Apoplastic fluids were prepared either from normally growing cells or

*Corresponding author. Fax: (44) (1784) 434326.
E-mail: UHBC006@vms.rhbnc.ac.uk

[1]*Permanent address:* Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznan, Poland.

Abbreviations: AOS, active oxygen species; FBP1, French bean peroxidase; HR, hypersensitive response; HRGP, hydroxyproline-rich glycoprotein; HRP, horse radish peroxidase; IDT, isodityrosine; PRP, proline-rich glycoproteins; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

PII S0014-5793(97)00166-X

from cells responding to biotic elicitation with an elicitor preparation from cell walls of *Colletotrichum lindemuthianum* prepared and quantified as described previously [17]. The intensity of the defence response was evaluated on the basis of the oxidative burst measured as a H_2O_2-inducible chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) using a luminometer fitted with a rotating cuvette holder and an injection port (model 1250, LKB Wallac, Bromma, Sweden) as described [19]. Following 10–12 min elicitor treatment (maximum of the oxidative burst), cells were filtered on a funnel, and washed with H_2O. After suspending the cells in 10% (v/v) ethanol in 20 mM acetate buffer, pH 5.5 for 1 min, the apoplastic fluid was recovered by gentle suction. No proteins were extracted by this procedure and cells remained intact. In all experiments the apoplastic fluids were used within 1 h after extraction.

2.4. In vitro cross-linking of cell wall glycoproteins

For the oxidative cross-linking reactions glycoproteins were dissolved (at 0.1 mg/ml) in 0.1 M Tris-HCl, pH 7.5, and 5-μl aliquots used per assay. In model experiments 1 μl of stock HRP solution was used or an FBP1 preparation (various amounts of the solution obtained in the last purification step). The reaction was initiated by the addition of H_2O_2 or cysteine to obtain end concentrations of 1 mM or 500 μM, respectively. Reactions were carried out for 30 min at room temperature. Complete reconstitution of the oxidative cross-linking involved the addition of 5-μl aliquots of the apoplastic fluids isolated from bean cells instead of H_2O_2/cysteine. Reactions were terminated by the addition of an equal volume of SDS-PAGE sample loading buffer and heating at 100°C for 2 min. Cross-linking of cell wall glycoproteins was analysed by mobility shift on SDS-polyacrylamide gels as described below. This criterion has been used previously to demonstrate the formation of high molecular weight cross-linked products [9,12]. Density gradient centrifugation was also carried out on the purified 42 kDa glycoprotein and following cross-linking in the presence of the French bean peroxidase and apoplastic fluid. 42 kDa or reaction mixtures were layered onto caesium chloride (initial density 1.44 g/ml) in a total volume of 1.2 ml and centrifuged for 24 h at 200 000 × *g* in a Beckman TL-100 ultracentrifuge. The shift in buoyant density of the proteins was determined by diluting 100 μl gradient fractions to 700 μl with H_2O_2 and determining absorbance at 280 nm as the 42 kDa protein is tyrosine-rich.

2.5. Characterisation of proteins

Protein content was determined using a BioRad assay system [20] with bovine serum albumin as a standard. Proteins were separated by SDS-PAGE on 10% (w/v) discontinuous gels [21], and detected by Coomassie staining. The identity of purified proteins was confirmed by N-terminal microsequencing using a gas-phase sequencer (model 477A, Applied Biosystems, Foster City, USA). For the amino acid analysis, cross-linked products were hydrolysed in 6 M HCl for 16 h at 95°C. Amino acid compositions were determined using ninhydrin detection on an Alpha plus amino acid analyser (LKB, Bromma, Sweden) according to the manufacturer's instructions. A standard sample of isodityrosine (IDT – obtained from Prof. S.C. Fry, Department of Botany, University of Edinburgh, UK) was added for comparative purposes.

3. Results and discussion

HRGP proteins of 230 and 136 kDa, and a 42 kDa chitin-binding protein were purified from cell wall extracts of French bean suspension cultured cells (Fig. 1), and their identity with previously characterised proteins [12,16] has been confirmed by N-terminal microsequencing. All three proteins, and a 22 kDa pathogenesis related-like protein, were previously demonstrated to be oxidatively cross-linked in cell walls of French bean cells within 15 min after application of an elicitor preparation from cell walls of *C. lindemuthianum* [12]. A similar situation was earlier noted in suspension-cultured soybean cells [9,11]. Although it was shown that this process is H_2O_2-driven and pH-dependent [12], the mechanism of cross-linking remained unsolved. One of the possible explanations is presented below.

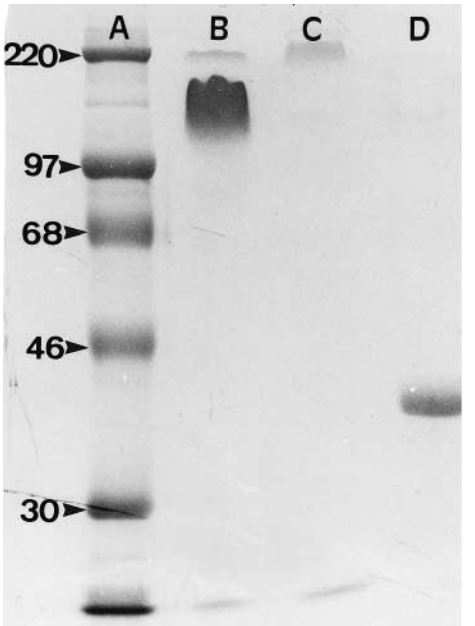

Fig. 1. SDS-PAGE of purified cell wall HRGP/PRP-type glycoproteins isolated from intact suspension-cultured French bean cells. (A) Molecular mass markers (kDa); (B) HRGP-type 136 kDa protein; (C) HRGP-type 230 kDa protein; (D) PRP-type 42 kDa chitin-binding protein.

Three purified proteins were used in single or mixed reactions in the presence of HRP and H_2O_2 under the conditions determined previously to be optimal for the oxidative burst [3]. The appearance of high molecular weight protein complexes, which did not enter the resolving gel, was observed (Fig. 2) indicating the oxidative formation of cross-links between (hydroxy)proline-rich O-linked glycoproteins. In the cases that included the 42 kDa PRP this always disappeared from its characteristic position in the gels. In the mixture of the two HRGPs aberrant running of some of the 136 kDa was observed, which may represent some loss of glycosylation under oxidising conditions, although the majority of it was cross-linked. In these mixed reactions it could not be determined whether cross-linking took place between the different structural proteins or whether it occurred only between like proteins.

To study this reaction in more detail, the properties for cross-linking of the 42 kDa proline-rich chitin-binding protein were determined. When using HRP and either H_2O_2 or Cys, the formation of high molecular weight complexes was again observed (Fig. 3). In the latter case, the complex formed was too large to enter the gel. The application of cysteine follows earlier propositions [22–25] and recent findings [3] that plant peroxidases are able to generate H_2O_2 in the presence of suitable reductant. Thus, the observation of cross-linking confirms that this process is driven by the oxidative burst when H_2O_2 is formed.

The other characteristic of the oxidative cross-linking is its pH dependence. It was demonstrated previously that upon elicitation the apoplastic pH of French bean cells rises transiently from about pH 6.5 to pH 7.2 [3]. This transient alkalinisation of extracellular matrix is also accompanied by a transient rise in the ability of the apoplastic fluid to support in vitro the production of H_2O_2 by peroxidases [26]. Previous data also implicated FBP1 peroxidase [15] as a putative source

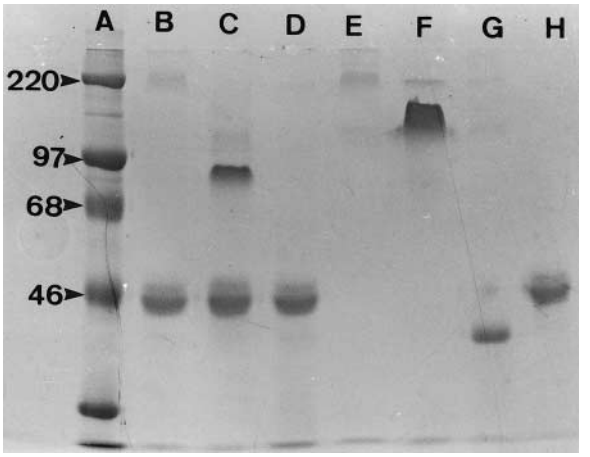

Fig. 2. Oxidative cross-linking of cell wall glycoproteins in mixed reactions in vitro in the presence of HRP and H_2O_2. (A) Molecular mass markers (kDa). Reaction mixtures: (B) 230 kDa +42 kDa glycoproteins (HRGP/PRP mixture); (C) 136 kDa +230 kDa proteins (HRGPs only); (D) 136 kDa +42 kDa glycoproteins (HRGP/PRP mixture). Protein standards: (E) 230 kDa protein; (F) 136 kDa protein; (G) 42 kDa chitin-binding protein; (H) horse radish peroxidase, all at the same concentrations as the reaction mixtures.

of H_2O_2 generated during the oxidative burst, with the pH profile of its activity being maximal at pH 7.5 [3]. When this homologous system (FBP1+apoplastic fluid from French bean cells) was tested for its ability to drive the oxidative cross-linking of a 42 kDa protein, the reaction was found to be dependent on the time course of elicitation. Only when the apoplastic fluid extracted from cells after 8 min and before the appearance of H_2O_2 in the oxidative burst (10–12 min after elicitation) was used was the formation of cross-linked high

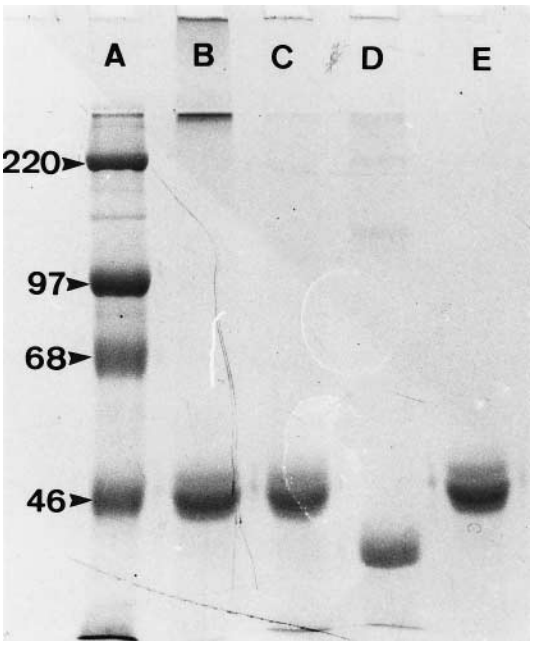

Fig. 3. Properties of the oxidative cross-linking of a 42 kDa protein in single reactions in vitro under various conditions. Reactions were carried out at pH 7.5. (A) Molecular mass markers (kDa); (B) 42 kDa+HRP+H_2O_2; (C) 42 kDa+HRP+cysteine. Protein standards: (D) 42 kDa chitin-binding protein; (E) horse radish peroxidase, at the same concentrations as the initial reaction mixture.

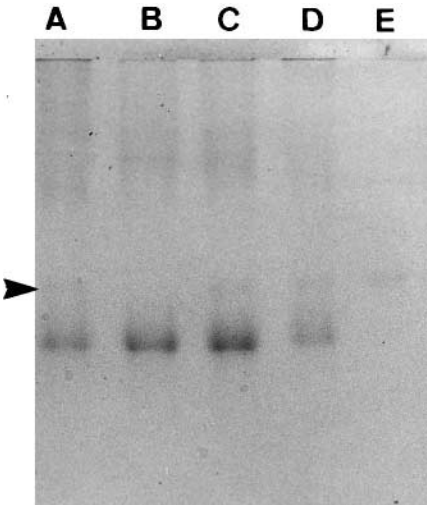

Fig. 4. Oxidative cross-linking of a 42 kDa chitin-binding protein in a fully homologous in vitro system consisting of the components isolated from suspension-cultured French bean cells. (A) Control mixture (42 kDa+FBP1+H_2O_2); (B) 42 kDa protein; (C) 42 kDa+FBP1+apoplastic fluid from non-elicited cells; (D) 42 kDa+FBP1+apoplastic fluid isolated from elicited cells before the peak of the oxidative burst; (E) FBP1 peroxidase. Arrows mark the position of the FBP1. Note much less peroxidase is required to bring about substantial immobility on gels for the 42 kDa PRP. The two control tracks are at the same concentration as in the reaction mixtures. Note less recovery of 42 kDa protein at its native molecular mass upon SDS-PAGE from the reaction mixtures.

molecular weight products observed (Fig. 4). Once again, these were too large to enter the gel. Attempts to demonstrate the existence of high molecular weight complexes by size exclusion chromatography were not possible since the heavily glycosylated protein bound to silica-, dextran- and acrylamide- based matrices and ran aberrantly. However, increased buoyant density following oxidative cross-linking, was observed on CsCl gradients (Fig. 5), consistent with the formation of high molecular weight complexes. It could therefore be possible that the alkalinisation of the cell wall is a prerequisite for FBP1 to use reductant appearing in the cell wall (released or synthesised) to produce H_2O_2 which is utilised in the oxidative cross-linking of structural proteins and other monomeric components such as phenolics. This result represents the first demonstration of the oxidative cross-linking of cell wall proteins in vitro in the presence of peroxidase, but without the addition of external H_2O_2.

It is interesting to note that the properties of FBP1 are different from those of a p*I* 4.6 peroxidase found to cross-link specifically monomeric extensin precursors in suspension-cultured tomato cells [27,28]. The latter is an acidic enzyme with a broad pH optimum (pH 5.5–6.5) for extensin cross-linking. Moreover, a p*I* 4.6 peroxidase does not cross-link minimally glycosylated PRP proteins indicating the requirement of glycosylated substrates for the enzyme to be active [28]. These differences seem to confirm previous suggestions [12] that plant cells possess several mechanisms for cross-linking of monomeric precursors in cell walls regulated either developmentally (e.g. a p*I* 4.6 peroxidase) or in response to external stress stimuli (e.g. FBP1 – an enzyme sensitive to cell wall alkalinisation). What is also of interest, an ovoperoxidase – enzyme responsible for the cross-linking of envelope pro-

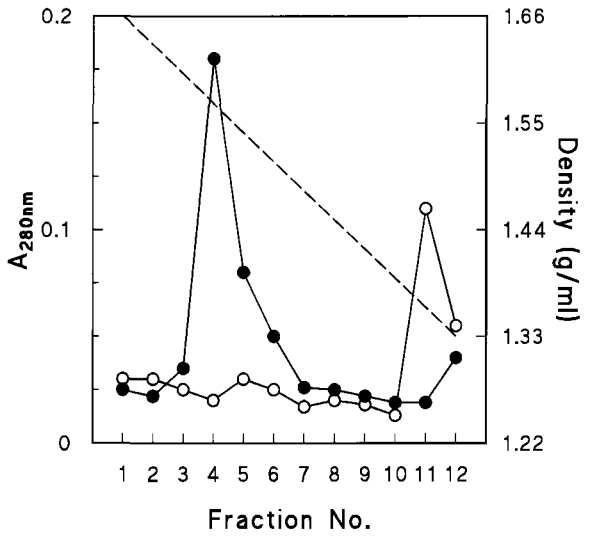

Fig. 5. Demonstration of increased buoyant density of the 42 kDa protein on CsCl gradients following oxidative cross-linking. Either 42 kDa protein (20 μg) or 42 kDa (50 μg)+FBP1+apoplastic fluid isolated from elicited cells before the peak of the oxidative burst were subjected to CsCl density centrifugation. Protein was detected by absorbance at 280 nm for (○) 42 kDa and (●) reaction mixture. (– – –) density gradient. Note increased buoyant density consistent with cross-linking.

teins in fertilised sea urchin egg – is similarly activated by the rise of pH in extracytoplasmic compartment [13].

The last question addressed is the nature of intermolecular cross-links in high molecular weight protein complexes. Although many putative intermolecular cross-links have been proposed (reviewed in [29–31], also [32,33]), no direct evidence, that is the isolation of a 'linkage group', have been presented yet. The in vitro formation of cross-links in a fully homologous system seemed to be a good model to study the nature of those intermolecular bonds. Thus, immobilised product of 42 kDa protein cross-linking was subjected to acid hydrolysis followed by amino acid analysis using a sample of isodityrosine (IDT) as a standard (IDT has been demonstrated to be a cross-linking component in previous studies [29]). However, no measurable appearance of IDT in cross-linked product has been observed (data not shown). It has already been proposed that IDT might serve only as an intra-, but not intermolecular linkage group (see [29,31]), and our result seems to confirm this view.

These and previous results suggest that immobilisation of HRGP/PRP-type proteins as a result of the oxidative burst is probably a primary event during the hypersensitive response where a fungal hypha entering a cell in an incompatible reaction could trigger the oxidative cross-linking of structural proteins and phenolics present [34] thus firstly slowing down the rate of penetration [11], and then forming a barrier (papillae) to further growth of the fungus [4,35]. The mechanism of the oxidative immobilisation is clearly different from the events observed during developmental processes. Moreover, it is also different from the immobilisation of proteins noted at later stages of the infection process, which is probably pH-independent and driven by lipid peroxides [12]. However, the nature of intermolecular cross-links formed in any of these processes remains an unsolved mystery.

Acknowledgements: We thank Prof. S.C. Fry for the kind gift of the isodityrosine sample. G.P.B. thanks the BBSRC for funding. J.B.T. is a recipient of a BBSRC research studentship.

References

[1] Atkinson, M.M. (1993) Adv. Plant Pathol. 10, 35–64.
[2] Mehdy, M.C. (1994) Plant Physiol. 105, 467–472.
[3] Bolwell, G.P., Butt, V.S., Davies, D.R. and Zimmerlin, A. (1995) Free Rad. Res. 23, 517–532.
[4] Aist, J.R. (1976) Annu. Rev. Phytopathol. 14, 145–163.
[5] Stuart, D.A. and Varner, J.E. (1980) Plant Physiol. 74, 247–251.
[6] Cooper, J.B. and Varner, J.E. (1984) Plant Physiol. 76, 414–417.
[7] Smith, J.J., Muldoon, E.P. and Lamport, D.T.A. (1984) Phytochemistry 23, 1233–1239.
[8] Bolwell, G.P. (1987) Planta 172, 184–191.
[9] Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Cell 70, 21–30.
[10] Bolwell, G.P. (1993) Int. Rev. Cytol. 146, 261–324.
[11] Brisson, L.F., Tenhaken, R. and Lamb, C.J. (1994) Plant Cell 6, 1703–1712.
[12] Wojtaszek, P., Trethowan, J.B. and Bolwell, G.P. (1995) Plant Mol. Biol. 28, 1075–1087.
[13] Shapiro, B.M. (1991) Science 252, 533–536.
[14] Waffenschmidt, S., Woessner, J.P., Beer, K. and Goodenough, U.W. (1993) Plant Cell 5, 809–820
[15] Zimmerlin, A. Wojtaszek, P. and Bolwell, G.P. (1994) Biochem. J. 299, 747–753.
[16] Millar, D.J., Slabas, A.R., Sidebottom, C., Smith, C.G., Allen, A.K. and Bolwell, G.P. (1992) Planta 187, 176–184.
[17] Dixon, R.A. and Lamb, C.J. (1979) Biochim. Biophys. Acta 586, 453–463.
[18] Stafstrom, J.P. and Staehelin, L.A. (1988) Planta 174, 321–332.
[19] Robertson, D., Davies, D.R., Gerrish, C., Jupe, S.C. and Bolwell, G.P. (1995) Plant Mol. Biol.27, 59–67.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Elstner, E.F. and Heupel, A. (1976) Planta 130, 175–180.
[23] Gross, G.G., Janse, C. and Elstner, E.F. (1977) Planta 136, 271–276.
[24] Halliwell, B. (1978) Planta 140, 81–88.
[25] Mader, M. and Amberg-Fisher, V. (1982) Plant Physiol. 70, 1128–1131.
[26] Bolwell, G.P. (1996) Biochem. Soc. Trans. 24, 438–442.
[27] Everdeen, D.S., Kiefer, S., Willard, J.J., Muldoon, E.P., Dey, P.M., Li, X.-B. and Lamport, D.T.A. (1988) Plant Physiol. 87, 616–621.
[28] Schnabelrauch, L.S., Kieliszewski, M., Upham, B.L., Alizedeh, H. and Lamport, D.T.A. (1996) Plant J. 9, 477–489.
[29] Fry, S.C. (1986) Annu. Rev. Plant Physiol. 37, 165–186.
[30] Showalter, A.M. (1993) Plant Cell 5, 9–23.
[31] Iiyama, K., Lam T.B.-T. and Stone, B.A. (1994) Plant Physiol. 104, 315–320.
[32] Kieliszewski, M.J. and Lamport, D.T.A. (1994) Plant J. 5, 157–172.
[33] Qi, X., Behrens, B.X., West, P.R. and Mort, A.J. (1995) Plant Physiol. 108, 1691–1701.
[34] Luck, G., Liao, H., Murray, N.J., Grimmer, H.R., Warminski, E.E., Williamson, M.P., Lilley, T.H. and Haslam, E. (1994) Phytochemistry 37, 357–371.
[35] O'Connell, R.J., Brown, I.R., Mansfield, J.W., Bailey, J.A., Mazau, D., Rumeau, D. and Esquerre-Tugaye, M.-T. (1990) Mol. Plant-Microbe Interact. 3, 33–40.