An Elicitor of the Hypersensitive Lignification Response in Wheat Leaves Isolated from the Rust Fungus *Puccinia graminis* f. sp. *tritici*
II. Induction of Enzymes Correlated with the Biosynthesis of Lignin

B. Moerschbacher, B. Heck, K. H. Kogel, O. Obst, and H. J. Reisener

Institut für Biologie III der RWTH Aachen, Worringer Weg, D-5100 Aachen, Bundesrepublik Deutschland

Z. Naturforsch. **41c,** 839–844 (1986); received April 4/June 9, 1986

Triticum aestivum, Puccinia graminis f. sp. *tritici,* Hypersensitive Response, Lignification, Elicitor

The genuine biotic elicitor from germ tube walls of *Puccinia graminis* f. sp. *tritici* (*Pgt*-elicitor) induces lignification, preceded by an increase in phenylalanine ammonia-lyase (PAL) activity, when injected into the intercellular space of primary wheat leaves. This increase in PAL activity is accompanied by an increase in other enzyme activities of the general phenylpropanoid pathway and the specific pathway of lignin biosynthesis: 4-coumarate : CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD) and peroxidase (PO).

Total extractable activities of 4CL, CAD and PO do not differ significantly in resistant and susceptible near isogenic wheat lines, whereas the dose response curve of induced PAL activity shows significantly higher values in the resistant isoline. This difference is not observed after injection of other biotic and abiotic elicitors of the lignification response.

Although total induced PO activities 24 h after elicitor treatment are equal in both isolines, the PO isoenzyme pattern of resistant plants differs markedly from that of susceptible plants. The patterns of PO isoenzymes in the compatible and incompatible interaction 48 h after inoculation with *Puccinia graminis* f. sp. *tritici* also show differences, which closely resemble those seen after injection of the *Pgt*-elicitor. The patterns observed after injection of an artificial biotic elicitor clearly differ from those of the natural interaction.

Introduction

In the preceding paper [1] we reported on the partial purification and characterization of an elicitor of the lignification response in wheat leaves isolated from germ tube walls of uredospores of *Puccinia graminis* PERS. f. sp. *tritici* ERICS. and E. HENN. The elicitor active compound(s) is thought to be a glycoprotein with a molecular weight of more than 100 kdalton. The carbohydrate moiety with a glucose : galactose : mannose ratio of 10 : 1 : 1 presumably carries the active residues. When injected into wheat leaves, the elicitor causes an increase in phenylalanine ammonia-lyase (PAL) activity and subsequent lignification, events characteristic for the hypersensitive cell death of infected, resistant wheat leaves [2–5].

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; DEAE, diethylaminoethyl; 4CL, 4-coumarate : CoA ligase; OMT, O-methyl transferase; PAL, phenylalanine ammonia-lyase; PO, peroxidase; *Pgt*-elicitor, elicitor-fraction from *Puccinia graminis* f. sp. *tritici.*

Reprint requests to B. Moerschbacher.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0900–0839 $ 01.30/0

PAL, the first enzyme of the general phenylpropanoid pathway, has been proposed to have a regulatory function in the biosynthesis of phenolics, such as flavonoids, stilbenes, benzoic acids and lignin [6]. In order to investigate whether the enhanced metabolic flux through the phenylpropanoid pathway, mediated by the enhanced PAL activity is correlated with lignification, we investigated other enzymes involved in the biosynthesis of lignin.

We measured the elicitor stimulated activities of 4-coumarate : CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD) and peroxidase (PO).

4CL, the last enzyme of the general phenylpropanoid pathway, leads, via different isoenzymes, to the different specific pathways, such as the biosynthesis of lignin [6]. CAD, an enzyme in the specific pathway of lignin biosynthesis, reduces the substituted cinnamyl aldehydes to the corresponding alcohols. These are oxidized by PO and the resulting radicals subsequently polymerize to lignin.

The activities of these enzymes increase in wheat leaves infected with *Puccinia graminis* f. sp. *tritici* [7, 8] (unpublished results of the authors). Induced PAL activity has also been reported after infection with *Erysiphe graminis* f. sp. *tritici* [3] and with the non-

pathogenic *Botrytis* [9]. In the latter case, an enhanced activity of 4CL was also reported [10]. Thorpe and Hall [11] reported on increased activities of PAL and PO in wheat leaves in response to wounding and wound-inoculation by *Botrytis cinerea*. They also found a weak additional activation of both enzymes in wounded wheat leaves after treatment with unidentified crude elicitor preparations from the non-pathogenic *Botrytis*. In spite of several studies concerning the role of PO isoenzymes in the interaction between wheat and stem rust, their participation in the resistance response is still controversial [8, 12]. The same difficulties in interpretation arose in a study of PO isoenzymes in the resistance response of wheat to the wheat leaf rust [13].

In this study we report on PAL, 4CL, CAD and PO activities in leaves of near isogenic wheat plants carrying either the *Sr5*-gene for resistance or the corresponding *sr5*-allele for susceptibility, induced by the injection of the genuine biotic elicitor from *Puccinia graminis* f. sp. *tritici* (*Pgt*-elicitor). A preliminary report of some of the presented results has been published previously [14].

Materials and Methods

Inoculation and elicitor treatment of plants

Reculturing of the fungus *Puccinia graminis* f. sp. *tritici* (race 32), growing and inoculation of wheat seedlings (cultivars Prelude and Marquis) and injection of the elicitor into the intercellular space of 7-day-old primary leaves were performed as described [1, 15].

Preparation of an elicitor active fraction from germinating uredospores

Elicitor active material was isolated from germ tube walls of uredospores. The term "*Pgt*-elicitor" in this paper refers to the elicitor active fraction, isolated as described [1].

Assay for enzyme activities

Induced PAL activity was determined using a modified procedure of Green *et al.* [3] as described [1]. 4CL and CAD activities were determined from a crude enzyme extract, prepared according to a modified procedure of Fuisting and Weissenböck [16].

Plants treated with elicitor or with water (controls) were harvested 24 h after injection and immediately frozen in liquid nitrogen. The frozen leaf sections were ground in a cold mortar containing 2 ml of phosphate buffer (0.1 M, pH 7.3, 40 mM Dithiothreitol), 50% (w/w) of abrasive and 10% (w/w) of both, insoluble polyvinylpyrrolidone and Dowex 1×2 (Cl^-). The homogenate was transferred to a centrifuge tube, mortar and pestle were rinsed with 2 ml of buffer. After stirring for 20 min particulate debris was removed by centrifugation for 20 min at $48000 \times g$. The supernatant was used as enzyme extract for the CAD assay or, after addition of 50% (v/v) of glycerol, for the 4CL assay.

4CL activity was assayed according to Gross and Zenk [17] in a modification of Knogge and Weissenböck [18]. 200 µl of the above enzyme extract were mixed with 250 µl of phosphate buffer (0.2 M, pH 7.3) containing 5 µmol $MgCl_2$, 0.5 µmol ATP, 1 µmol dithiothreitol and 0.1 µmol 4-coumarate as substrate. After incubation at 30 °C for 1 min the enzyme reaction was started by adding 0.1 µmol CoA in 50 µl of phosphate buffer and the absorbance at 333 nm recorded for 10 min. Enzyme activity was calculated using the molar extinction coefficient of 4-coumaryl:CoA (2.3×10^7 cm^2/mol [19]) and expressed as pkat/g fresh weight. CAD activity was measured according to Wyrambik and Grisebach [20] with some modifications. 350 µl of Tris-HCl (0.2 M, pH 9.25) containing 0.1 µmol $NADP^+$, and 100 µl of the above enzyme extract were kept at 30 °C for 1 min. Addition of 0.1 µmol coniferyl alcohol in 50 µl of Tris buffer started the reaction and the absorbance at 400 nm was recorded for 5 min. The molar extinction coefficient of coniferyl aldehyde is 2.0×10^7 cm^2/mol [20], enzyme activity was calculated as pkat/g fresh weight.

PO activity was determined using the same crude enzyme extract as prepared for the PAL assay. Activity was measured according to a modified procedure of Bergmeyer [21]. 50 µl of the above enzyme extract were added to 2.95 ml of phosphate buffer (0.1 M, pH 7.0) containing 0.9 µmol of guaiacol and 0.36 µmol of H_2O_2. The absorbance at 470 nm was recorded for 2 min at 25 °C. The molar extinction coefficient of tetraguaiacol at 470 nm is 2.66×10^7 cm^2/mol [22]. Enzyme activity was expressed as pkat/g fresh weight.

PO isoenzymes

Soluble protein was extracted from 3 g of primary leaves by grinding them in a mortar containing 6 ml

of borate buffer (0.1 M, pH 8.8) and an abrasive. The supernatant, after a 30 min centrifugation at $10000 \times g$, was diluted with borate buffer to 0.2% protein as determined by the Biuret reaction. 37.5 µl of the above protein extract were submitted to a gradient polyacrylamide gel electrophoresis under non-denaturing conditions (125 mM Tris-borate buffer pH 8.9, 4–25% acrylamide, 500 V, 24 h) and stained for PO activity with o-dianisidine as substrate according to methods described by Stegemann *et al.* [23].

Protein and carbohydrate content

Protein was determined by the methods of Lowry *et al.* [24] and Gornall [25], carbohydrate with the anthrone reagent by the method of Morris [26].

Results

All enzyme activities were determined 24 h after the elicitor had been injected into the intercellular space of primary leaves of the near isogenic wheat line Prelude carrying the *Sr5*-gene for resistance or the corresponding *sr5*-allele for susceptibility. At that time all enzyme activities are increased 3 to 20-fold compared to water injected controls (Table I). Injection of water alone did not enhance enzyme activities.

4CL, CAD and PO activities are induced to the same extent in both isolines, whereas PAL activity is induced more strongly in the resistant as compared to the susceptible line. In order to corroborate this specific effect, dose response curves of the induced PAL activity were measured (Fig. 1). PAL activity in resistant plants consistently showed significantly higher values than in susceptible plants.

Dose response curves of induced PAL activity after injection of an abiotic and a biotic elicitor (DEAE-dextran, chitosan, see [1]) showed no significant differences between resistant and susceptible near isogenic lines.

In a single experiment dose response curves of the three other enzyme activities after treatment with the abiotic elicitors as well as with the *Pgt*-elicitor all showed a monophasic shape with a maximum at relatively high concentrations without any specific differences between the two isolines.

These results were confirmed in experiments with the near isogenic wheat line Marquis carrying either the *Sr5*- or *sr5*-allele.

The isoenzyme pattern of peroxidases was determined 24 h after injection of elicitors or 48 h after

Table I. Increased activities of enzymes involved in the biosynthesis of lignin, after injection of 0.04% (glucose equivalents) of the genuine elicitor from *Puccinia graminis* f. sp. *tritici* into the intercellular space of 7-day-old primary leaves of the near isogenic wheat line Prelude, carrying the *Sr5*-gene for resistance or the corresponding *sr5*-allele for susceptibility. Data given are the average values of at least 4 independent experiments. As shown for PAL, injection of water alone did not affect enzyme activities. Only the PAL activities after injection of the *Pgt*-elicitor differ significantly in resistant and susceptible isolines (paired sample t-test, $P = 0.05$).

Treatment	Enzyme	Enzyme activity in pkat/g f. wt	
		Sr5	*sr5*
untreated	PAL	62	40
water		46	42
elicitor		1175	696
water	4CL	138	114
elicitor		739	750
water	CAD	136	112
elicitor		1792	1779
water	PO	81000	72000
elicitor		230000	238000

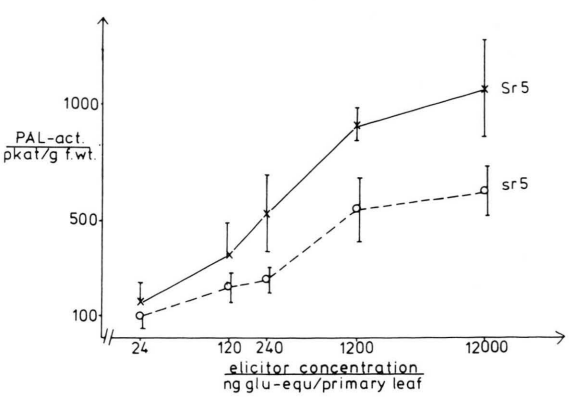

Fig. 1. Dose response curves of elicitor induced PAL activity in 7-day-old primary leaves of the near isogenic wheat line Prelude carrying either the *Sr5*-gene for resistance or the corresponding *sr5*-allele for susceptibility. PAL activity was determined 24 h after injection of the genuine elicitor from *Puccinia graminis* f. sp. *tritici* into the intercellular spaces of the leaves. Data shown are the average of 5 independent experiments, bars represent SD. Resistant plants show significantly higher values than susceptible plants (t-test, $P < 0.05$ (24 ng: $P = 0.1$)).

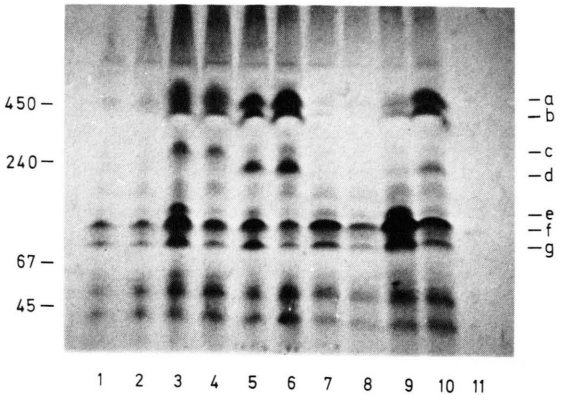

Fig. 2. PO isoenzyme patterns in primary leaves of the near isogenic wheat line Prelude, carrying the resistance gene *Sr5* or the corresponding *sr5*-allele for susceptibility. Enzyme extracts were prepared 48 h after inoculation or 24 h after injection of elicitors, and aliquots containing 75 µg of protein were submitted to gradient polyacrylamide gel electrophoresis under non-denaturing conditions. Lanes designated with even numbers are from *Sr5*-plants, lanes with uneven numbers from *sr5*-plants. The numbers on the left margin indicate the molecular weight in kdalton.

5 gels with independently prepared samples using different preparations of elicitors were run with the same results, the plate shows a typical separation.

lanes 1 and 2:	injection of water;
lanes 3 and 4:	injection of chitosan oligomers;
lanes 5 and 6:	injection of the *Pgt*-elicitor;
lanes 7 and 8:	inoculation control without uredospores;
lanes 9 and 10:	inoculation with uredospores from *Pgt;*
lane 11:	intercellular washing fluid of *Sr5*-plants (5 µg of protein).

inoculation with uredospores (Fig. 2). Infection with uredospores induces a set of PO isoenzymes, with resistant and susceptible isolines reacting differently. The higher molecular weight isoenzymes a and b, as well as isoenzyme d are induced more strongly in the incompatible interaction. In contrast, the isoenzymes e, f and g show up more intensely in the compatible interaction. After treatment with the *Pgt*-elicitor, the same isoenzymes appear, showing the same though less pronounced differences between the isolines. Injection of an other biotic elicitor (chitosan oligomers), produces a different isoenzyme pattern. Isoenzyme c is strongly induced, but apparently does not play an important role in the natural interaction, whereas isoenzyme d can hardly be detected. Moreover, the differences between the two isolines, that are typical for the natural interaction, do not appear consistently upon injection of chitosan. However, the appearance of isoenzyme e preferently in the compatible interaction corresponds well with the natural interaction.

Discussion

Changes in the phenolic compounds of host cells have been proposed as a mechanism of disease resistance more than 50 years ago [27]. The finding that lignin is a polyphenolic compound [28, 6] led to the hypothesis, that the formation of lignin may be involved in such resistance responses [29, 30].

Enzymes of the general phenylpropanoid pathway, leading to the biosynthesis of phenolics, have been investigated in a number of studies on lignification as a resistance response. Increased activity of PAL, possibly the main regulatory step in the pathway [6], has been shown for a wide range of host parasite interactions [3, 7, 9, 31–34]. Considerably less studies deal with enhanced activities of *o*-methyl transferases (OMT) and/or 4CL [9, 10, 33–35]. However, the general phenylpropanoid pathway leads to the formation not only of lignin, but also of other metabolites, such as flavonoids, stilbenes and benzoic acids, and most of the reports on pathogen induced activities of these enzymes concern host parasite interactions, where the accumulation of a phytoalexin is involved in the expression of the resistance response [36]. Thus, enhanced activities of these enzymes are thought to be a necessary but not a sufficient prerequisite for lignification. Investigations on enzymes specific for the biosynthesis of lignin (cinnamyl-CoA oxidoreductase, CAD, specific PO isoenzyme(s)) are required in order to confirm, that the enhanced enzyme activities of the general phenylpropanoid pathway are correlated with the de-novo synthesis of lignin.

As far as we know, neither cinnamyl-CoA oxidoreductase nor CAD have yet been investigated in correlation with resistance phenomena.

Following injection of the genuine *Pgt*-elicitor, PAL, 4CL and CAD activities increased up to 20, 6 and 15-fold, respectively. In rust infected resistant wheat leaves, a 3-fold increase in PAL [7] and 2 and 1.5-fold increases in 4CL and CAD, respectively, were observed (unpublished results). The higher activities obtained with elicitor presumably originate from the fact, that the elicitor solution reaches almost every cell, whereas only few cells are involved in the resistance response in the natural interaction.

The small increases in PAL and 4CL activities, 1.5 and 4-fold, respectively, in wounded wheat leaves

upon treatment with an unidentified biotic elicitor preparation from the non-pathogenic *Botrytis,* reported by Thorpe and Hall [11], probably are due to the different test system, as the activities were already stimulated by wounding alone.

The enzyme activities of healthy wheat leaves, recorded in this paper, are in the range of those reported by others for wheat or other monocotyledons [3, 37, 38].

The last enzyme of the lignin biosynthesis is PO, which activates the monomers for polymerization to lignin. The appearance of multiple isoenzymes of PO with different biological functions may account for the controversial discussion about their role in the expression of resistance. Different stimulation of isoenzymes or even a mere shift in the pattern of isoenzymes could be of great importance for the metabolism of the plant.

The patterns of PO isoenzymes, observed after injection of the *Pgt*-elicitor, more closely resembled those seen after inoculation (particularly in respect to isoenzyme d) as did the patterns, caused by the injection of chitosan. Interestingly, the differences between the isoenzyme patterns in resistant and susceptible plants after treatment with the genuine *Pgt*-elicitor resemble those observed after inoculation, although they are less marked.

Similar results were reported by Seevers *et al.* [8], who detected 14 isoenzymes, of which increase in only one was associated consistently with the development of resistant disease reaction.

Dose response curves of PAL activity after injection of the *Pgt*-elicitor showed an interesting specific effect in the two near isogenic lines, the resistant line showing significantly higher activities than the susceptible at 24 h after injection. This, together with the different stimulation of PO isoenzymes and a similar effect on isolated wheat protoplasts [14], may point to a differential activity of the *Pgt*-elicitor in near isogenic wheat lines, which was never observed with any of the other elicitors tested, and which corresponds to different host reactions in compatible and incompatible interactions. Such quantitatively rather than qualitatively different reactions are typical for most [39], though not all [40], elicitors showing specific effects, and are consistent with the fact, that in most host parasite interactions, intermediate infection types with more or less marked resistance responses occur in between the extreme resistant and the extreme susceptible reactions.

However, we are far from knowing, whether there is a causal relationship between the observed specific effects of the *Pgt*-elicitor and compatibility or incompatibility in the wheat stem rust system. Further work including other races of the stem rust fungus differing in their spectrum of avirulence genes, especially the *P5*-gene, is required to further substantiate the significance of these observations.

Acknowledgements

The authors gratefully acknowledge the perfect technical assistance of U. Noll. B. M. is currently in receipt of a Graduiertenstipendium des Landes Nordrhein-Westfalen. This work was supported in part by the Deutsche Forschungsgemeinschaft.

[1] B. Moerschbacher, K. H. Kogel, U. Noll, and H. J. Reisener, Z. Naturforsch. **41c,** 830 (1986).
[2] J. Beardmore, J. P. Ride, and J. W. Granger, Physiol. Plant Pathol. **22,** 209 (1983).
[3] N. E. Green, L. A. Hadwiger, and S. O. Graham, Phytopathol. **65,** 1071 (1975).
[4] J. P. Ride, Physiol. Plant Pathol. **5,** 125 (1975).
[5] R. Tiburzy, PhD thesis, RWTH Aachen, FRG (1984).
[6] K. Hahlbrock and H. Grisebach, Ann. Rev. Plant Physiol. **30,** 105 (1979).
[7] K. Achenbach-Blasberg, PhD thesis, RWTH Aachen, FRG (1986).
[8] P. M. Seevers, J. M. Daly, and F. F. Catedral, Plant Physiol. **48,** 353 (1971).
[9] A. J. Maule and J. P. Ride, Phytochemistry **15,** 1661 (1976).
[10] A. J. Maule and J. P. Ride, Phytochemistry **22,** 1113 (1983).
[11] J. R. Thorpe and J. L. Hall, Physiol. Plant Pathol. **25,** 363 (1984).
[12] V. Macko, W. Woodbury, and M. A. Stahmann, Phytopathol. **58,** 1250 (1968).
[13] L. B. Johnson and B. A. Cunningham, Phytochemistry **11,** 547 (1972).
[14] K. H. Kogel, B. Heck, G. Kogel, B. Moerschbacher, and H. J. Reisener, Z. Naturforsch. **40c,** 743 (1985).
[15] K. H. Kogel, F. Schrenk, N. Sharon, and H. J. Reisener, J. Plant Physiol. **118,** 343 (1985).
[16] K. Fuisting and G. Weissenböck, Z. Naturforsch. **35c,** 973 (1980).
[17] G. G. Gross and M. H. Zenk, Eur. J. Biochem. **42,** 453 (1974).
[18] W. Knogge and G. Weissenböck, Planta **187,** 196 (1986).
[19] G. G. Gross and M. H. Zenk, Z. Naturforsch. **21b,** 683 (1966).

[20] D. Wyrambik and H. Grisebach, Eur. J. Biochem. **59,** 9 (1975).
[21] H. U. Bergmeyer, Methoden der enzymatischen Analyse, Verlag Chemie (1970).
[22] A. C. Maehly and B. Chance, in: Methods of Biochemical Analysis, **Vol. 1,** p. 357 (D. Glick, ed.), Interscience Publishers 1954.
[23] H. Stegemann, W. Burgermeister, H. Francksen, and E. Krögerrecklenfort, PAGE-Manual, Biologische Bundesanstalt, Braunschweig, FRG (1984).
[24] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265 (1951).
[25] A. G. Gornall, C. J. Bardaville, and M. M. David, J. Biol. Chem. **177,** 751 (1949).
[26] D. L. Morris, Science **107,** 254 (1948).
[27] R. Newton and J. A. Anderson, Can. J. Res. **1,** 86 (1929).
[28] K. Freudenberg and A. C. Neish, Constitution and biosynthesis of lignin, in: Molecular Biology, Biochemistry and Biophysics (A. Kleinzeller, G. F. Springer, and H. G. Wittmann, eds.), Springer Verlag 1968.
[29] T. Hijwegen, Neth J. Plant Pathol. **69,** 314 (1963).
[30] C. P. Vance, T. K. Kirk, and R. T. Sherwood, Ann. Rev. Phytopathol. **18,** 259 (1980).
[31] J. Friend, S. D. Reynolds, and M. A. Aveyard, Physiol. Plant Pathol. **3,** 495 (1973).
[32] J. A. Glazener, Physiol. Plant Pathol. **20,** 11 (1982).
[33] M. Legrand, B. Fritig, and L. Hirth, Phytochemistry **15,** 1353 (1976).
[34] C. P. Vance, J. O. Anderson, and R. T. Sherwood, Plant Physiol. **57,** 920 (1976).
[35] C. Grand and M. Rossignol, Plant Sci. Lett. **28,** 103 (1982/83).
[36] J. A. Bailey, in: Phytoalexins (J. A. Bailey and J. W. Mansfield, eds.), p. 289, Blackie 1982.
[37] G. G. Gross, R. L. Mansell, and M. H. Zenk, Biochem. Physiol. Pflanzen **168,** 41 (1975).
[38] R. L. Mansell, G. R. Babbel, and M. H. Zenk, Phytochemistry **15,** 1849 (1976).
[39] J. A. Callow, in: Encyclopedia of Plant Physiology, New Series, **Vol. 17,** p. 212 (H. F. Linskens and J. Heslop-Harrison, eds.), Springer Verlag 1984.
[40] P. J. G. M. De Wit, A. E. Hofman, G. C. M. Velthuis, and J. A. Kuc, Plant Physiol. **77,** 642 (1985).