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Chitooligosaccharide-Induced Activation of *o*-Phenylenediamine Oxidation by Wheat Seedlings in the Presence of Oxalic Acid

R. M. Khairullin*, L. G. Yarullina, N. B. Troshina, and I. E. Akhmetova

Institute of Biochemistry and Genetics, Ufa Scientific Center, Russian Academy of Sciences, pr. Oktyabrya 69, Ufa, 450054 Russia; fax: (3472) 35-6100; E-mail: phyto@anrb.ru

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Abstract—A method for determination of oxidation of phenolic compounds by intact wheat seedlings using *o*-phenylenediamine (OPD) was developed. The reaction is initiated by the addition of oxalic acid to the incubation medium. It is suggested that an endogenous peroxidase and hydrogen peroxide formed during oxidation of oxalic acid by endogenous oxalate oxidase are involved in OPD oxidation. Treatment of plants with chitooligosaccharides (1-10 mg/liter) with acetylation degree of 65% and molecular masses of 5-10 kD significantly activated OPD oxidation by wheat seedlings.

Key words: wheat seedlings, oxalic acid, oxalate oxidase, *o*-phenylenediamine, oxidation, chitooligosaccharides, induction

Oxidation of phenolic compounds in the presence of hydrogen peroxide (H_2O_2) and peroxidase plays an important role in plant defense against pathogenic microorganisms [1, 2].

It is generally accepted that one of the mechanisms leading to the production of H_2O_2 in plants consists in oxidation of NADPH and NADH catalyzed by a cell wall peroxidase. It cannot be excluded that NADPH oxidase, which is similar in functions to a membrane-bound NADPH oxidase found in animal leukocytes with polymorphous nuclei, is also involved in the formation of hydrogen peroxide [3].

One of the alternative mechanisms of H_2O_2 production in plants induced by pathogen infection may be oxidation of oxalic acid catalyzed by oxalate oxidase (EC 1.2.3.4) [4]. The involvement of oxalate oxidase in a cascade of defense reactions was shown in wheat leaves infected by powdery mildew (*Erysiphe graminis f. sp. tritici*) [5].

It is known that defense reactions involving peroxidase and H_2O_2 may be triggered by such cell wall components of phytopathogenic fungi as chitin, chitosan, and chitooligosaccharides, which are known as elicitors [6-8]. However, the involvement of oxalate oxidase and oxalic acid as alternative sources of hydrogen peroxide is poorly studied.

The communication of Dumas et al. [9] attracted our attention. They revealed oxalate oxidase activity on the surface of roots of intact barley seedlings by α-naphthol oxidation in the presence of endogenous peroxidase. Oxidation was induced by the addition of oxalic acid to the incubation medium.

However, α-naphthol is unsuitable for quantitative measurements of oxalate oxidase activity because in its oxidized form it is insoluble in water. In addition, in this system the oxidation of α-naphthol occurs in the presence of endogenous peroxidase. Thus, the rate of phenol oxidation also depends on the peroxidase activity.

We supposed that activation of phenol oxidation in the presence of oxalic acid, oxalate oxidase, and peroxidase can be used as a general characteristic of the ability of elicitors to induce defense mechanisms in plants.

The goals of this work were to develop a simple method of quantitative determination of phenol oxidation in the presence of oxalic acid and oxalate oxidase in intact wheat seedlings and to investigate the ability of chitooligosaccharides to initiate these reactions, including oxalate oxidase activation.

MATERIALS AND METHODS

Seedlings of spring common wheat *Triticum aestivum* L. (cv. Zhnitsa) were used. Seeds were sterilized in 80% ethanol for 5 min and germinated in distilled water in the dark at 22°C.

Endosperm was gently removed from seedlings, and roots were washed in distilled water. Seedlings were trans-

* To whom correspondence should be addressed.

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ferred to Petri dishes (ten seedlings per dish) with 0.01 M KCl solution (10 ml) and incubated at least 4 h to eliminate the stress induced by injury. Then the medium was replaced with fresh medium containing 0.05% *o*-phenylenediamine (OPD). The reaction was immediately initiated by the addition of 1 ml 0.025 M oxalic acid; the reaction mixture was stirred gently, and 0.2-ml aliquots were taken at 2-min intervals. The aliquots were applied to the wells of a flat-bottom plate for immunoassay (Linbro, UK) preliminarily filled with 0.05 ml 4 M H_2SO_4. Absorption of the samples was measured at 492 nm on a vertical photometer (Titertek Uniskan, UK). The OPD oxidizing activity was expressed in absorption units per g wet root weight.

To determine the localization of oxalate oxidase, seedlings were incubated in the medium containing 0.01 M KCl, 0.025 M oxalic acid, and α-naphthol (0.6 mg/liter) [9].

Chitooligosaccharides used to elicit defense reactions in wheat were obtained as follows. Chitin of crabs was purified with 2 M HCl and 1 M NaOH as described [10]. The chitin was hydrolyzed in 65% H_2SO_4 according to Maksimov et al. [11] at 40°C. The hydrolyzate was precipitated with cold acetone. The pellet was dissolved in water and centrifuged; the supernatant was precipitated with five volumes of cold acetone. To remove sulfate ions, the chitooligosaccharides were repeatedly precipitated from aqueous solution with acetone to pH 5. The solution was neutralized with 10 M KOH and washed from salts by repeated precipitation in acetone (six times) and finally in 96% ethanol. The preparation was freeze-dried. The degree of acetylation was determined by the method of Pliska et al. [12]; molecular masses were estimated by gel filtration on Sephadex G-15 and G-25 (Serva, Germany) and Acrylex P-10 (Reanal, Hungary) according to the manufacturers' protocols.

White powder of chitooligosaccharides with ash content below 0.5% was dissolved in water (at least 100 mg in 100 ml). The elemental composition (C 46.33%, N 7.48%, H 6.62%) was close to that of chitin [13] with 2.5% sulfur. This was probably due to low ash content in the form of potassium sulfate and to the presence of small amounts of sulfo groups tightly associated with amino groups of carbohydrates, since the deacetylation degree of chitooligosaccharides was 35%. The molecular masses of the chitooligosaccharides varied from 5 to 10 kD. ^{13}C-NMR spectra showed that the oligomers contained only chitosan and chitin fragments, the latter component considerably prevailing. The structure, degree of polymerization, and deacetylation of the chitooligosaccharides were similar to those of biologically active chitin derivatives used as elicitors of plant defense reactions [14].

To evaluate the ability of elicitors to induce OPD oxidation, 3-day-old seedlings were incubated in 0.01 M KCl in the presence of the elicitor (1 mg/liter) for 10 min. Measurements of OPD oxidation were described above.

The amount of oxalic acid (% of the original concentration) remaining in the medium after incubation of roots was measured by titration with KOH solution [15].

To study the ability of elicitors to activate endogenous peroxidases, hydrogen peroxide was added to the medium (final concentration of 0.01 mM) instead of oxalic acid.

All chemicals used were of domestic production and of analytical grade. The experiments were conducted in three biological replicates. Absorption of samples was measured in three replicates. Mean values obtained in three replicate experiments ± standard deviation are given in the tables. Statistical analysis was performed according to Rokitskii [16].

RESULTS AND DISCUSSION

Since in barley seedlings oxalate oxidase is located on the surface of roots [9], we suggested that by substitution of α-naphthol for soluble OPD we would be able to develop a quantitative method for determination of oxidation of phenolic compounds by intact wheat seedlings in the presence of oxalic acid. The addition of OPD and oxalic acid to the seedlings led to the development of yellowish orange staining of the medium after 1-2 min of incubation. The root surface also gradually stained. If OPD and/or oxalic

Table 1. OPD oxidation by wheat seedlings in the presence of oxalic acid (units per g wet mass)

Variant	Time after reaction initiation, min			
	2	4	6	8
(Medium + OPD) + OA	16.3 ± 1.2	17.3 ± 0.9	19.3 ± 1.1	24.0 ± 1.2
(Medium + OPD) + SA*	11.3 ± 0.7	10.3 ± 0.7	11.7 ± 1.0	11.0 ± 0.8
(Medium + OPD) +AA*	10.0 ± 0.6	9.7 ± 0.6	10.3 ± 0.9	10.7 ± 0.9
(Medium + H_2O) + OPD	12.0 ± 1.0	11.0 ± 0.8	11.0 ± 0.8	10.0 ± 0.6
(Medium + H_2O) + OA	9.7 ± 0.8	9.7 ± 0.7	9.3 ± 0.6	10.0 ± 0.7

Note: After the addition of OPD or H_2O to seedlings, the reaction was initiated by oxalic acid.
* Instead of oxalic acid (OA), succinic (SA) or aspartic (AA) acid was added.

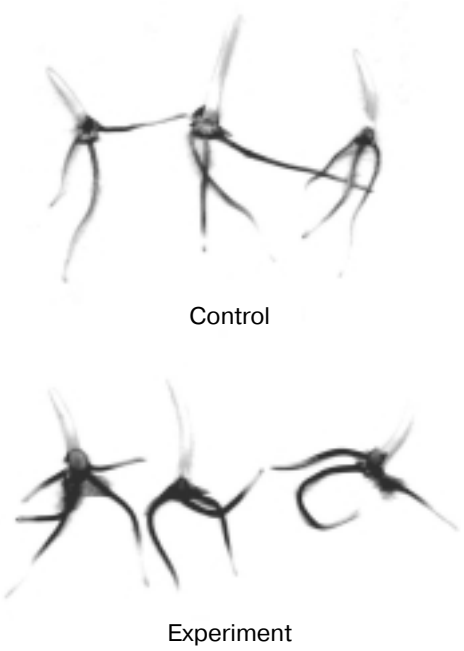

Fig. 1. Oxidation of α-naphthol on the surface of roots of wheat seedlings in the presence of oxalic acid. The staining developed after 8 min.

acid were replaced by an equal volume of growth medium, the staining did not develop. This method allowed us to quantitatively estimate differences in the intensity of staining (Table 1). The specificity of the reaction was confirmed by the absence of staining in the presence of succinic or aspartic acids.

In parallel experiments with an addition of α-naphthol, the oxalic acid induced the development of violet color of roots (Fig. 1).

It is common knowledge that root growth is accompanied by lignification of epidermal cell walls that reduces their permeability for various molecules [17]. On the other hand, oxalate oxidase and peroxidase activities may change in ontogeny [2, 9]. Therefore, we measured OPD oxidation in roots of wheat seedlings of different age. As seen from Fig. 2, OPD oxidation was the highest in roots of 2-day-old seedlings; it was about four times higher than in 6-day-old seedlings.

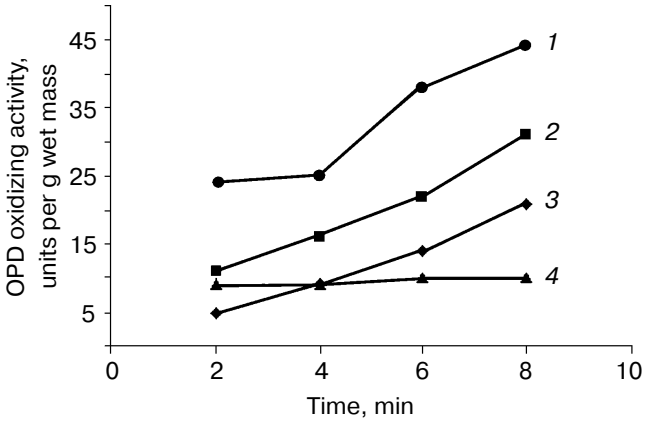

Fig. 2. OPD oxidation by wheat seedlings of different age: 2- (*1*), 3- (*2*), 4- (*3*), and 6-day-old (*4*).

Statistical analysis of the measurements of OPD oxidation in six independent biological replicates showed that, despite a limited number of plants analyzed in one experiment (10) and a large volume of the incubation medium (10 ml), the confidence interval was ±5% of the mean value at a probability level of $t_{0.05}$.

As we have already mentioned, chitin, chitosan, and their derivatives are well-known inducers of peroxidase-mediated defense reactions [6-8]. However, the involvement of oxalic acid and oxalate oxidase in these processes has not been shown. To investigate this problem, we studied the effect of chitooligosaccharides on OPD oxidation by 3-day-old seedlings. Two elicitor concentrations were taken, 10 and 1 mg/liter. It appeared that even 10-min treatment of plants with the preparation was sufficient for the induction of OPD oxidation. Thus, at chitooligosaccharide concentration of 10 mg/liter, OPD oxidizing activity was 42.4 ± 2.8 units, while at a concentration of 1 mg/liter it was 35.7 ± 2.3 units; in control seedlings it was 31.0 ± 2.1 units. Thus, the intensity of oxidative processes correlated with the concentration of elicitors in the medium.

Chitooligosaccharide-induced OPD oxidation could be due either to activation of oxalate oxidase or of endogenous peroxidases localized on the surface of roots. To detect activation of endogenous peroxidases, seedlings were treated with chitooligosaccharides and after 10 min only hydrogen peroxide was added to the medium to a final concentration of 0.01 mM (below this value, the staining did not develop). The addition of H_2O_2 drastically stimulated OPD oxidation in comparison to the variant in which oxalic acid was added (Table 2). However, the difference between the absorption values in control and experimental variants (when only H_2O_2 was added) was insignificant.

The titration of the original oxalic acid solution followed by 10-min incubation of control and chitooligosaccharide-treated (1 μg/ml) plants showed that acid concentration decreased by 39 ± 4% of its original concentration in the control and by 52 ± 1% in the experimental variant. The difference was statistically significant. Accordingly, treatment with the elicitor significantly activated oxalic acid oxidation in wheat seedlings.

Our method and the original procedure, using OPD and α-naphthol, respectively, are based on the finding that both oxalate oxidase and peroxidase are localized on the surface of roots in barley and wheat seedlings [9]. OPD oxidation is initiated by the addition of oxalic acid generating H_2O_2 during oxidative degradation [4]. In contrast to the original procedure, we used OPD; a colored product formed in this instance is released into the solution that

Table 2. Effect of chitooligosaccharides on OPD oxidation by wheat seedlings in the presence of oxalic acid (OA) and H_2O_2* (units per g wet mass)

Experiment number	Variant	2 min	4 min	6 min	8 min
1	control + OA	7.3 ± 0.5	11.2 ± 1.3	18.1 ± 1.5	24.0 ± 1.9
2	experiment + OA	9.2 ± 0.8	16.2 ± 1.5	25.8 ± 2.1	44.7 ± 3.7
3	control + H_2O_2	88.3 ± 7.2	141.4 ± 12.4	213.1 ± 20.7	265.8 ± 25.1
4	experiment + H_2O_2	92.9 ± 8.0	165.2 ± 10.8	228.9 ± 21.4	279.7 ± 26.4

* In the control, seedlings were incubated in 0.01 M KCl for 10 min; in the experimental variant, in the same medium supplemented with the elicitor (1 mg/liter), whereupon the medium was discarded and OPD and OA (1, 2) or OPD and H_2O_2 (3, 4) were successively added to the seedlings. After incubation for the time indicated, activity was measured.

makes it possible to quantitatively estimate the rate of the reaction as an integral characteristic of the processes involving oxalate oxidase, hydrogen peroxide, and peroxidase.

It is well known that cell walls are less differentiated in young than in old roots [17], this making them more susceptible to pathogens. At the same time, it seems likely that more intensive oxidation of OPD in the presence of oxalic acid and oxalate oxidase in 2-day-old seedlings, namely in less differentiated young roots, represents an alternative defense response of plants to pathogen attack.

Our results on the induction of OPD oxidation in the presence of oxalic acid by treatment of seedlings with elicitors are of particular interest. Analysis of the OPD oxidation mechanisms leads to two hypotheses. First, the induction of OPD oxidation on the surface of roots treated with elicitors, which is accompanied by more rapid decrease in the concentration of oxalic acid in the medium, provides evidence for the specific role of oxalate oxidase in this reaction. This suggestion is supported by the observation that staining of the medium did not develop in the presence of succinic and aspartic acids (Table 1). On the other hand, it seems impropriate to associate the degree of OPD oxidation only with the oxalate oxidase activity. Although peroxidase activity in chitooligosaccharide-treated seedlings did not significantly differ from the control (Table 2), it seemed possible that activation of OPD oxidation in wheat seedlings induced by chitooligosaccharides was associated both with oxalate oxidase and peroxidase.

The involvement of methyl salicylate, abscisic acid, and auxin in induction of expression of the oxalate oxidase gene in barley seedlings was reported. However, this process requires several hours [18]. Our results indicate that the effect of elicitors on oxalate oxidase activity is more rapid and not associated with expression of a particular gene; elicitors may interfere with other regulation mechanisms, such as conformational rearrangements in the enzyme molecule or activation of protein synthesis from the preexisting mRNA. The role of elicitors in modifying the activity of oxalate oxidase involved in rapid production of hydrogen peroxide by plant cells requires further investigations.

REFERENCES

1. Zaprometov, M. N. (1993) *Fiziol. Rast.*, **40**, 921-929.
2. Andreeva, V. A. (1988) *Peroxidase. Role in Plant Defense against Viral Infection* [in Russian], Nauka, Moscow.
3. Baker, C. J., Deahl, K., Domek, J., and Orlandi, E. W. (1998) *Biochem. Biophys. Res. Commun.*, **252**, 461-464.
4. Azarashvili, T. S., Evdotienko, V. Yu., and Kudzina, L. Yu. (1996) *Fiziol. Rast.*, **43**, 196-200.
5. Hurkmann, W. J., and Tanaka, C. K. (1996) *Plant Physiol.*, **111**, 735-739.
6. Lyon, G. D., Reglinski, T., and Newton, A. C. (1995) *Plant Pathol.*, **44**, 407-427.
7. Kauss, H., and Jeblik, W. (1996) *Plant Physiol.*, **111**, 755-763.
8. Pearce, R. B., and Ride, J. P. (1982) *Physiol. Plant Pathol.*, **20**, 119-123.
9. Dumas, B., Freyssinet, G., and Pallet, K. (1995) *Plant Physiol.*, **107**, 1091-1096.
10. Khairullin, R. M., Shakirova, F. M., Bezrukova, M. V., and Yamaleev, A. M. (1992) *Prikl. Biokhim. Mikrobiol.*, **28**, 468-474.
11. Maksimov, V. I., and Smirnova, Yu. V. (1993) *Biotekhnologiya*, **10**, 26-30.
12. Plisko, E. A., Nul'ga, L. A., and Danilov, S. N. (1977) *Uspekhi Khim.*, **46**, 1470-1487.
13. Feofilova, E. P. (1986) *Fungal Cell Walls* [in Russian], Nauka, Moscow.
14. Vander, P., Varum, K. M., Domard, A., Gueddari, N. E., and Moerschbacher, B. M. (1998) *Plant Physiol.*, **118**, 1353-1359.
15. Babko, A. K., and Pyatnitskii, I. V. (1968) *Quantitative Analysis* [in Russian], Vysshaya Shkola, Moscow, pp. 371-372.
16. Rokitskii, P. F. (1961) *Principles of Variational Statistics for Biologists* [in Russian], Belorussian State University, Minsk.
17. Rodchenko, O. P., Maricheva, E. A., and Akimova, G. P. (1988) *Adaptation of Growing Root Cells to Low Temperatures* [in Russian], Nauka, Moscow.
18. Hurkman, W. J., and Tanaka, C. K. (1996) *Plant Physiol.*, **110**, 991-997.