Phytochemistry, Vol. 26, No. 1, pp. 21–24, 1987.
Printed in Great Britain

0031-9422/87 $3.00 + 0.00

TRANSPORT PROPERTIES OF BEAN VESICLES IN THE PRESENCE OF FUNGAL COMPONENTS

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(*Revised received* 27 *May* 1986)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; *Colletotrichum lindemuthianum*; Fungi imperfecti, H^+ transport; membrane ATPase.

Abstract—Sealed membrane vesicles were isolated from hypocotyls of two varieties of French bean (*Phaseolus vulgaris*). The preparations were shown to contain vesicles in which H^+ transport and ATPase were sensitive to nitrate but insensitive to vanadate. These sensitivities suggest the vesicles were enriched for tonoplast. Fractions prepared from the α and β races of the fungus *Colletotrichum lindemuthianum* inhibited H^+ transport in the vesicles isolated from cvs. Dark Red Kidney and Great Northern bean tissue. These data are discussed in terms of the biochemical mechanisms operating in plant/pathogen interactions.

INTRODUCTION

Membrane transport has been studied in several higher plant systems [1]. Sealed membrane vesicles isolated from various plant tissues [1–3] demonstrate that membrane associated ATPases can directly transport protons. Proton-transporting vesicles have been isolated from the plasma membrane [4] and the tonoplast [5]. The two types of vesicles can be distinguished by differences in ion stimulation of proton transport, inhibitor sensitivity and intrinsic membrane density [3 7]. A major goal of this laboratory is to understand at the biochemical level the responses observed when *Phaseolus vulgaris* is challenged by various races of the fungus *Colletotrichum lindemuthianum*. When certain bean cultivars are challenged by some races of the fungus a hypersensitive response, characteristic of plant resistance, is observed [8–10]. Occurring concurrently at the plant cell level are major metabolic changes which result in the production and accumulation of oxidized polyphenols and phytoalexins [8, 9]. These phytoalexins have antifungal properties [11] and are only produced rapidly and in large quantities in hypersensitive type reactions [8]. Fungal and non-fungal compounds have been shown to induce phytoalexin production in many systems [11–14]. These compounds have been termed 'elicitors'. Studies performed with plant/pathogen systems which involve elicitors [12] indicate that plant membranes are involved. In the present paper, we report that culture filtrates from two races of *C. lindemuthianum* and fungal wall material isolated from the β race effect the proton transport of membrane vesicles isolated from the bean cvs. Dark Red Kidney and Great Northern. These data are discussed in relation to cultivar/race specificity.

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RESULTS AND DISCUSSION

Isolation of sealed vesicles

The membranes isolated from cv. Dark Red Kidney had ATPase activity sensitive to nitrate and vanadate (Table 2). The membranes isolated from cv. Great Northern had ATPase activity sensitive to nitrate but less sensitive to vanadate (Table 3) or oligomycin (data not shown). Nitrate caused substantial inhibition of H^+ transport with membranes from cv. Dark Red Kidney (Fig. 1, Table 1) and complete inhibition with the membranes from cv. Great Northern. Transport in membranes from cvs. Great Northern and Dark Red Kidney was

Table 1. H^+ transport in membrane vesicles from *P. vulgaris* cvs. Dark Red Kidney and Great Northern

Interphase vesicles in the presence of inhibitors and fungal fractions	H^+ transport (Reduction in % quench/mg protein) Dark Red Kidney bean*	Great Northern bean†
Control	0.0	0.0
α F1	2.4	1.5
α F2	34.7	2.5
β F1	10.3	3.5
β F2	11.3	40.5
β wall F1	7.0	36.0
KNO_3	87.7	100.0
Na_3VO_4	2.7	15.5
Se	2.4	2.9

* Values given are the mean of three independent estimate.
† Values given are the mean of two independent estimates.
Se = standard error.

Table 2. Phosphohydrolase activities of membrane vesicles from *P. vulgaris* cv. Dark Red Kidney hypocotyls in the absence and presence of fungal fractions

Interphase vesicles and fungal fractions	ATPase activity (μmol Pi/hr/mg) Control	Δ KNO_3*	Δ Na_3VO_4*
Vesicles alone	12.6 (100%)	7.6 (100%)	7.3 (100%)
Vesicles and α F1	10.9 (86%)	9.4 (123%)	6.2 (85%)
Vesicles and α F2	5.6 (44%)	5.1 (67%)	4.0 (55%)
Vesicles and β F1	10.7 (84%)	7.9 (104%)	7.0 (96%)
Vesicles and β F2	10.4 (83%)	6.9 (91%)	6.4 (88%)
Vesicles and β wall F1	9.6 (76%)	7.2† (95%)	5.4 (74%)

Values given are the mean of three independent estimates. Percentages are in parentheses.

*Inhibitor-sensitive components of ATPase, see Experimental for further details.

†Mean of two values.

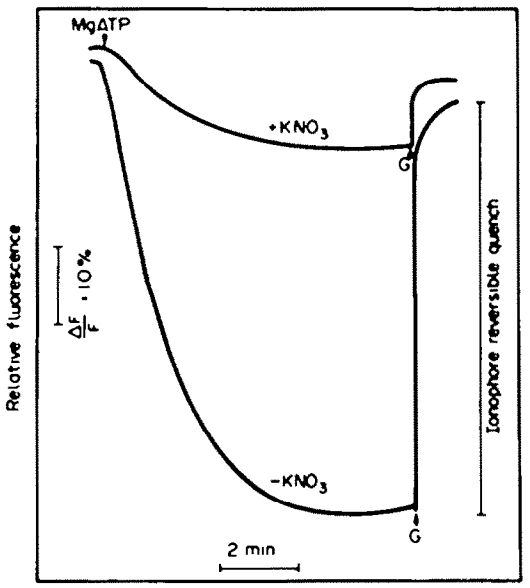

Fig. 1. Transport of H^+ by sealed vesicles isolated from *P. vulgaris* cv. Dark Red Kidney hypocotyls. Fluorescence quenching was reversed by the addition of 5 μM gramicidin D at time denoted by G.

hardly affected by vanadate (Table 1). Studies performed with membranes from many different plants [1, 3] strongly suggest that ATPase activity that is nitrate sensitive but insensitive to vanadate, azide or oligomycin is associated with tonoplast. In contrast, ATPase activity and transport that is sensitive to vanadate but insensitive to nitrate may represent vesicles derived from the plasma membrane [3]. In quantitating H^+ transport, measured optically by the quenching of quinacrine fluorescence [15], we have expressed the data in terms of the steady state pH gradient established across the vesicle membrane. This is taken as the fluorescence (%) restored from the steady state level of fluorescence by the addition of gramicidin D, a channel-forming ionophore (ionophore reversible quench, Fig. 1). From the ATPase-inhibition studies, the vesicles isolated from both cvs. Dark Red Kidney and Great Northern represent a mixed population most likely derived from both the plasma membrane and tonoplast. The H^+ transport data, however, suggest that the sealed vesicles which are competent in H^+ transport are of tonoplast origin. Research currently in progress on characterizing these H^+ transporting vesicles (Rogers, personal communication) reaffirms that for both cultivars they are derived mainly from the tonoplast.

Isolation of extracellular products and cell wall fractions for Colletotrichum lindemuthianum

The race–cultivar specificity observed when certain bean cultivars are inoculated with spores of specific races of

Table 3. Phosphohydrolase activities of membrane vesicles from *P. vulgaris* cv. Great Northern hypocotyls in the absence and presence of fungal fractions

Interphase vesicles and fungal fractions	ATPase activity (μ mol Pi/hr/mg) Control	Δ KNO_3*	Δ Na_3VO_4*
Vesicles alone	5.3 (100%)	2.5 (100%)	1.7 (100%)
Vesicles and α F1	4.9 (92%)	2.7 (108%)	2.5 (147%)
Vesicles and α F2	4.4 (83%)	3.6 (144%)	1.6 (94%)
Vesicles and β F1	4.2 (79%)	2.7 (108%)	1.2 (71%)
Vesicles and β F2	3.8 (72%)	3.1 (124%)	2.0 (118%)
Vesicles and β wall F1	5.3 (100%)	2.7 (108%)	1.7 (100%)

Values given are the mean of two independent estimates. Percentages are in parentheses. Standard error = 0.25.

*Inhibitor-sensitive components of ATPase, see Experimental for further details.

C. lindemuthianum was apparent when cotyledons from cvs. Dark Red Kidney and Great Northern were treated with the culture filtrate preparations. Elicitor activity of α F1 and F2 was evident when filtrate preparations were applied to cotyledons from cv. Dark Red Kidney. No browning or phytoalexin production was observed when the β F1 and F2 fractions were applied to Dark Red Kidney cotyledons. No elicitor activity was observed on treatment of Great Northern cotyledons with α or β race culture filtrate products. In contrast, the β cell wall preparation had elicitor activity on both Great Northern and Dark Red Kidney. No phosphate was detected in the culture filtrate or cell wall fractions.

Effect of fungal fractions on proton transport

Proton transport in membrane vesicles from cvs. Dark Red Kidney and Great Northern was inhibited by certain fungal fractions from the α and β races. Strongest inhibitions were observed with the α F2 fraction on Dark Red Kidney vesicles and the β F2 and wall F1 on Great Northern vesicles. This specificity in the inhibition of proton transport is interesting. Inoculation with α race spores resulted in a resistant response in Dark Red Kidney, and susceptibility in Great Northern. Spores of the β race produced large lesions on Dark Red Kidney and limited lesions on Great Northern. It is clear that the ability to inhibit proton transport in tonoplast vesicles does not directly correlate with elicitor activity of the preparations. However, the transport inhibition may participate in the complex metabolic changes that accompany hypersensitivity.

The mechanism by which H^+ transport is inhibited is unresolved currently. Initial data (Tables 2 and 3) suggested that ATPase activity was inhibited by certain fractions. However, later studies (Rogers and Anderson, in preparation) do not demonstrate that the fungal fractions which inhibit proton pumping cause reduction in fungal preparations on ATPase activity. Rather these studies support data (Tables 2 and 3) that ATPase activity may be slightly stimulated. Consequently, the effect of fungal products on proton pumping in the tonoplast may not be a simple inhibition of the ATPase activity.

EXPERIMENTAL

Plant material. Bean seeds (*Phaseolus vulgaris* cv. Dark Red Kidney and cv. Great Northern) were surface sterilized in 20% commercial Clorox, washed with H_2O and sown in vermiculite. The seeds were germinated and grown in darkness at 22°. After 7 days the hypocotyls were harvested for membrane vesicle isolation.

Isolation of membrane vesicles. The bean hypocotyl tissue was cut into sections and homogenized for 1 min in a pre-cooled Waring blender using 100 g of plant tissue to 200 ml of homogenization medium [1]. Sealed membrane vesicles were isolated by the method of Briskin *et al.* [1] with a modification at the vesicle purification step. The membrane pellet was suspended in 4.0 ml of vesicle suspension medium [1] and layered over a cushion of 10% dextran in vesicle suspension medium. The dextran step gradient was centrifuged as described by Briskin *et al.* [1] and the sealed vesicles present at the 10% dextran interphase were removed with a Pasteur pipette.

Protein assay. Protein was determined by the method of Bradford [16] using bovine serum albumin as a standard.

Isolation of extracellular products and cell walls from Colletotrichum lindemuthianum. Extracellular products were isolated from α and β races of *C. lindemuthianum* using methods described by Anderson [13]. The fractions obtained from chromatography on Sepharose 6B were pooled to give three fractions; fraction 1, the void volume of the column, fraction 2, the intermediate volume, and fraction 3, the fully included volume. All the fractions were lyophilized to dryness for storage. Fractions 1 and 2 from the α and β culture filtrates were used in this study. Cell walls were isolated from the β race using the methods previously described [14]. Elicitor activity [14] and phosphate assays [17] were performed on the cell wall and culture filtrate fractions.

Enzyme assay. ATPase assays were performed using a modification of the method described by Briskin *et al.* [1]. Phosphate hydrolysing activity of the vesicles was measured in a 1.0 ml reaction volume in the presence of 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 30 mM Tris Mes, 5 μM Na_2MoO_4, pH 7.2, and 30–50 μg of membrane protein. The released Pi was determined by the method of Ames [17]. The reactions were carried out for 30 min at 25°. Inhibitor sensitive components of ATPase represented the difference between the control activity and the activity in the presence of 50 mM KNO_3 (Δ KNO_3) or 50 μM Na_3VO_4 (Δ Na_3VO_4). The effect of the fungal preparation on ATPase activity was examined by adding 0.1 mg (dry wt) of the fungal extract to the reaction mixtures, 10 min before commencing the enzyme assay.

Optical measurements of the vesicle pH gradient. Proton transport in membrane vesicles was measured in the presence of 250 mM sorbitol, 5 mM ATP, 5 mM $MgSO_4$, 50 mM KCl, 5 μM quinacrine, 25 mM bis-tris propane/Mes pH 7.2 and 5–6 mg of membrane protein. Nitrate (KNO_3) when present was at 50 mM and vanadate (Na_3VO_4) was at 50 μM [18]. The fungal extracts were used as 10 mg/l. solns and were added to the reaction mixture 10 min before commencing proton transport measurements. Fluorescence quenching was reversed by the addition of 5 μM gramicidin D. The fluorescence measurements were made at room temp. (25°) with a Perkin Elmer LS-5 spectrofluorimeter with the excitation monochromator set at 430 nm and the emission monochromator set at 500 nm.

Acknowledgements—A.J.A. gratefully acknowledges grants from USDA-ARS competitive grant program and National Science Foundation (PCM 0810-5128). The technical assistance of W. R. Thornley is gratefully acknowledged. We thank B. Gandy for typing the manuscript. Utah State Agricultural Experiment Station paper no. 3019.

REFERENCES

1. Briskin, D. P., Thornley, W. R. and Wyse, R. E. (1985) *Plant Physiol.* **78**, 865.
2. Sze, H. (1980) *Proc. Natn. Acad. Sci. U.S.A.* **77**, 5904.
3. Sze, H. (1985) *Ann. Rev. Plant Physiol.* **36**, 175.
4. Sze, H. (1984) *Physiol. Plant.* **61**, 683.
5. Poole, R. J., Briskin, D. P., Kratky, Z. and Johnstone, R. M. (1984) *Plant Physiol.* **74**, 549.
6. Churchill, K. A. and Sze, H. (1983) *Plant Physiol.* **71**, 610.
7. Mettler, I. J., Mandala, S. and Taiz, L. (1982) *Plant Physiol.* **70**, 1738.
8. Bailey, J. A. and Deverall, B. J. (1971) *Physiol. Plant Path.* **1**, 435.
9. Mansfield, J. W. (1983) in *Biochemical Plant Pathology* (Callow, J. A., ed.) pp. 251–253. Wiley, London.
10. Theodorou, M. K., Scanlon, J. C. M. and Smith, I. M. (1982) *Phytopath. Z.* **103**, 189.

11. Darvill, A. G. and Albersheim, P. (1984) *Ann. Rev. Plant Physiol.* **35**, 243.
12. Yoshikawa, M. (1983) in *Biochemical Plant Pathology* (Callow, J. A., ed.) pp. 267 298. Wiley, London.
13. Anderson, A. J. (1980) *Can. J. Microbiol.* **26**, 1473.
14. Anderson, A. J. (1978) *Can. J. Botany* **56**, 2247.
15. Lew, R. and Spanswick, R. M. (1985) *Plant Physiol.* 77, 352.
16. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 243.
17. Ames, B. N. (1966) *Methods Enzymol.* **8**, 115.
18. Sze, H. (1983) *Biochim. Biophys. Acta* **732**, 586.