

# Tetrazolium Reduction by Guard Cells in Abaxial Epidermis of *Vicia faba*: Blue Light Stimulation of a Plasmalemma Redox System<sup>1</sup>

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## ABSTRACT

The stomata in the abaxial epidermis of *Vicia faba* were examined for the location of redox systems using tetrazolium salts. Three distinct redox systems could be demonstrated: chloroplast, mitochondrial, and plasmalemma. The chloroplast activity required light and NADP. Mitochondrial activity required added NADH and was suppressed by preincubation with KCN. The plasmalemma redox system in guard cells also required NADH, but was insensitive to KCN and was stimulated by blue light. The involvement of an NADH dehydrogenase in the blue light stimulated redox system in guard cells was suggested by the sensitivity to plantanetin, an inhibitor of NADH dehydrogenase. The redox system of mitochondria was the most active followed by that of plasmalemma. The activity of chloroplasts was the least among the three redox systems. The plasmalemma mediated tetrazolium reduction was stimulated by exogenous flavins and suppressed by KI or phenylacetate, inhibitors of flavin excitation. We therefore conclude that an NADH-dependent, flavin mediated electron transport system, sensitive to blue light, operates in the plasmalemma of guard cells.

Changes in the turgor potential during stomatal opening and closure are caused by 'active' movement of potassium into and out of guard cells (6, 26) and associated malate-starch-sugar interconversions (12, 17, 25). The energy necessary for the processes has been proposed to be derived from at least three different processes—photosynthesis, respiration, and a blue light sensitive system (27).

The involvement of several energy sources for stomatal function implicates the operation of different redox reactions in guard cells. Guard cells contain mitochondria, chloroplasts, and other organelles, but the operation of an electron transport system has been well documented only in chloroplasts (28). Other organelles have not been critically examined.

We have attempted to locate and demonstrate redox reactions in guard cells using tetrazolium salts, which have been traditionally used to study oxidoreductases in plant and animal systems (14). Our results indicate that a distinct redox system exists in the plasmalemma in guard cells of *Vicia faba*. The plasmalemma redox system requires NADH, is insensitive to KCN, and is stimulated by blue light.

## MATERIALS AND METHODS

### Plant Material

*Vicia faba* was raised from seeds (supplied by National Bureau of Plant Genetic Resources, Indian Agricultural Research Institute Campus, New Delhi) in 25 cm diameter earthen pots, on soil supplemented with farmyard manure. Plants were grown in the field under a natural photoperiod of approximately 12 h and mean temperature of 30°C day/20°C night. Plants were watered daily. Third and fourth leaves were picked from 4- to 6-week-old plants, usually between 9:00 and 9:30 AM.

### Epidermal Strips

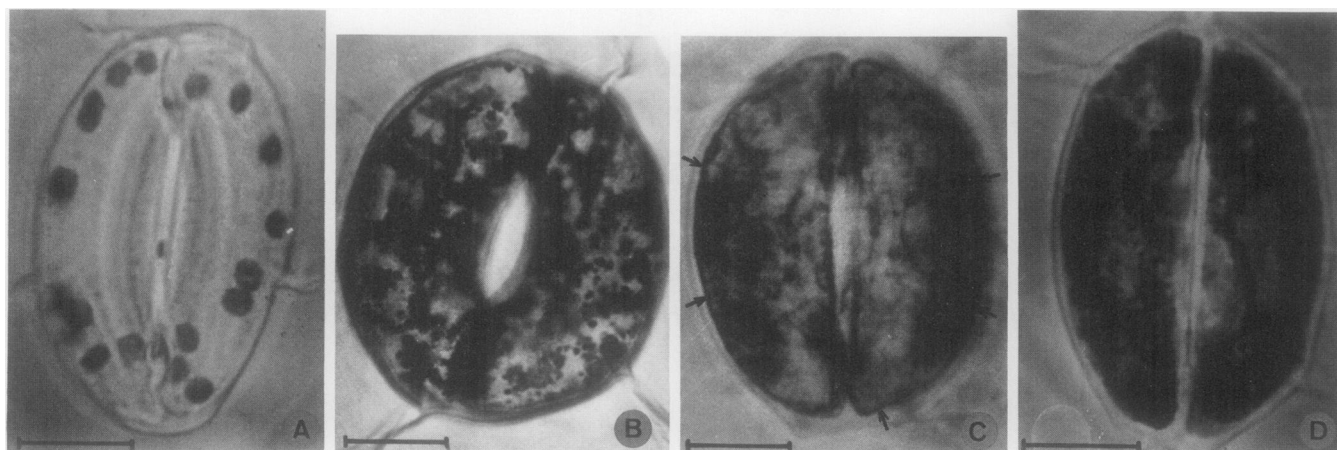
Strips (0.5 × 0.5 cm) were prepared from abaxial (lower) epidermis as described previously (15). Fifteen to 20 strips were put in 20 mL of 25 mM Tris-maleate buffer (pH 7.2) and 0.05 mM Ca(NO<sub>3</sub>)<sub>2</sub> in a 3.5 × 11 cm test tube kept on an ice-cushion in a beaker. The strips were subjected to ultrasonication for 15 sec in a 150 W Ultrasonic Disintegrator (MK 2, MSE Instruments, U.K.) at 40% of its maximum amplitude (60 W), using a probe with a diameter of 9.5 mm. After a break of 30 s, the strips were sonicated again for 15 s. The strips were rinsed and kept in fresh suspension medium, until use. The viability of cells in the epidermal strips before and after sonication was routinely checked with 0.01% (w/v) neutral red. The guard cells remained alive, whereas the epidermal cells were killed by the ultrasonication treatment.

### Tetrazolium Reduction

Ten strips were incubated in 2 mL of reaction medium, containing 25 mM Tris-maleate buffer (pH 7.2), 0.5 mM MgCl<sub>2</sub>, 0.05 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.4 mM NADH, and 0.05% (w/v) tetrazolium salt, as described. The above medium resulted in a rapid reduction of tetrazolium by mitochondria. A 60 to 70 min preincubation in 25 mM Tris-maleate buffer (pH 7.2), with 1 mM KCN and addition of 1 mM KCN or NaN<sub>3</sub> to the above reaction medium suppressed mitochondrial activity and resulted in a diffuse-type reduction. All the histochemical tests were conducted at 30 ± 1°C.

For examining the tetrazolium reduction by guard cell chloroplasts *in vivo* (i.e. in epidermal strips), the duration of preincubation of epidermal strips was limited to 15 s. The components of the reaction medium for chloroplast PSII activity were 25 mM Tris-maleate buffer (pH 7.2), 60 mM

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**Figure 1.** Location of redox systems in different compartments of *V. faba* guard cells: chloroplasts (A), mitochondria (B), and plasmalemma indicated by diffuse location (C, D). The occurrence of reduced tetrazolium can be clearly seen at the protoplast boundary, *i.e.* plasmalemma (indicated by arrows, in C). The intensities of tetrazolium reduction in (C) and (D) correspond to scores of 1 and 8, respectively. The bar represents 10  $\mu\text{m}$ .

sorbitol, 0.05% (w/v) NBT<sup>2</sup>, and 0.67 mM NADP and for PSI activity; additionally, 10  $\mu\text{M}$  DCMU, 2  $\mu\text{M}$  DCPIP, and 60  $\mu\text{M}$  sodium ascorbate. The strips in reaction mixture were illuminated, as described below. The guard cell chloroplasts were quite delicate. Either an increase in the period of sonication (of epidermal strips) or omission of sorbitol in the incubation medium affected the integrity of chloroplasts as indicated by their appearance under the microscope. Under these conditions there was marked decrease in the extent of NBT reduction by guard cell chloroplasts.

Addition of NADH was essential for mitochondrial and plasmalemma activity. The presence of NADP, though not essential, hastened tetrazolium reduction by chloroplasts. During histochemical demonstration of soluble or membrane bound oxidoreductases, nucleotides are added to the reaction medium (7, 24; also see pp. 907–908 and pp. 1342–1344 in Ref. 14).

### Scoring

The tetrazolium reduction in guard cells was scored using an arbitrary scale of 0 to 10. A score of '0' represents no reduction, while '10' indicates that the dark reaction product occupied the entire guard cell area (samples of guard cell activity corresponding to the scores of '1' and '8' are shown in Fig. 1). Fifteen to 20 stomata were scored in each epidermal strip (chosen at random from three different field areas under the microscope) and three strips were examined. Thus, each score represents an average of 45 to 60 measurements. The experiments were repeated at least three times.

### Illumination

Light was not necessary for mitochondrial activity. For diffuse type reduction, incandescent bulbs in combination

<sup>2</sup> Abbreviations: NBT, nitroblue tetrazolium chloride; BTC, blue tetrazolium chloride; DCPIP, 2,6-dichlorophenol indophenol; FMN, flavin mononucleotide; INT, iodonitrotetrazolium violet; MTT, thiazolyl blue; NTC, neotetrazolium chloride; Plantanetin, 3,5,7,8-tetrahydroxy, 6-isoprenylflavone; TNBT, tetranitroblue tetrazolium chloride; TTC, triphenyl tetrazolium chloride.

with cellophane filters (Petri dishes covered with 2–3 layers) to give 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of blue or red light, or for chloroplasts, white light of 900  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was used. Water filters, between light source and Petri dishes were used to prevent heat buildup.

### Chemicals

The sources of chemicals were: neutral red and FMN, Sigma Chemical Co; DCMU, ICN Pharmaceuticals Inc., Life Sciences Group, New York; NBT, NTC, TTC, riboflavin, Sisco Research Laboratories, Bombay; BTC, INT, MTT, and TNBT, Loba Chemie, Bombay. All other chemicals and reagents were from BDH or Sisco Research Laboratories or S. Merck, Bombay, India. Plantanetin was a gift from Dr. P. Ravanel, La Tronche (France).

### RESULTS

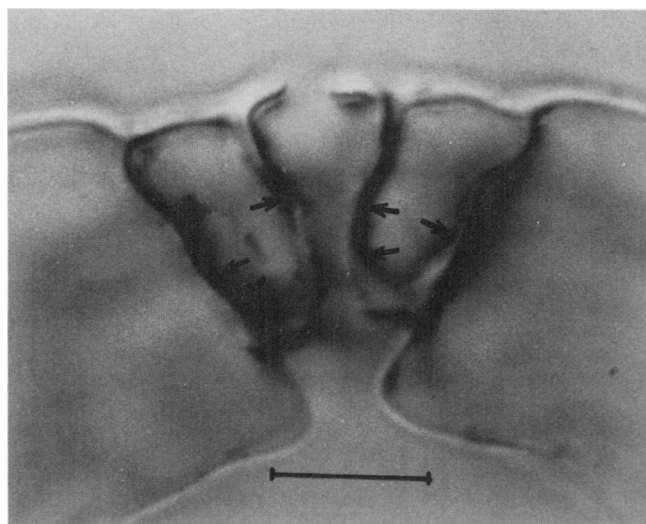
Among the seven different tetrazolium salts used in the present study, the reduction of TTC by guard cells was very poor. Maximum reduction occurred with INT, the order of activity being INT/MTT > NBT/TNBT > NTC > BTC > TTC. The tetrazolium salts were initially light yellow and their reaction products (reduced formazan compounds) were brightly colored. On reduction by guard cells, the product of INT appeared pink, MTT black, and NBT purple-black.

Under appropriate conditions, the redox activity (indicated by tetrazolium reduction) could be localized in the chloroplasts, mitochondria, or plasmalemma. In the presence of added NADP and illumination, the reduction of tetrazolium occurred in chloroplasts after 30 to 45 min (Fig. 1A). The activity in chloroplasts was low if NADP was not included in the reaction mixture. When NADH was added to the incubation medium, the reduced formazan could be located in the mitochondria, even in darkness, within 5 to 10 min (Fig. 1B). The diffuse reduction, if any, was not discernible, because of the predominance of reduced formazan in mitochondria.

The mitochondrial activity could be suppressed by preincubation in 1 mM KCN for 60 to 90 min. In the presence of

KCN, a diffuse tetrazolium reduction appeared in 10 to 15 min. The location of reduced formazan, near the protoplast boundary (Fig. 1C) indicated that the redox activity was in plasmalemma. If the epidermal tissue was incubated for more than an hour, the intensity of reduction increased but remained diffuse (Fig. 1D). Since the cell was being viewed from the surface, the location of tetrazolium reduction was apparently diffuse, although the redox activity was on the plasmalemma. Cross-sectional views of guard cells confirmed the location of this redox activity on the plasmalemma (Fig. 2).

A quantitative evaluation of tetrazolium reduction indicated that the diffuse-type (KCN-insensitive) tetrazolium reduction increased upon illumination, the stimulation being more under blue than that under red light, particularly after 15 min (Table I). The difference in the extent of tetrazolium reduction with blue or red illumination or darkness was masked with increased incubation time, as reduced formazan accumulated in the guard cells. The tetrazolium reduction was greater with INT than that with NBT. But INT could not be used during incubation periods longer than 10 to 15 min, because the tendency of the reduced formazan product of INT to crystallize made quantitative evaluation difficult.



**Figure 2.** Cross-sectional view of guard cells. The location of redox activity on plasmalemma, demonstrated by tetrazolium reduction, is indicated by arrows. The bar represents 10  $\mu\text{m}$ .

**Table I.** Diffuse Type (Plasmalemma—Located) Reduction of INT or NBT by Guard Cells at Different Times of Incubation Under Red or Blue Light ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and Darkness

The values ( $\pm\text{SD}$ ) represent the extent of tetrazolium reduction within an arbitrary scale of 0 to 10.

Tetrazolium	Treatment	Time of Incubation (min):		
		15	30	45
INT	Darkness	$1.9 \pm 0.03$	$2.6 \pm 0.07$	$2.6 \pm 0.07$
	Red	$2.1 \pm 0.21$	$3.7 \pm 0.04$	$5.3 \pm 0.12$
	Blue	$2.8 \pm 0.30$	$3.6 \pm 0.14$	$5.6 \pm 0.21$
NBT	Darkness	$1.7 \pm 0.08$	$2.8 \pm 0.04$	$3.8 \pm 0.09$
	Red	$1.9 \pm 0.05$	$2.8 \pm 0.03$	$3.7 \pm 0.12$
	Blue	$2.5 \pm 0.02$	$3.0 \pm 0.03$	$3.8 \pm 0.07$

The presence of riboflavin or FMN stimulated the diffuse-type tetrazolium reduction, particularly under blue illumination (Table II). The stimulatory effect of blue light on the diffuse-type NBT reduction was inhibited by plantanetin, potassium iodide, or phenyl acetate (Table III).

## DISCUSSION

Our observations demonstrate that significant redox reactions can occur at three different levels of organization in guard cells: chloroplasts, mitochondria, and on the plasmalemma (Fig. 1). These components differed not only in location but also in their rate of reaction. Mitochondria were the most active, since formazan appearance could be established in 5 min, compared to 10 to 15 min for the plasmalemma located activity. Chloroplasts were the least active, requiring 30 to 45 min.

The ability of guard cell chloroplasts to carry out photosynthetic electron transport, photophosphorylation, and carbon fixation has recently been reviewed (13, 28). The rates of photophosphorylation by isolated guard cell chloroplasts were sufficient to support ion uptake in the light (22). The presence of mitochondria/respiratory enzymes in guard cells is well documented (10, 26), but the electron transport activities of guard cell mitochondria have not been reported. However, respiratory  $\text{O}_2$  uptake by guard cell protoplasts has been measured by several groups (1, 4, 20).

We have demonstrated visually for the first time, a third redox system in guard cells, on the plasmalemma (Figs. 1 and 2). This supports earlier suggestions in the literature (21, 27). The operation of an electron transport system on the plasmalemma, which may aid in the generation of a proton gradient and subsequent ion uptake, has been proposed in several plant and animal systems (3). Plasmalemma bound-redox systems are capable of reducing externally supplied

**Table II.** Stimulation by Flavin Compounds of Diffuse Type (Plasmalemma-Located) NBT Reduction ( $\pm\text{SD}$ ) Under Light ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or Darkness

The values represent the score for tetrazolium reduction on a scale of 0 to 10.

Treatment	Incubation (15 min)		
	Darkness	Red	Blue
Control	$1.2 \pm 0.15$	$1.5 \pm 0.10$	$1.7 \pm 0.06$
+3 $\mu\text{M}$ Riboflavin	$1.2 \pm 0.20$	$2.0 \pm 0.10$	$2.3 \pm 0.18$
+3 $\mu\text{M}$ FMN	$1.1 \pm 0.19$	$2.0 \pm 0.26$	$2.6 \pm 0.20$

**Table III.** Effect of Metabolic Inhibitors on the Plasmalemma Mediated NBT Reduction in Light (red or blue,  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or in Darkness

The values represent the score for tetrazolium reduction ( $\pm\text{SD}$ ) on a scale of 0 to 10.

Treatment	Incubation (15 min)		
	Darkness	Red	Blue
Control	$1.0 \pm 0.18$	$1.8 \pm 0.17$	$2.4 \pm 0.34$
+1 mM KI	$1.8 \pm 0.07$	$1.6 \pm 0.14$	$1.9 \pm 0.15$
+100 $\mu\text{M}$ phenyl acetate	$1.4 \pm 0.08$	$1.8 \pm 0.12$	$1.7 \pm 0.17$
+10 $\mu\text{M}$ plantanetin	$1.4 \pm 0.08$	$2.0 \pm 0.11$	$1.8 \pm 0.06$

ferricyanide or Cyt *c*, with concomitant oxidation of NADH (for a model, see Ref. 8). Whether a membrane bound redox system capable of reducing ferricyanide or Cyt *c* exists in guard cells/stomata is yet to be examined.

The addition of NADH was essential to observe the plasmalemma bound system in guard cells. Further, the sensitivity of tetrazolium to plantanetin, an inhibitor of NADH dehydrogenase (16) suggests that this redox system involves an NADH dehydrogenase.

Current models of plasmalemma redox systems envisage the flow of electrons from exogenous or endogenous NADH through an NADH dehydrogenase, flavoprotein, and Cyt components (11). The role of flavins in the guard cell plasmalemma redox system is suggested by the inhibition by KI and phenyl acetate, inhibitors of flavin excitation (2, 23). Although it is intriguing that exogenously added flavin can stimulate the membrane bound activity (Table II), several reports on the stimulation of blue light effects in intact cells by exogenous flavins have appeared (5, 18).

The role of NADH and flavins in regulation of stomatal movements is further demonstrated by stimulation of stomatal aperture in isolated epidermis by FMN/riboflavin and suppression by KI/phenyl acetate (T Vani, AS Raghavendra, unpublished data). We therefore suggest that an NADH-dependent, flavin mediated electron transport system operates in the plasmalemma of guard cells, which is sensitive to blue light.

The relative intensity of tetrazolium reduction at different sites within the cell may throw light on their significance *in vivo*. Oxidative phosphorylation appears to be the prime source, not only because it reacts most rapidly with tetrazolium but also gives the extreme sensitivity of stomatal aperture to KCN or anoxia (9, 19). Even in terms of organelle number, mitochondria far exceed chloroplasts in the guard cell (26). Further experiments on guard cells should be directed to characterize mitochondrial and plasmalemma redox reactions.

The plasmalemma type NBT reduction was not entirely dependent on blue light since there was some activity even under red light (Tables I–III). Further, stomata are known to open in response to red light (26, 27). It is possible that the plasmalemma located redox system is a sensory transducing mechanism rather than the main driving force of proton efflux.

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