Role of Light and Malate in the Decreased Sensitivity of cms-T Cytoplasm Maize Leaves to *Bipolaris maydis* Race T Toxin

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ABSTRACT

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Leaf segments from Texas male sterile (cms-T) cytoplasm maize isolines exposed to light ($50 \mu\text{M s}^{-1} \text{ m}^{-2}$) for 8 h or more before or after being infiltrated with the *Bipolaris maydis* race T toxin (T-toxin) leaked significantly less electrolytes when immersed in distilled water (DW) for 24 to 48 h than did dark-treated leaf segments. No comparable effect of light on toxin-induced electrolyte leakage was observed with normal (N) cytoplasm isolines. Toxin-treated cms-T leaf segments incubated in DW for three consecutive 12-h periods of alternating light and dark showed significantly greater electrolyte leakage than leaf segments incubated in continuous light for 36 h and sig-

nificantly less leakage than segments incubated in continuous dark for 36 h. Exposure of cms-T, but not N, cytoplasm leaves to 25 or 50 μM malic acid decreased their sensitivity to T-toxin in the dark to a level similar to that observed when leaves were incubated in the light without malic acid. The potency of T-toxin appeared to be unaffected by its exposure to light. The loss of electrolytes from T-toxin-treated cms-T cytoplasm leaf segments was at approximately the level seen with light or malate when 25 μM H_2O_2 was added to the DW bathing solution. Evaluation of the data points to the possibility that H_2O_2 might be involved with the altered sensitivity of cms-T cytoplasm leaves to T-toxin caused by either light or malate.

Additional keywords: *Cochliobolus heterostrophus*, disease resistance, hydrogen peroxide, N cytoplasm maize.

The effects of light on the responses of normal (N) and Texas male sterile (cms-T) cytoplasm maize (*Zea mays* L.) leaves to the host-selective toxin from *Bipolaris maydis* race T (T-toxin) were first noted by Bhullar et al. (6). Light caused enhanced bleaching of toxin-treated cms-T cytoplasm maize leaves. Also, Daly and Barna (9) noted that the potency of toxin, as measured by CO_2 fixation by cms-T leaves, was increased by light. The significance of these observations is somewhat obscured by the various effects of light on the sensitivity of cms-T maize to T-toxin noted by others. For example, MacRae and Yoder (35) observed that protoplasts from etiolated leaves were insensitive to T-toxin when left in the dark, but became sensitive if the etiolated leaves were exposed to light prior to the assay of the protoplasts. On the other hand, protoplasts from green leaves that were preincubated in the dark were sensitive to T-toxin, but became insensitive when the leaves were exposed to light before the protoplasts were used in the toxin assay.

Differing explanations have been given for the stimulatory (6,9) and inhibitory (35,50) effects of light on the sensitivity of cms-T leaves to T-toxin. Because we have observed that light decreases the sensitivity of cms-T leaves to T-toxin (4), the studies of Walton et al. (50) are of interest. They showed that the level of ATP in toxin-treated mesophyll protoplasts declined less rapidly in the light than in the dark, and they suggested that light could decrease the sensitivity of cms-T maize leaves to toxin by light-driven phosphorylation. However, the contrasting observations of the effects of light (6,9,35) indicate that the mechanisms by which it affects the sensitivity of cms-T maize to T-toxin have been neither completely understood nor fully explained.

Molecular geneticists and biologists have shown that cms-T-induced male sterility associated with maternal inheritance of sensitivity to T-toxin is due to the presence of a mitochondrial gene designated T-urf 13 (33,47). A 13-kDa polypeptide is the product of the

gene, and it is a component of the inner mitochondrial membrane (11,52). Reports that the T-urf 13 gene confers sensitivity to the toxin when it is expressed in *Escherichia coli* (12) or in *Saccharomyces cerevisiae* (20), together with tissue culture studies involving cms-T revertants (49,53), provide the molecular basis for the link of cms-T-induced male sterility and cms-T-induced sensitivity to toxin (33). Since the 13-kDa protein of the mitochondrion of cms-T maize is the site of action of T-toxin, light suppression would be assumed to be due to chloroplast compensation for defective mitochondria in supplying ATP and reducing potential to the cell (50). However, this interpretation does not explain the stimulatory effect of light on toxin action reported by Bhullar et al. (6) and Daly and Barna (9) or by MacRae and Yoder (35) with etiolated protoplasts.

The T-toxin causes multiple biochemical effects in susceptible cms-T cytoplasm maize tissues that are associated with its mitochondrial site of action. In the presence of T-toxin, mitochondria isolated from cms-T cytoplasm maize display increased leakage of NAD^+ and coenzyme A (5,37), altered permeability to anions and cations (24,30,38), reversible swelling (19,31), depolarization of the membrane (5,39), changes in the rate of substrate oxidation (3,40,45), and uncoupling of $\text{NADH} + \text{H}^+$ from ATP synthesis (3,5,20).

Since the organelle primarily affected by the action of light in green leaves is the chloroplast (15,23,28), it is possible that the influence of light on the sensitivity of maize leaves to T-toxin involves, in addition to ATP and $\text{NADPH} + \text{H}^+$, metabolites derived directly or indirectly from light-activated processes in the chloroplast. In plants such as maize, CO_2 is transferred via the C-4 carboxyl of C_4 acids to the reactions of the photosynthetic carbon reduction (PCR) cycle and is there reduced to the level of carbohydrate (23,29). The distinctive biochemical features of this process, known as C_4 photosynthesis, are the carboxylation and associated reactions leading to the synthesis of C_4 acids and those concerned with subsequent decarboxylation of C_4 acids to supply CO_2 for the PCR cycle (23). Another distinctive feature of the C_4 pathway of photosynthesis is that it involves the transport of intermediates between intracellular compartments such as chloroplasts and mitochondria, as well as between cells (23).

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The studies of Gengenbach et al. (19) support the idea that malate and related metabolites could play a mediating role in the light-modulated response of cms-T maize to T-toxin. They measured the effect of T-toxin on state 4 oxygen uptake by mitochondria from cms-T and N cytoplasm maize. With NADH as the substrate, O₂ uptake by mitochondria from cms-T maize was enhanced in the presence of T-toxin, presumably by promoting uncoupling. On the other hand, with malate as a substrate, O₂ uptake by isolated mitochondria was inhibited in the presence of toxin, presumably by preventing uncoupling. No such effects of these substrates on O₂ uptake in response to toxin were seen with mitochondria from N cytoplasm maize. These effects of malate on state 4 oxygen uptake have been observed by others (43). Thus, C₄ metabolites may mediate the effect of light on the sensitivity of cms-T maize to T-toxin through an effect on mitochondrial metabolism.

The production and accumulation of active oxygen species (AOS) has been shown to occur in a variety of plant-pathogen systems. Also, AOS may accumulate under stress associated with toxic metabolites produced by infectious pathogens (1,32) or by environmental stresses such as high light (7,41) and extremes of temperature (14,22,27). Therefore, the possibility that AOS might be involved with the effect of light on the response of cms-T maize leaves to toxin is of interest.

Based on the above considerations and on our previous observations (4), the objectives of this study were to determine the effect of light on the sensitivity of N and cms-T cytoplasm maize leaves to T-toxin as measured by electrolyte leakage; evaluate the effect of the C₄ metabolites, malic and aspartic acids, on this sensitivity; and propose an explanation for the action of either light or malate on the decreased sensitivity of cms-T maize to T-toxin.

MATERIALS AND METHODS

Host. Maize inbreds B37, OH43, and W64A with N or cms-T cytoplasms were grown in 24-cm-diameter pots, three plants per pot, in a steam-sterilized mixture of perlite/peat moss/loam (1:1:1). The plants were maintained in the greenhouse at a temperature of 28°C during the day and 24°C at night. Plants were watered and fertilized alternately with a soluble 20-20-20 fertilizer at the rate of 200 ppm. Plants used for the experiments were 4 to 5 weeks old. Leaves of similar age were detached from each isolate, washed with distilled water (DW), and then cut into strips 16 by 1.5 cm and used in the experiments described below.

Toxin preparation. A single-spore isolate of *Cochliobolus heterostrophus* (Drechs.) Drechs. (*Bipolaris maydis* (Nisikado & Miyake) Shoemaker) race T recovered from an ear of maize from a field in Franklin County, OH, in 1970 (ATCC 36180) was grown for 7 days on a xylose-glucose-L-asparagine-mineral salts agar medium as previously described (16,17). The toxin solution was prepared by the procedure of Bednarski et al. (3) with the following modifications. Agar cultures were homogenized in two volumes of methanol. The homogenate was filtered, with pH of the filtrate adjusted to 4.5, and then concentrated in a rotary evaporator at reduced pressure to 10% of the original volume. Two volumes of methanol was then added, and the preparation was held overnight at 4°C. A precipitate was removed followed by the removal of methanol at reduced pressure to 20% of the original volume. The resulting aqueous solution was partitioned three times with equal volumes of chloroform. Chloroform extracts were pooled, concentrated in vacuo, and then dissolved in 50% ethanol. This partially purified soluble toxin preparation was stable in storage (–15°C). The toxin was purified further by applying 2.0-ml aliquots to a Sephadex LH-20 column (particle size 25 to 100 µm, column dimensions 1.5 × 30 cm; Amersham Pharmacia Biotech, Piscataway, NJ) and eluting with 80% ethanol at a flow rate of 2.0 ml/min. The toxin-containing aliquots prepared in this way were stored in 80% ethanol and referred to as T-toxin. They were several times more potent than the partially purified toxin preparation, as measured by electrolyte leakage from toxin-treated cms-T maize

leaves. Also, by measuring differences in electrolyte leakage from toxin-treated leaves, it was possible to distinguish N from cms-T maize with similar results to those of others (43,44).

Toxin assay. Leaf strips were placed cut-end down into vials containing T-toxin in 2% aqueous ethanol. Infiltration of leaf strips was carried out by allowing them to take up the toxin solutions in the transpiration stream in the dark at 28°C for 24 h, except for those treatments to be described later in which the effect of the light regime during infiltration was evaluated. The concentrations of T-toxin used in the study were estimates and were designated as 0.04, 0.1, or 0.2 µg/ml. These estimates were based on the increments of electrolyte loss from cms-T maize leaves treated with a series of T-toxin dilutions in 2% aqueous ethanol. The quantitative results were calibrated against the published findings of Payne and Yoder (45), who worked with highly purified preparations of T-toxin. Following infiltration, the leaves were rinsed in DW and the immersed ends cut off. The trimmed leaves were then cut into five 3 × 1.5-cm segments, and each was immersed in a bathing solution of 25 ml of DW as previously described (17,18). In two of the experiments reported below on the effects of light, malic acid, and H₂O₂, leaf segments, instead of being infiltrated with 0.2 µg of T-toxin per ml of 2% ethanol solution, were immersed directly into a DW bathing solution of 0.04 µg of T-toxin per ml of 0.5% ethanol solution. This altered protocol was adopted because cms-T tissues were more sensitive to T-toxin when assayed with this method. Also, although the results were more variable, this approach involved fewer manipulations and facilitated the more efficient acquisition of data from the very large number of treatments involved in the study.

The initial conductivity of the bathing solution was measured with a conductivity cell (model 4973) attached to a conductivity bridge (model 4959; Leeds and Northrup Co., Philadelphia). The immersed segments were incubated either in the dark or the light or in alternating light and dark for up to 36 h. The conductivity of the bathing solution was measured at 6-, 12-, and 24-h intervals. The change in conductivity was computed, and the values were used as an indication of electrolyte leakage from leaves. At the end of each experiment, the leaf segments were dried at 80°C and the dry weights determined.

The protocols described above were used in a series of experiments designed to test the potency and selectivity of the toxin preparations and the effects of light, malate, aspartate, and H₂O₂ on the sensitivity of cms-T cytoplasm maize to T-toxin as measured by electrolyte leakage. All experiments were performed at least three times, with five replicates in each. Data were calculated as the means ± the standard deviations of five replicates and were obtained from three separate experiments. Differences between treatments were considered significant when the standard deviation values did not overlap. Also, treatment comparisons were significant at the 99% confidence level based on Student's *t* test.

RESULTS

Effect of T-toxin on leakage from N and cms-T cytoplasm maize. This study was undertaken to insure that the toxin preparations used had similar selectivity to that prepared by the method of Bednarski et al. (3) or Payne and Yoder (45). The T-toxin caused a significant increase in electrolyte leakage from cms-T, but not N, cytoplasm leaf segments. Moreover, an increase in the toxin concentration in the infiltration solution from 0.1 to 0.2 µg/ml caused an increase in the electrolyte leakage from cms-T leaves. Moreover, the amount of electrolytes lost increased in magnitude with increasing concentrations of T-toxin in the 2% ethanol solution used to infiltrate the cms-T maize leaves (Fig. 1). These observations are consistent with other studies involving the use of purified or partially purified preparations of T-toxin (44,45,51).

Effect of preincubation of cms-T cytoplasm leaves in light on T-toxin-induced electrolyte leakage. For this study, cms-T cytoplasm leaves from cultivar W64A were detached from 4-week-old maize plants, kept overnight in the dark, cut into 16 × 1.5-cm

strips, and then the ends were immersed in vials with 5 ml of DW. The leaf strips were exposed to either 0, 2, or 8 h of light ($50 \mu\text{M s}^{-1} \text{m}^{-2}$) during 8 h of preincubation at 28°C . The preincubated strips were then infiltrated in the dark for 24 h with solutions of 2% ethanol or 2% ethanol containing approximately 0.1 or 0.2 μg of T-toxin per ml. Three-centimeter segments from these leaf strips were immersed in 25 ml of DW and then incubated for up to 48 h in the dark at 28°C . T-toxin caused a significant increase in electrolyte leakage from cms-T leaves whether or not they were exposed to light prior to infiltration (Fig. 2). However, leaf segments that were exposed to light lost less electrolytes than those that were

Fig. 1. Effect of *Bipolaris maydis* race T toxin (T-toxin) on electrolyte leakage from 'W64A' maize leaf segments in normal (N) and Texas male sterile (cms-T) cytoplasm. Leaf strips were infiltrated with T-toxin for 24 h in the dark at 28°C at the concentrations indicated, and then 3-cm leaf segments were immersed in 25 ml of distilled water (DW) and incubated in the dark for another 24 h at 28°C . The change in conductivity ($\Delta\mu\text{S/g}$ of dry weight) of the DW after the first 24 h is reported. N and cms-T cytoplasm maize isolines are resistant and susceptible, respectively, to *B. maydis* race T and T-toxin. Vertical bars delimit $\pm$ standard deviation.

Fig. 2. Effect of a prior exposure to light on the sensitivity of 'W64A' Texas male sterile (cms-T) cytoplasm maize to *Bipolaris maydis* race T toxin (T-toxin) as measured by electrolyte leakage. Leaf strips were infiltrated with distilled water (DW) for 0, 2, or 8 h in the light ($50 \mu\text{M s}^{-1} \text{m}^{-2}$) prior to infiltration with T-toxin for 24 h in the dark at 28°C , and then 3-cm segments were floated on 25 ml of DW for 24 to 48 h in the dark. The changes in conductivity after 24 and 48 h are reported as $\mu\text{S/24 h/g}$ of dry weight. The 'W64A' cms-T isolate of maize is susceptible to T-toxin. Vertical bars delimit $\pm$ standard deviation.

not exposed (0 h of light). Moreover, an 8 h of light pretreatment was more effective in reducing electrolyte loss than was a 2 h of light pretreatment, which produced a small but insignificant response relative to the 0 h of light pretreatment. Although this phenomenon was seen with both of the toxin concentrations used, the inhibitory effect of light on toxin-enhanced electrolyte leakage was attenuated when the concentration of toxin used to infiltrate cms-T leaves was increased. Also, the effect of light was nullified when immersed leaf segments were incubated for 48 h in the dark (Fig. 2).

The cultivars B37 and OH43 in cms-T cytoplasm, which differed in their sensitivity to T-toxin in the dark, were compared to determine whether they also differed when exposed to different light regimes. For this study, leaf strips were preincubated for 8 h, during which time they were exposed to 0, 2, or 8 h of light and then infiltrated in the dark for another 24 h in solutions of 2% ethanol or 2% ethanol containing approximately 0.2 μg of T-toxin per ml. Three-centimeter segments from these strips were immersed in 25 ml of DW and incubated at 28°C for a further 48 h in either dark or light. Leaf segments from 'OH43' cms-T leaked consistently more electrolytes than those from 'B37' cms-T, regardless of the light regime to which they were exposed (Fig. 3). However, for either maize cultivar, preincubation of leaf strips for 8 h of light, followed by incubation of immersed leaf segments for 48 h in the dark, resulted in no significant reduction in T-toxin sensitivity as measured by electrolyte loss. In contrast, immersed leaf segments that were postincubated in light for 48 h were strikingly less sensitive to T-toxin than those postincubated in the dark for all preincubation treatments. The segments from leaf strips that were preincubated in light for 8 h were the least sensitive to T-toxin (Fig. 3). These data indicate that the reduced sensitivity to T-toxin caused by an initial preincubation period in light may persist for an extended postincubation period in the presence or absence of light. The variation in the sensitivity of cms-T maize to T-toxin has been shown by others to involve variation in the nuclear genome (44).

Time course of electrolyte leakage from cms-T cytoplasm leaves exposed to various light regimes after infiltration with T-toxin. For

Fig. 3. Electrolyte leakage from Texas male sterile (cms-T) cytoplasm maize leaves exposed to light before and after infiltration with *Bipolaris maydis* race T toxin (T-toxin). Replicate leaf strips from 'OH43' cms-T and 'B37' cms-T cultivars were exposed for 0, 2, or 8 h in the light ($50 \mu\text{M s}^{-1} \text{m}^{-2}$) prior to infiltration with 0.2 μg of T-toxin per ml for 24 h in the dark at 28°C , and then 3-cm leaf segments were immersed in 25 ml of distilled water (DW) and postincubated for another 48 h in either the light or dark. The changes in conductivity ($\Delta\mu\text{S/24 h/g}$ of dry weight) after 48 h are reported. The changes in conductivity of DW with leaves from the 'OH43' cms-T and 'B37' cms-T cultivars not infiltrated with T-toxin were 65 and 42 ± 10 , respectively. cms-T cytoplasm isolines of maize are susceptible to T-toxin. Vertical bars delimit $\pm$ standard deviation.

this study, leaf strips were infiltrated with 2% ethanol solutions containing 0.2 μg of T-toxin per ml for 24 h in the dark. They were then immersed in DW and incubated at 28°C for 36 h in the dark, 12-h intervals in a light-dark-light sequence, 12-h intervals in a dark-light-dark sequence, or 36 h in the light. Electrolyte leakage from leaves incubated in the dark was significantly higher after 12, 24, and 36 h than from leaves incubated in the light (Fig. 4). Leaves incubated under a light-dark-light sequence showed an electrolyte leakage trend similar to that for light for the first 18 h and one similar to that for dark from 18 to 36 h. In contrast, leaves incubated under a dark-light-dark sequence showed an electrolyte leakage trend that resembled that for dark for the first 18 h and that for light from 18 to 36 h (Fig. 4). These data indicated that the inhibitory effect of light on toxin-induced electrolyte leakage from maize leaf segments could be reversed in the dark. Also, the toxin-induced electrolyte leakage that occurred when cms-T leaves were incubated in the dark could be inhibited by their transfer to the light.

Effect of preincubation of T-toxin in light on its potency. We sought to determine whether the decrease in sensitivity to T-toxin observed when leaf segments are incubated in light could be due to inactivation of the T-toxin by the light alone. This was done to validate our assumption that the light effect involved mediating factors and processes in cms-T maize and not a direct inhibition of the T-toxin. For this study, three 5-ml aliquots of toxin (0.2 μg per ml

Fig. 4. Time course of electrolyte leakage from Texas male sterile (cms-T) cytoplasm maize leaves exposed to various light regimes following their infiltration with *Bipolaris maydis* race T toxin (T-toxin). Leaf strips of 'W64A' cms-T maize were infiltrated with 0.2 μg of T-toxin per ml for 24 h in the dark at 28°C, and then 3-cm segments were immersed in 25 ml of distilled water (DW) for 36 h as follows: continuous dark, 12-h dark + 12-h light + 12-h dark, 12-h light + 12-h dark + 12-h light, and continuous light. Changes in the conductivity ($\mu\text{S/g}$ of dry weight) of the DW containing the leaf segments were measured every 6 h. Changes in the conductivity of DW with leaf segments that were not treated with T-toxin was $115 \pm 22 \mu\text{S/g}$ of dry weight after 36 h of incubation in either the dark or light. Each point on the graph is the mean of 10 replications. The standard error was less than 8% of the mean for each data point.

of 2% ethanol solution) were put into separate vials and preincubated for 24 h at 28°C in either the light ($50 \mu\text{mol s}^{-1} \text{m}^{-2}$) or the dark. Thereafter, leaf strips prepared as described previously were placed in these preincubated T-toxin solutions and incubated for 24 h in the dark at 28°C. Leaf segments ($3 \times 1.5 \text{ cm}$) from strips that were treated with this preincubated toxin were then immersed in DW and incubated for another 24 or 48 h in the dark at 28°C. Electrolyte leakage was comparable from segments exposed to either light-preincubated or dark-preincubated toxin. Also, the increase in electrolyte leakage between 24 and 48 h was comparable for leaf segments exposed to either light- or dark-preincubated T-toxin (Fig. 5). These results indicated that exposure to light for extended periods does not affect T-toxin potency and suggest that light-inactivation of T-toxin may not be the cause of the reduced sensitivity observed when cms-T cytoplasm leaves are exposed to T-toxin in the light.

Effect of malic and aspartic acids on the sensitivity of maize leaves to T-toxin. We evaluated the effect of malate and aspartate on toxin-induced leakage of the electrolytes from N and cms-T cytoplasm leaves. The impetus for this study came from previous reports that light causes a several-fold increase in the fixation of CO_2 into organic and amino acids in plants (2,25). Eighty percent of this CO_2 is fixed into C_4 acids like malate, aspartate, and oxaloacetate (2,25,26). Therefore, we considered the possibility that metabolites produced by maize leaves in light could affect T-toxin sensitivity. For this reason, the C_4 acids malate and aspartate were compared. We immersed T-toxin-infiltrated leaf segments from N or cms-T cytoplasm maize cultivar W64A in a DW bathing solution with or without 25 or 50 μmol of either malate or aspartate and then measured electrolyte leakage after incubating for 24 h in the dark at 28°C. The presence of malic acid, but not aspartic acid, in the bathing solution inhibited toxin-induced leakage of electrolytes from cms-T cytoplasm leaf segments (Table 1). The inhibitory effect of malic acid was seen with two different concentrations of T-toxin (Table 2). In contrast, leakage of electrolytes from T-toxin-treated N cytoplasm leaves was not significantly altered by either malic or aspartic acid (Tables 1 and 2). An increase in the aspartic acid concentration in the DW bathing solution to 100

Fig. 5. Effect of preincubation of *Bipolaris maydis* race T toxin (T-toxin) in either the light or dark on its ability to stimulate electrolyte leakage in normal (N) and Texas male sterile (cms-T) cytoplasm maize leaves. Leaf strips from 'W64A' N and 'W64A' cms-T cytoplasm isolines were infiltrated for 24 h in the dark with 0.2 μg of T-toxin-per-ml solutions that was preincubated for 24 h at 28°C in either the light ($50 \mu\text{mol s}^{-1} \text{m}^{-2}$) or dark. Three-centimeter segments from these leaves were then immersed in 25 ml of distilled water (DW) and incubated in the dark for 24 to 48 h at 28°C. Changes in the conductivity ($\Delta\mu\text{S}/24 \text{ h/g}$ of dry weight) of the DW containing the leaf segments were measured at 24 and 48 h. N and cms-T cytoplasm maize isolines are resistant and susceptible, respectively, to *B. maydis* race T and T-toxin. Vertical bars delimit $\pm$ standard deviation.

or 200 μmol caused a similar response in cms-T cytoplasm leaves to that seen for 25 or 50 μmol of malic acid (data not shown).

We examined the effect of light and malic acid, alone and in combination, on toxin-induced electrolyte leakage from leaf segments. For this study, three maize cultivars ('B37', 'OH43', and 'W64A') in N or cms-T cytoplasm were used. Also, the protocol followed was noticeably different from that used in the experiments reported above. Segments were prepared from leaf strips that were first infiltrated only with DW for 24 h in light at 28°C. The segments were then immersed into a DW bathing solution containing 0.5% ethanol and 0.04 μg of T-toxin per ml with or without 50 μmol of malic acid. The leaf segments immersed in these dilute T-toxin solutions were then incubated for another 24 h at 28°C in either the light or the dark. This altered protocol was adopted because cms-T leaves appeared more sensitive (3 to 5 $\times$) under these assay conditions, and fewer procedures were needed to generate the experimental data. As noted previously, T-toxin caused a several-fold increase in the electrolyte leakage from cms-T cytoplasm leaf segments that were incubated in the dark. The three maize cultivars studied had differing sensitivities to T-toxin. However, they all showed approximately 50% reduction in electrolyte loss when T-toxin-treated segments were incubated in either the light without malic acid in the bathing solution or the dark with malic acid in the bathing solution (Table 3). Moreover, the stimulatory effect of

T-toxin on electrolyte leakage, seen in the dark, was completely suppressed when T-toxin-treated leaf segments that were immersed in a toxin-containing bathing solution with malic acid were also exposed to light (Table 3). No such responses to light or malic acid were seen in T-toxin-treated N cytoplasm segments.

Effect of H_2O_2 on T-toxin-induced electrolyte leakage from cms-T cytoplasm leaves. We were motivated to do this experiment because preliminary tests showed that, after 48 h in bathing solutions containing cms-T cytoplasm maize leaf segments, residual levels of hydrogen peroxide (H_2O_2) were up to 80% higher in the light than in the dark. Also, leaf segments had decreased sensitivity to T-toxin when they were immersed in DW containing 25 or more μmol of H_2O_2 , but not when immersed in DW containing H_2O_2 plus 8×10^{-4} μg of catalase per ml. These observations were taken to mean that alterations in H_2O_2 levels might be involved in the altered sensitivity of cms-T cytoplasm maize leaves to light and malic acid. H_2O_2 is one of the AOS that are typically produced when plant tissues are exposed to light (7,41) or to stress metabolites associated with plant-microbe interactions (1,32). Also, malate, which accumulates in plant leaves in response to light,

TABLE 1. Effects of malate and aspartate on *Bipolaris maydis* race T (T-toxin)-enhanced leakage of electrolytes from normal (N) and Texas male sterile (cms-T) cytoplasm maize leaf segments

Malate or aspartate concentration (μM)	Electrolyte leakage from T-toxin-infiltrated leaves ^a ($\Delta\mu\text{S/g}$ of dry weight in 24 h)	
	N cytoplasm ^b	cms-T cytoplasm ^b
Malate 0	163 $\pm$ 34	580 $\pm$ 104
Malate 25	152 $\pm$ 28	368 $\pm$ 55
Malate 50	129 $\pm$ 35	194 $\pm$ 21
Aspartate 0	143 $\pm$ 22	562 $\pm$ 120
Aspartate 25	145 $\pm$ 24	440 $\pm$ 95
Aspartate 50	180 $\pm$ 38	393 $\pm$ 84

^a Leaf strips of maize cultivar W64A were infiltrated with T-toxin (0.2 $\mu\text{g/ml}$) for 24 h in the dark at 28°C.

^b Leaf segments (3 $\times$ 1.5 cm) were prepared from toxin-infiltrated leaf strips and then immersed in a distilled water bathing solution with or without either malate or aspartate and incubated for 48 h at 28°C in the dark. The initial pH of the malate and aspartate solutions were adjusted to 6.0 with 0.1 M NaOH. The changes in conductivity per 24 h are reported for each treatment as $\Delta\mu\text{S/g}$ of dry weight. Data are the means $\pm$ standard deviations of five replicates and are representative of at least three separate experiments. Electrolyte leakage from N and cms-T leaf segments not infiltrated with toxin was 89 $\pm$ 31 $\mu\text{S/g}$ of dry weight per 24 h of incubation.

TABLE 2. Effect of preinfiltration with two *Bipolaris maydis* race T (T-toxin) dilutions on leakage of electrolytes from normal (N) and Texas male sterile (cms-T) cytoplasm maize leaf segments that were subsequently immersed for 48 h in distilled water (DW) bathing solutions with or without 50 μM malate

Toxin concentration ($\mu\text{g/ml}$) ^a	Electrolyte leakage from T-toxin-infiltrated leaves ^a ($\Delta\mu\text{S/g}$ of dry weight in 24 h)			
	N cytoplasm ^b		cms-T cytoplasm ^b	
	– Malate	+ Malate	– Malate	+ Malate
Control	104 $\pm$ 20	88 $\pm$ 17	122 $\pm$ 14	106 $\pm$ 28
0.1	116 $\pm$ 25	101 $\pm$ 23	230 $\pm$ 42	118 $\pm$ 22
0.2	131 $\pm$ 28	95 $\pm$ 24	620 $\pm$ 85	264 $\pm$ 59

^a Leaf strips of maize cultivar W64A were infiltrated with either DW or toxin solutions for 24 h in the dark at 28°C.

^b Leaf segments (3 $\times$ 1.5 cm) were prepared from leaf strips that were infiltrated with DW or toxin solutions and then immersed in a DW bathing solution and incubated for 48 h at 28°C in the dark. The initial pH of the malate solution was adjusted to 6.0 with 0.1 M NaOH. The changes in conductivity per 24 h are reported for each treatment as $\Delta\mu\text{S/g}$ of dry weight. Data are the means $\pm$ standard deviations of five replicates and are representative of at least three separate experiments.

TABLE 3. Effect of light on leakage of electrolytes from leaf segments of three maize cultivars in normal (N) and Texas male sterile (cms-T) cytoplasm after they were immersed in a distilled water (DW) bathing solution containing 0.04 μg of *Bipolaris maydis* race T (T-toxin) per ml with or without malate

Treatment ^a	Maize cultivar	Electrolyte leakage from toxin-treated leaf segments ^b ($\Delta\mu\text{S/g}$ of dry weight in 24 h)			
		N cytoplasm		cms-T cytoplasm	
		Dark	Light	Dark	Light
Control	B37	135 $\pm$ 34	214 $\pm$ 56	510 $\pm$ 88	243 $\pm$ 41
Malate (50 μM)	B37	171 $\pm$ 36	168 $\pm$ 31	255 $\pm$ 47	124 $\pm$ 27
Control	OH43	216 $\pm$ 31	281 $\pm$ 54	721 $\pm$ 102	357 $\pm$ 56
Malate (50 μM)	OH43	247 $\pm$ 52	226 $\pm$ 44	340 $\pm$ 74	165 $\pm$ 25
Control	W64A	183 $\pm$ 31	246 $\pm$ 54	620 $\pm$ 146	284 $\pm$ 63
Malate (50 μM)	W64A	204 $\pm$ 42	195 $\pm$ 34	289 $\pm$ 64	154 $\pm$ 43

^a Leaf strips from maize cultivars B37, OH43, and W64A were infiltrated with DW for 24 h in the light (50 $\mu\text{M s}^{-1} \text{ m}^{-2}$) at 28°C, cut into 3 $\times$ 1.5-cm segment, and then immersed in a DW bathing solution containing 0.04 μg of toxin per ml with or without malate (50 μM). The initial pH of the malate solution was adjusted to 6.0 with 0.1 M NaOH.

^b Leaf segments (3 $\times$ 1.5 cm) in the bathing solutions were incubated for 48 h at 28°C in either the dark or the light. The changes in conductivity ($\Delta\mu\text{S/g}$ of dry weight) per 24 h are the means $\pm$ standard deviations of five replicates and are representative of at least three separate experiments. Conductivity changes in solutions with N and cms-T cytoplasm leaf segments not treated with T-toxin were the same as those for toxin-treated N cytoplasm ones.

TABLE 4. Effects of malate, hydrogen peroxide, and light on leakage of electrolytes from leaf segments of two maize cultivars in Texas male sterile (cms-T) cytoplasm after they were immersed in distilled water (DW) bathing solutions with and without 0.04 μg of *Bipolaris maydis* race T (T-toxin) per ml

Treatment ^a	Maize cultivar	Electrolyte leakage from leaf segments ^b ($\Delta\mu\text{S/g}$ of dry weight in 24 h)			
		Dark		Light	
		– Toxin	+ Toxin	– Toxin	+ Toxin
Control	B37	220 $\pm$ 31	564 $\pm$ 64	184 $\pm$ 29	214 $\pm$ 42
Malate (50 μM)	B37	170 $\pm$ 28	310 $\pm$ 33	168 $\pm$ 32	195 $\pm$ 24
H_2O_2 (25 μM)	B37	145 $\pm$ 28	176 $\pm$ 32	136 $\pm$ 31	168 $\pm$ 27
Control	OH43	287 $\pm$ 41	688 $\pm$ 73	280 $\pm$ 36	374 $\pm$ 51
Malate (50 μM)	OH43	240 $\pm$ 37	360 $\pm$ 44	215 $\pm$ 32	290 $\pm$ 42
H_2O_2 (25 μM)	OH43	229 $\pm$ 35	256 $\pm$ 37	160 $\pm$ 26	188 $\pm$ 17

^a Leaf strips from maize cultivars B37 and OH43 were infiltrated with DW for 24 h in the light (50 $\mu\text{M s}^{-1} \text{ m}^{-2}$) at 28°C, cut into 3 $\times$ 1.5-cm segments, and then immersed in a DW bathing solutions with or without 0.04 μg of T-toxin malate (50 μM) or H_2O_2 (25 μM) per ml. The initial pH of the solution was 6.0.

^b Leaf segments (3 $\times$ 1.5 cm) in the bathing solution were incubated for 48 h at 28°C in either the dark or the light. The changes in conductivity ($\Delta\mu\text{S/g}$ of dry weight) per 24 h are the means $\pm$ standard deviations of five replicates and are representative of at least three separate experiments.

could serve as a precursor for intermediates whose metabolism is accompanied by the formation of H_2O_2 (46). Therefore, the possibility that light- and malate-generated H_2O_2 might be involved with the reduced susceptibility of cms-T maize to T-toxin is of interest. For this study, segments from cms-T cytoplasm leaf strips were immersed into a bathing solution containing a very dilute solution of T-toxin (0.04 μg per ml of 0.5% ethanol solution) with or without 25 μmol of H_2O_2 or 50 μmol of malate, and then incubated for 48 h at 28°C in either the dark or the light. For both cultivars B37 and OH43, the loss of electrolytes caused by T-toxin treatment in the dark was significantly reduced when either H_2O_2 or malate was in the bathing solution (Table 4). There was a similar response when T-toxin-treated segments were incubated in the light without either H_2O_2 or malate. Moreover, with either maize cultivar, the combination of light and H_2O_2 or light and malate appeared to be more effective in reducing the T-toxin-induced loss of electrolytes than either treatment alone (Table 4).

DISCUSSION

We have demonstrated that the exposure of cms-T cytoplasm maize leaves to light reduces their sensitivity to *B. maydis* race T toxin. cms-T leaves treated with T-toxin and kept in light lost less electrolytes than similar leaves that were kept in the dark. Also, cms-T leaves that were exposed to intermittent 12-h periods of light and dark, following T-toxin treatment in the dark, showed intermediate levels of sensitivity to T-toxin, as measured by the quantity of electrolyte leakage. These findings, together with our observation that the toxin is not inactivated by direct exposure to light of the intensity used in our study, leads to the conclusion that physiological changes in cms-T leaves, rather than light-induced changes in the potency of the toxin, might be involved. The results are consistent with those of others who reported on the protective effect of light on the action of T-toxin. For example, the collapse of T-toxin-treated protoplasts was prevented by light (13). Similarly, protoplasts from green leaves that had been exposed to light before T-toxin treatment became insensitive to the toxin (35). In related studies, Walton et al. (50) noted that the T-toxin-induced reduction in ATP in protoplasts incubated in the dark was alleviated in the light. This response was abolished in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosynthetic electron transport. Based on these findings, they concluded that light-driven ATP synthesis compensated for that which was lost due to inhibition by T-toxin of oxidative phosphorylation in mitochondria.

Previous reports have shown that light stimulates the fixation of CO_2 into malate and other organic acids in C_4 plants like maize (2,26,29), as well as plants with crassulacean acid metabolism (8, 42,48). Also, the level of C_4 acids in mesophyll cells enhances the light-stimulated fixation of CO_2 into these same acids, indicating that C_4 acids and light produce additive effects (26). Further, the level of C_4 organic acids in leaves fluctuate in parallel with the diurnal periodicity of light (42,48) in a way that is reminiscent of the patterns of sensitivity to toxin seen when toxin-treated cms-T cytoplasm leaves were exposed to intermittent 12-h periods of light and dark (Fig. 4). Moreover, it is worth noting that, in C_4 plants, the organic acid pool is typically 1.0 $\mu mol/g$ of fresh weight or more. Also, in plants with crassulacean acid metabolism, the malate pool may peak at 15.0 $\mu mol/g$ of fresh weight (42). We noted that infiltration of cms-T cytoplasm maize leaves with a solution containing physiological concentrations of malate (Tables 1 to 4) caused a reduction in their sensitivity to T-toxin in the dark. This is consistent with our view that malate may have played a mediating role in reducing the sensitivity of cms-T maize to T-toxin at the relatively low intensities or dosages of light used in this study.

One possible explanation of the light effect is that it raises the levels of reduced cofactors and Calvin cycle intermediates and causes respiratory rates to exceed the demands of the maize leaf cells for ATP. As a result, electrons may spill over into other path-

ways, some of which involve the formation of AOS. It is assumed that one or more of these AOS might, by mechanisms that are not yet understood, interfere with the ability of T-toxin to disrupt key functions of cms-T mitochondria.

We observed that malate mimics the light effect. Also, aspartate appeared to produce a similar response to malate, but at two- or threefold higher concentrations (data not shown). Others (19,43) have reported that state 4 respiration is inhibited when toxin-treated mitochondria from cms-T maize are incubated with malate. This could mean there is a diversion of the flow of electrons from their usual path to oxygen as a terminal acceptor. Therefore, the explanation for the light effect proposed above may apply to that for malate and other C_4 metabolites as well. Alternatively, malate itself may contribute to H_2O_2 formation if physiological conditions are favorable (21,46). Interestingly, the mechanism of the light-induced insensitivity of cms-T maize to T-toxin proposed here is not incompatible with that enunciated by Walton et al. (50), who suggested that the protection of maize leaves from the action of toxin in light is associated with light-driven phosphorylation that compensates for toxin-induced inactivation of oxidative phosphorylation. However, this is the first time consideration has been given to a possible role for AOS in the light response of cms-T maize to T-toxin. Further studies involving the interaction of H_2O_2 with the sensitivity of cms-T maize to T-toxin are in progress.

It is noteworthy that H_2O_2 can substitute for light and malate to reduce the sensitivity of cms-T cytoplasm maize leaves to T-toxin (Table 4). This is consistent with the idea that AOS might be involved. Since AOS are often implicated with disease resistance responses in plants (1,32,34,54), follow-up studies with H_2O_2 could lead to new insights into the mechanism(s) of light- and malate-induced insensitivity of cms-T maize to T-toxin. At any rate, the results of this study point to the possibility that, in addition to light-driven phosphorylation, AOS, particularly H_2O_2 , may be involved with various effects of environmental factors on the sensitivity of cms-T maize to T-toxin.

Also, the work presented could set the stage for more critical studies on the implications for plant-microbe interactions of light effects and altered levels of C_4 metabolites. In this connection, Daly and Livne (10) noted that more ^{14}C from ^{14}C -labeled bicarbonate was incorporated into organic acids when either healthy or rusted wheat leaves were incubated for 5 h in the light than when similar leaves were incubated in the dark. Also, Malca and Zscheile (36) noted that malic acid levels were higher in infected leaves of seedlings of a maize inbred (Pr_1) that was resistant to race 1 of *H. carbonum* than in an infected inbred that was susceptible (Pr). They observed a higher malic acid content in seedlings of the Pr inbred of maize that were inoculated with race 2 of *H. carbonum* (incompatible) instead of race 1 (compatible).

The results of the current study and the interpretations presented could add new insights into the large body of knowledge that already exist on the mode of action of T-toxin and on the nature of the responses of maize to it. Also, these studies could give renewed significance to earlier investigations on the effects of light and organic acids on host-parasite interactions including those of Malca and Zscheile (36), Daly and Livne (10), Bhullar et al. (6), Daly and Barna (9), Walton et al. (50), and MacRae and Yoder (35).

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