The pathogenic site of the C-toxin derived from *Bipolaris maydis* race C in maize (*Zea mays*)

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Abstract *Bipolaris maydis* race C strain 523 (C523) induces severer leaf blight on cytoplasmic male sterility (CMS)-C maize than on normal (N) maize. Previously, a pathotoxin isolated from C523 (C-toxin) was shown to be responsible for the disease. To understand the basis of the differential responses between CMS-C and N maizes to this fungus, protein synthesis *in vitro* by mitochondria from N and CMS-C cytoplasms was monitored after their incubation in a solution containing the toxin (0.3%). Similar protein products were detected between the two alloplasmic lines, indicating that the toxin does not directly act on the mitochondrial membrane, nor inhibits the expression of mitochondrial genes. To further locate the action site of the toxin, intact leaves from both N and several subtypes of CMS-C lines were treated by 0.3% toxin. Analysis of electrolyte leakage of leaf cells showed that the leakage rates were similar to one another among the alloplasmic maize lines. In contrast, at a lower concentration of the toxin (0.05%), the leaf cells from CMS-C line were more susceptible to the toxin than those of the other lines. All these results indicate that the target of the toxin action appears to be the cellular-membrane rather than mitochondria, suggesting that the variable susceptibilities to *B. maydis* between the alloplasmic maize lines might be related to a difference in their cellular-membranes.

Keywords: maize, *Bipolaris maydis* race C, specificity.

The purity of hybrid seeds is a standing problem in China's corn industry, which leads to a yield loss of around 5×10^9 kg per year, equivalent to losing 6×10^6 hm^2 farm lands. The main reason for this is that a strict emasculation cannot be carried out. To solve this problem, male sterile maize lines are widely used in hybrid seed production, which eliminates the need for expensive hands emasculation, and ensures the purity of hybrid seeds as well as the use of the hybrid vigor. However, potential dangers are associated with this technology. For example, from 1969 to 1970, an epidemic of southern corn leaf blight struck a sterile U.S. maize line, causing a heave yield loss of about 165×10^8 kg. At present, two improved strategies are used in hybrid seed production to avoid the spread of a specific disease. First, breeders mix cytoplasmic male sterility (CMS) seeds with seeds produced by hand emasculation for planting. Second, several CMS maize lines in stead of one are used as parents for seed production. CMS-C maize lines are divided into three subtypes, C I (C), C II (BB, RB) and CIII (ES). C I subtype is susceptible to *Bipolaris maydis* race C strain 523 (C523) whereas C II and CIII are not[1]. Currently, CMS-C maize line is extensively used in hybrid seed production in China. To prevent potential heavy yield loss due to the use of a single CMS-C line, the knowledge of pathogenesis of *B. maydis* on the maize lines is urgently required. Previously, a pathotoxin derived from C523 (C-toxin) was found to be involved in corn leaf blight. Here, we investigated the pathogenic site of the C-toxin and found that it acts on the cellular membrane. Furthermore, the variable susceptibilities to the toxin by alloplasmic maize lines might be related to differential stability of their cellular membranes.

1 Materials and methods

(i) Materials. *B. maydis* race C strain 523 was given by the Resistance Physiology Group, Institute of Physiology and Biochemistry, the Academy of Agricultural and Forestry Sciences, Hebei Province. Inbred alloplasmic maize lines with *zong3* as the nuclear background were provided by the Genetics Group, Department of Plant Genetics and Breeding, China Agricultural University. ^{35}S-Met was purchased from Amersham (UK). The conductivity detector is of Model DDS-11A with an electrode type of DJS-1 manufactured by the Shanghai No. 2 Analytic Instruments Factory.

(ii) Extraction of the C-toxin secreted by *B. maydis* and identification of its pathogenicity. Fungal spores were inoculated in a Fries liquid medium and incubated at 25℃ with shaking for 2 weeks. The crude toxin was obtained by filtering the culture fluid of fungi and evaporating at 50℃ until a yellow thick liquid was formed. After methanol/chloroform extraction and further evaporation, the oily liquid containing the pathotoxin was diluted with heated water[2].

The fourth leaves were cut off from the five-leaf maize seedlings. Three holes were pricked on each point and inoculated with a cotton ball soaked with diluted toxin. The leaves were left in a warm and moist condition for 16 h. The size of wilting spots was measured for the pathogenicity.

(iii) Measurement of electric conductivity of maize leaves inoculated by the toxin. Leaves at the same age and weight were cut down and washed with water. After surface drying by absorbing with tissue paper, the leaves were soaked in a 0.3% toxin-containing water solution. The conductivity of the solution was detected after the leaves were vacuumed for 5 min. The first measurement of conductivity represented the basic conductivity. The leaves soaked in water without toxin were used as control. The conductivity was further tested in every 2 h. 20 h later, the solution containing leaves was boiled. The conductivity of the boiled solution represented the total conductivity. The following formula was used to calculate the relative conductivity:

$$\text{Relative conductivity (\%)} = \frac{\text{Conductivity} - \text{Basic conductivity}}{\text{Total conductivity} - \text{Basic conductivity}}.$$

(iv) Analysis of the toxin action on *in vitro* translation of mitochondria. Isolation and *in vitro* translation of mitochondria were performed as described[3,4]. Mitochondria were isolated from yellowing buds of the maize lines. Mitochondria were pre-incubated for 30 min in the toxin-containing buffer before being allowed to initiate protein synthesis for 2 h.

2 Results

(i) The toxin derived from *B. maydis* causes serious corn leaf blight. Large wilting leaf spots were produced after the spore inoculation, indicating that the race C shows a strong pathogenicity. The 3% toxin inoculation caused the leaves to wither. Sixteen hours after inoculation, wilting spots on the leaves of maize line *zong3* were detected and they spread quickly. No symptom difference was found between the maize lines with N or CMS-C cytoplasms. However, 1% toxin inoculation induced larger spots on CMS-C maize leaves than on normal ones. When the concentration of toxin was reduced to 0.3%, it caused light symptoms on CMS-C maize leaves whereas no symptoms developed on normal maize leaves. These results suggest that the higher concentration toxin causes strong symptoms on both CMS-C and normal maize leaves. In addition, CMS-C and normal maize lines showed variable susceptibilities to the toxin at a lower concentration.

(ii) The C-toxin has no effect on *in vitro* protein synthesis of mitochondria from the alloplasmic maize lines. A host specific infection on CMS-T maize has been shown for T-toxin derived from *B. maydis* race T with a pathogenic site on mitochondria. To investigate if the C-toxin is specific to mitochondria from CMS-C maize, we observed the effect of the toxin on *in vitro* translation of mitochondria. Isolated mitochondria synthesize proteins *in vitro* under an optimal culture condition with sodium succinate as substrate using ATP produced from oxidative phosphorylation. Synthesized peptides vary with mitochondria from different cytoplasms.

By using this method, we are able to differentiate the three alloplasmic maize lines with *zong3* nuclear background (N, C and ES) (fig. 1). The synthesized peptides from them have an average of 30 bands, with molecular weights varying between 1 ku and 130 ku. An 86-ku band was detected only in C and ES maize lines. An additional 21.5 ku band was found specific to the ES line. These polypeptides can be used to identify the three maize lines. This result also showed that the *in vitro* translation system worked well.

To analyze if the toxin directly acts on mitochondria, 0.3% toxin solution was added to the *in vitro* translation system of mitochondria from the alloplasmic lines. The results showed that mitochondria synthesis proceeded normally (fig. 2), suggesting that the toxin does not affect the integrity of mitochondria or inhibits the *in vitro* translation of mitochondria.

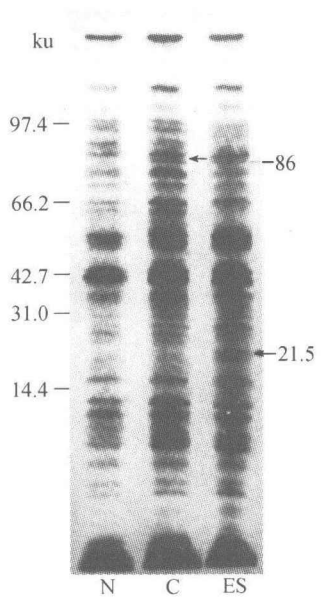

Fig. 1. Polypeptide synthesis *in vitro* by mitochondria from three alloplasmic maize lines. Mitochondria were isolated from the maize lines with normal (N), CMS-C and CMS-ES cytoplasms, respectively. After *in vitro* translation, the proteins were separated by polyacrylamide gel eletrophoresis and detected by autoradiography. Polypeptides specific to CMS cytoplasms are indicated by arrows.

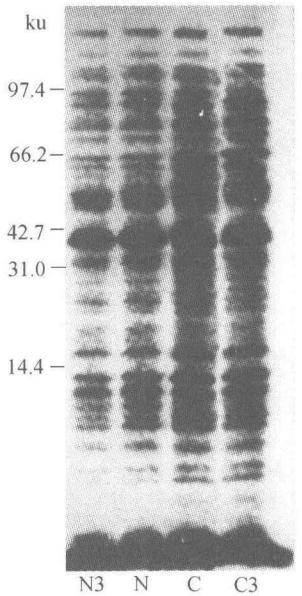

Fig. 2. Polypeptide synthesis *in vitro* by mitochondria from three alloplasmic maize lines in the presence of C-toxin. N and C represent the polypeptides synthesized from normal and CMS-C mitochondria, respectively. N3 and C3 indicate the polypeptides synthesized in the presence of 0.3% of C-toxin. The rest illustrations are the same as in fig. 1.

(iii) The toxin increases the permeability of cellular membrane of maize leaf rapidly. To further locate the possible pathogenic site of the toxin, we measured the leaf electric conductivity after the toxin treatment. Changes of the conductivity reflect the permeability alterations of cellular membranes. The cellular membranes of the leaves from the three alloplasmic lines displayed differential permeabilities in water (fig. 3(a)). The progressive change of conductivity was detected. During the first 5 h, the conductivity increased rapidly, but after 5 h, it did not change markedly. Interestingly, a drop was detected during this period, reflecting a rapid decrease of free ions in the solution. 14 h later, the conductivities of all the three lines showed an increase with the biggest increase found in the CMS-C line. Overall, the permeability of cellular membranes of the CMS-C maize showed a stronger and quicker change, suggesting that its cellular membrane is more fragile and less stable.

The conductivity of intact leaves treated with a 0.3% toxin solution from the three alloplasmic lines showed a rapid growth in one hour, indicating that their cellular membranes were damaged (fig. 3(b)). The toxin solutions with the leaves from the three lines did not show a continuous rise in conductivity. All of them showed a drop after 5 h, which again reflected a rapid change in free conductive ions. The leaves were also treated with a 0.05% toxin solution. The low concentration toxin appeared to have a negative effect on the permeability of cellular membranes (fig. 3(c)). At this concentration of the toxin, the leaf cells from the lines with N, C or ES cytoplasms did not display a marked increase in the conductivity within a period of 13 h. However, the N and ES leaves displayed a drop in the conductivity. 13 h later, all of the three lines showed a sudden rise in the conductivity. No significant difference was found between N and ES, while C showed a slight difference from N and ES. These results indicated that C showed a higher susceptibility to the toxin than N and ES.

In summary, the 0.3% toxin caused a rapid increase of the permeability of cellular membrane of the leaves but has no effect on mitochondria, suggesting that the leaf cellular membranes are the primary action site of the toxin.

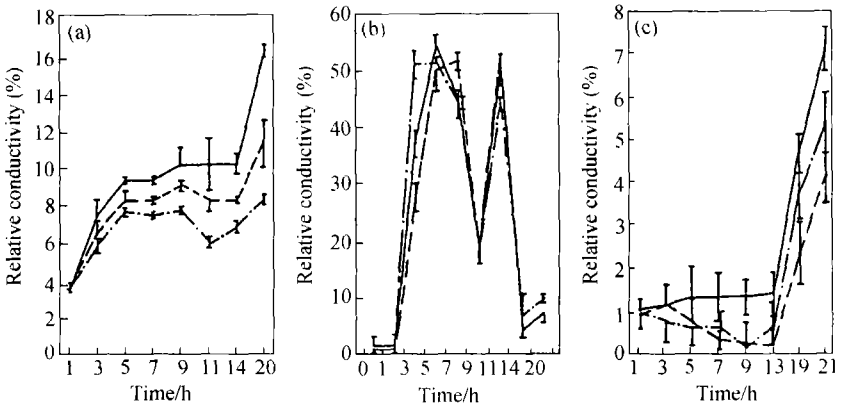

Fig. 3. Changes of the electric conductivity of intact leaves from the alloplasmic maize lines. (a) Conductivity changes of the leaves from the maize lines with N, CMS-C or CMS-ES cytoplasms in water. (b) The effect of 3% toxin on the conductivity of the leaves from the alloplasmic maize lines. (c) The effect of 0.05% toxin on the conductivity of the leaves from the alloplasmic maize lines. ---, N; ——, C; - • -, ∞ES.

3 Discussion

B. maydis race C elicits strong leaf blight on several maize lines. In this study, we have shown that a variable symptom is induced by the C-toxin on the three alloplasmic lines (N, C and ES) and the primary effective site of the toxin is on cellular membranes rather than mitochondria. This finding implies that the worry about a specific toxicity of *B. maydis* race C to CMS-C maize is unfounded.

Pathogenesis of *B. maydis* race C to CMS-C maize differs from the race T to CMS-T maize. The T-toxin shows a specific toxicity to CMS-T maize and induces mitochondria from T cytoplasm to swell. CMS-T mitochondria can encode a unique protein URF13, which is a hydrophobic polypeptide that resides in the inner mitochondrial membrane as an oligomer. URF13 is a ligand-gated, pore-forming receptor that binds the T toxin. When the toxin binds to the URF13, a pore is formed in the inner mitochondrial membrane, resulting in the dissipation of the membrane potential and uncoupling of oxidative phosphorylation[5]. Previous studies suggested that mitochondria from CMS-C cytoplasm were the primary effective sites of the C-toxin. The results presented here showed that *B. maydis* race C toxin does not specifically affect CMS-C maize, and the pathogenic site of C-toxin lies on cellular membrane in stead of mitochondria. Therefore, the possibility of a wide-spread of race C similar to that of race T is minimized. However, as the race C is pathogenic on normal maize, proper control of the race C should be implemented.

We observed that the race C infected maize leaves from the stomas of the infection site or directly through the intercellular space. The C-toxin secreted by the fungus spread along the leaf vein, and subsequently, many wilting spots emerge on the surface of leaves. The C-toxin causes an increase of permeability of cellular membranes and increases the outflux of cellular solutes. The fungi thereby assimilate nutrients from the solutes. The more susceptible to the toxin a leaf cell is, the more rapidly the cellular solutes flow outward, and the faster fungal filaments extend, resulting in larger wilting spots. A similar pathogenesis process was also found during host infection of *Cercospora beticola*. Non-peptide fungal toxins could incorporate themselves into transmembrane pores in the presence of Mg^{2+}, which results in an increase in conductance of biological membranes. This channel-like activity might be related to the deleterious effect of the toxin through a collapse of ionic and electrical gradients across biological membranes together with a Ca^{2+} influx and disruption of normal cellular signaling[6].

When the leaves were treated with the high concentration toxin, the conductivity of the solution containing them changed dramatically. The changes may be caused by cell breakdowns at different time points. The initial rise of the conductivity could be generated by the breakdown of a few surface cells. The ions released cannot maintain their free status because some of them are quickly neutralized or absorbed, resulting in a subsequent loss of the conductivity. The second rise of the conductivity appears

to come from the breakdown of the cells beneath the surface. After being boiled for 20 h, the leaves will release all their ions. The leaf-containing water showed a sharp increase in conductivity, whereas the conductivity of the leaf-containing toxin solution showed little changes, suggesting that the majority of the cells from the toxin-treated leaves were dead with losing the ability to release ions.

The low concentration toxin (0.05%) did not cause a rapid change in the permeability of the cellular membrane, but a decrease of the conductible ions was detected over a short period of time. One possibility is that cells detect the existence of the toxin and initiate a defensive response by closing their cellular pores. The low concentration toxin might not be enough to open the pores. Another possible explanation is that the toxin includes a substance that can neutralize the electric charge of ions, producing free conductible ions resulting in a loss of electric conductivity. No obvious difference in response to the higher concentration toxin (0.3%) was found among the alloplasmic maize lines. The variable susceptibility after leaf surface inoculation of the toxin may be correlated to a differential stability of cellular membranes in connection with different cytoplasms.

Previous work on the isolated C-toxin indicated that it is of a mixture containing several components toxic to leaf cells. One of the toxic components was proven to be a lipid with a structure similar to sesquiterpenoids[7]. Many fungal toxins contain this class of substances, which can induce leaves to form wilt spots. However, how the toxins affect the leaf cells is still unclear. Some toxins are found to be potential inhibitors on protein synthesis or act as a key to a cellular channel. Available evidence shows that this class of sesquiterpenoids is not host specific toxin showing pathogenicity to both host and non-host[8]. The isolated C-toxin likely contains a derivative from the sesquiterpenoids.

In summary, our results suggest that the alloplasmic maize lines display a difference in the stability of their cellular membranes. Because the components of the cellular membrane are generally encoded by nuclear genes, we postulate that a nuclear gene(s) plays an important role in pathogenesis of *B.maydis* race C on CMS-C maize. Furthermore, mitochondrial genes can modify the expression of nuclear genes. The interaction between nuclear and cytoplasmic genes may generate the difference in the stability of cellular membranes of the maize lines eventually leading to the variable susceptibility to the pathogenic fungi[9]. Further investigation is required to clarify how and what nuclear and cytoplasmic genes interact with each other to produce such a membrane difference among the alloplasmic maize lines.

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