Acetylcholine as a Signaling System to Environmental Stimuli in Plants

I. Contribution of Ca^{2+} in heat-stressed *Zea mays* seedlings*

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Abstract : Plant growth is influenced by transport of water, ions and hormones under various environmental stresses. If acetylcholine (ACh) mediates the opening and/or closing of ion channels in plants just as in animal systems, it is important to detect such evidence. The effect of heat stress on the acetylcholinesterase (AChE) activity and Ca^{2+} as a trigger of ACh release can be determined in selected organs of dark grown *Zea mays* seedlings. The enzyme activity in the coleoptile node was 3-7 fold higher than that of other organs. By heat stress, the AChE activity increased about 20% in the node. Cytochemical evidence for AChE activity was found only in the node. This reaction appeared in the cortical cells around the vascular system. After heat stress, the localization of AChE was radically changed in the node. AChE activity was detected in all endodermal cells surrounding the vascular system. Further, fluorescent labeled Ca^{2+} in the node was identified in cortex cells around the vascular system, epidermis and adhering peripheral cortical cells with a laser scanning confocal microscope. Following heat stress, Ca^{2+} was found more in the cortical cells and whole endodermal cells between the cortex and stele. The appearance of AChE and Ca^{2+} in endodermal cells after heat stress seems to be correlated with ACh function controlling ion channels.

Key words : AChE activity, ACh response, Ca^{2+} , Heat stress, Ion channels, *Zea mays*.

植物の刺激応答反応系におけるアセチルコリン 第1報 熱ストレス下のトウモロコシ芽生えにおける Ca^{2+} の関与: 桃木芳枝・桃木徳博**・Joanne H. WHALLON*** (東京農業大学生物産業学部・畜産試験場**・Michigan State University***)

要 旨 : 種々の環境ストレスは、植物における水、イオン、ホルモンなどの物質輸送に影響を与える。もし、植物細胞間の接合部で、動物と同様、刺激が起こす生体電気の伝達に起因するアセチルコリン (ACh) が、物質の輸送を制御するイオンチャネルの開閉に関与するならば、刺激を与えた植物の器官部位でアセチルコリン分解酵素 (AChE) 活性および ACh を細胞内に遊離させる Ca^{2+} の分布に変化が認められるはずである。本報では、暗条件で生育させたトウモロコシ芽生えに熱ストレスを与え、AChE 活性と Ca^{2+} 分布に及ぼす影響をおもに組織化学的に検討した。AChE 活性は、子葉鞘節が最も高く、他の器官の 3-7 倍であった。また、熱ストレス後、AChE 活性は、この節で約 20% 増加した。AChE の組織化学的反応は、無処理の植物では、この節の維管束の周囲の皮層細胞のみに認められたが、熱ストレス後、子葉鞘節における AChE 活性は、著しく変化し、中心柱と皮層の間に存在する内皮細胞全体に維管束系に沿って円周状に検出された。さらに、共焦点走査型レーザー生物顕微鏡を用いて、子葉鞘節における蛍光標識 Ca^{2+} を検出すると、維管束周辺の皮層、表皮および皮層外周部に認められた。熱処理後、 Ca^{2+} は、皮層細胞での増加と共に、新たに内皮細胞全体に検出された。これらの結果から、熱ストレス後、内皮細胞に検出された AChE と Ca^{2+} は、イオンチャネルを制御する ACh 作用に関与すると考えられる。

キーワード : アセチルコリンの応答, アセチルコリン分解酵素, イオンチャネル, カルシウムイオン, トウモロコシ芽生え, 熱ストレス。

Acetylcholine is a chemical transmitter serving to propagate an electric stimulus across the synaptic junctions of animals^{8,9,13)}. In previous work, Momonoki¹⁴⁾ proposed a working hypothesis of ACh in plants. Acetylcholine

may function as a membrane trigger for depolarization at the junction between cortex and vascular stele. Acetylcholinesterase could exist to hydrolyze the released free ACh around the vascular system. Then, the ion channels can be controlled by the binding of ACh to its receptors, just as in the animal system. Consequently, transport of hormones

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and substances can be regulated by open and/or close of the ion channels.

To function in this manner, the ACh-receptor-AChE system would require the presences of AChE and Ca^{2+} as a trigger of ACh release at the plasmodesmatal cell-cell interface in plants. On the other hand, it is known that an environmental stimuli cause a membrane depolarization in plants by a change in the bioelectric potential^{19,20}.

In the present work, AChE activity was detected by histochemical localization of AChE with light microscope and identification of Ca^{2+} labeled with fluorescent calcium green was done in selected organs of 4-d old dark grown maize following heat stress at the level of laser scanning confocal microscope.

Materials and Methods

1. Plant material

Kernels of *Zea mays* L. cv Silver Honey Bantam sweet corn were soaked 24 h in running water and germinated in rolled moist paper towels for 4-d old seedlings in a dark room at 25°C.

2. Heat stress

Ten maize seedlings were pinned to a Styrofoam sheet placed in a vertical position. Then, seedlings were exposed to a jet of warm air (30°C) for 1.5 min at room temperature. The air jet was generated by a dryer positioned at a distance of 20 cm from the plants. Plants were reduced by about 15% of fresh weight because of water loss.

3. Determination of AChE activity by SH appearance

Determination of AChE activity was based on the colorimetric SH reagent, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), as used by Asahi et al.¹⁾ and Ellman et al.⁴⁾ Harvested organs were incubated for 30 min¹⁴⁾ in a vial containing 500 μl of 100 mM sodium phosphate buffer (pH 7.5) and 500 μl of acetylthiocholine (AThCh) chloride (12.5 mM in 100 mM sodium buffer, pH 7.5) as a substrate. After incubation, 200 μl of solution was transferred to a vial, and 950 μl of the sodium phosphate buffer (pH 7.5) and 50 μl of DTNB (10 mM in 100 mM sodium phosphate, pH 7.0) were added. The absorbance at 412 nm was read after 1 min using a molar extinction coefficient of 13,600 and an identical tube without plant organs as a control.

Neostigmine bromide (35 μM) was used as a inhibitor of AChE. The regions of selected organs of maize seedlings are shown in Fig. 1. Harvested samples were 10 mm of coleoptile (from 2 mm above the coleoptile node), 10 mm of mesocotyl closed to the node (from 4 mm below the coleoptile node), 10 mm of mesocotyl closed to root (from 10 mm above the junction between mesocotyl and root), 10 mm of root (from 10 mm below the junction between mesocotyl and root) and 2.5–3 mm of coleoptile node.

4. Histochemical detection of AChE

The cross sections of selected maize organs were made 80–100 μm in thickness with a Microslicer (Dosaka, DTK-1000). Reagents for detection of AChE were prepared by the modified methods of Koelle^{10,11)}. The preparation of reagents and enzyme reaction using acetylthiocholine (AThCh) iodide, as a substrate of AChE was carried out by the same

Fig. 1. The regions of organs measuring AChE activity in 4-d old dark grown *Zea mays* seedlings.

Mesocotyl closed to node was taken a 10 mm segment from 4 mm below the coleoptile node. Mesocotyl closed to root was taken a 10 mm segment from 10 mm above the junction between mesocotyl and root.

methods described in our previous paper¹⁷⁾. Di-isopropylfluorophosphate (DFP) was used as a inhibitor of nonspecific esterases¹⁰⁾ because ACh is hydrolyzed by both specific cholinesterase (AChE) and nonspecific ester-

ases¹⁰⁾. The control sections were used without DFP for pretreatment and incubated in a medium, without AThCh, to which was added distilled water instead of substrate. AChE in plant cells was detected at the level of light

Table 1. AChE activity in heat-stressed *Zea mays* seedlings as measured by SH appearance following hydrolysis of acetylthiocholine.

Plant organ	AChE activity (pmol SH g ⁻¹ fresh weight h ⁻¹)	
	Control	Heat-stressed
<u>Coleoptile</u>		
Enzyme	185 ± 5	195 ± 7
Enzyme + Inhibitor	(37 ± 2)	(49 ± 3)
<u>Coleoptile node</u>		
Enzyme	612 ± 37	723 ± 48
Enzyme + Inhibitor	(182 ± 15)	(181 ± 12)
<u>Mesocotyl (closed to node)</u>		
Enzyme	88 ± 3	91 ± 4
Enzyme + Inhibitor	(18 ± 2)	(18 ± 1)
<u>Mesocotyl (closed to root)</u>		
Enzyme	101 ± 4	108 ± 5
Enzyme + Inhibitor	(21 ± 2)	(22 ± 1)
<u>Root</u>		
Enzyme	85 ± 7	96 ± 3
Enzyme + Inhibitor	(21 ± 2)	(23 ± 3)

Mean of 3 experiments ± SE.

Ten plants were used for each experiment.

Neostigmine (35 μM) was used as a AChE inhibitor.

Table 2. Effect of heat stress on the AChE activity in selected various organs in *zea mays* seedlings.

	Multiple comparison between T				
M.D. (0.05) ^{a)}	T _O	T _H ^{b)}			
	214	243			
	Multiple comparison between T at each O level				
	0 _C	0 _N	0 _{M-N}	0 _{M-R}	0 _R ^{c)}
T _O	185	612	88	101	85 ^{d)}
T _H	195	723	91	108	96
(M.D. 0.05)	T _O T _H	T _O T _H	T _O T _H	T _O T _H	T _O T _H

a) Minimum difference by Turkey's procedure (q-test) for the significance at the 5% level.

Any two means underscored by the same line are not significantly different.

Any two means not underscored by the same line are significantly different.

b) Treatment level. T_O, Non-heat stress; T_H, Heat stress.

c) Organ level. O_C, Coleoptile; O_N, Coleoptile node; O_{M-N}, Mesocotyl closed to node; O_{M-R}, Mesocotyl closed to root; O_R, Root.

d) Average of enzyme activity.

microscope.

5. Detection of Ca^{2+}

Ca^{2+} in node sections was detected by the modified method of Read et al.¹⁸⁾ Five hundred micro grams of calcium green (fluorescent label for calcium, Molecular Probes, Inc. Oregon USA) dissolved in 50 ml of distilled water was used as stock solution. For label loading, cross sections were incubated in 2 μM calcium green in KH_2PO_4 (0.01 M, pH 4.5) buffer for 2 h at room temperature in the

dark. After incubation the cells were transferred into KH_2PO_4 (0.01 M, pH 4.5) buffer solution. Sections were rinsed several times with fresh same buffer solution, then, cells were mounted on the slide glass with water. The control sections were incubated in the buffer without calcium green. Fluorescent Ca^{2+} labeled with calcium green was detected by laser scanning confocal microscope (Carl Zeiss, Inc. Thornwood, New York USA). The 488 nm line of an argon ion was used for

Fig. 2. Histochemical evidence of AChE in coleoptile node of non-heated *Zea mays* seedlings.

A, Control (without substrate, $\times 30$);

B, AChE-positive spots ($\times 30$);

C, Control (without substrate, $\times 75$);

D, AChE-positive spots ($\times 75$).

The dark residues indicated by arrows show AChE-positive spots of copper sulfide after cytochemical reaction in tissues.

single-wavelength of calcium green. Fluorescent barrier filter was used at 520–560 nm.

Results

The AChE activity in the coleoptile node was 3–7 fold higher than other organs in non-heated control seedlings (Table 1). Significant change in AChE activity after heat stress was found in the node (Table 2). By heat stress, AChE activity was increased about

10% in the node (Table 1). Further, the AChE activity was inhibited about 75% by an inhibitor of AChE, neostigmine (Table 1).

The cytochemical reaction of AChE in non-heated seedlings was found only in node within various selected organs. The AChE reaction was observed in the cortex cells around the vascular systems (Fig. 2). After heat stress, the localization of AChE in the node was extremely changed. The AChE reac-

Fig. 3. Histochemical evidence of AChE in coleoptile node of heat-stressed *Zea mays* seedlings.

A, Control (without substrate, $\times 30$) ;

B, AChE-positive spots in endodermal cells ($\times 30$) ;

C, Control (without substrate, $\times 75$) ;

D, AChE-positive spots in endodermal cells ($\times 75$).

The dark residues indicated by arrows show AChE-positive spots of copper sulfide after cytochemical reaction in tissues.

tion was found in the cortical cells and the whole endodermal cells between cortex and stele in the node (Fig. 3). In roots, AChE reaction was observed in cortex cells and endodermal cells around the lateral root (Fig. 4). The AChE in both of the organs appeared in plasma-membrane associated with cell wall and in intercellular spaces (Fig. 3 and Fig. 4). Further, labeled fluorescent Ca^{2+} in non-

heated node was found mainly in cortex cells, epidermis and adhering peripheral cortical cells (Fig. 5). By heat stress, labeled Ca^{2+} appeared in the endodermal cells between cortex and stele and increased Ca^{2+} contribution in the cortex cells (Fig. 5). In figures of 5-C and 5-D, white spots indicated by fluorescent Ca^{2+} were estimated in the square area. From the inserted figures, that showing the

Fig. 4. Histochemical evidence of AChE in root of heat-stressed *Zea mays* seedlings.

A, Control (without substrate, $\times 60$);

B, AChE-positive spots in cortex cells around lateral root ($\times 60$);

C, Control (without substrate, $\times 120$);

D, AChE-positive spots in cortex cells around lateral root ($\times 120$);

E, Control (without substrate, $\times 200$);

F, AChE-positive spots in endodermal cells around lateral root ($\times 200$).

The dark residues indicated by arrows show AChE-positive spots of copper sulfide after cytochemical reaction in tissues.

amount of fluorescent Ca^{2+} , amount of Ca^{2+} in the area including whole vascular system and cortex cells increased from 43% to 64% after heat stress (Fig. 5).

Discussion

Bandurski et al.²⁾ proposed the potential-

gating theory to provide a mechanism for asymmetric hormone distribution by gravity stimulus. We proposed a more detailed working hypothesis for explaining the mechanism, the voltage-gating theory by ACh function¹⁴⁾. In ACh function, Ca^{2+} acts as a trigger for releasing ACh just as in the animal sys-

Fig. 5. Localization of labeled fluorescent Ca^{2+} in coleoptile node of *Zea mays* seedlings.

A, Control (non-heated, without fluorescent Ca^{2+}) ($\times 30$);

B, Control (heat-stressed, without fluorescent Ca^{2+}) ($\times 30$);

C, Localization of Ca^{2+} in the node of non-heated seedlings ($\times 30$);

D, Localization of Ca^{2+} in the node of heat-stressed seedlings ($\times 30$);

E, Localization of Ca^{2+} in endodermal cells between cortex and stele of the node in non-heated seedlings ($\times 200$);

F, Localization of Ca^{2+} in endodermal cells between cortex and stele of the node in heat-stressed seedlings ($\times 200$).

White spots show existence of fluorescent Ca^{2+} .

White arrows show endodermal cells between cortex and stele of the node.

tem^{3,8,9,12,13}).

To clarify the function of ACh in plants, the effect of heat stress on AChE activity, histochemical localization of AChE in various selected organs and identification of Ca^{2+} were examined in coleoptile node of maize seedlings. Increase of AChE activity and significant changes in enzyme activity were obtained after heat stress especially in the node. By heat stress, increase of AChE activity in cucumber node¹⁶⁾ and in primary pulvinus of leguminous plants¹⁵⁾ has been reported. The activity of AChE in these organs were 4–7 fold higher than that of other organs. By histochemical observations, significant evidence of AChE activity was detected in the primary pulvinus of leguminous plant¹⁷⁾. This is also true in the maize node. The dark-brown residues of copper sulfide were found in cortex cells around the vascular system of the node. After heat stress, the AChE activity increased in whole endodermal cells between cortex and stele cells around the vascular system. The AChE was localized in the plasma-membrane associated with cell wall and in intercellular spaces. Fluck and Jaffe⁶⁾ suggested that AChE was located in the area between the cell and cell membrane. Tretyn et al.²¹⁾ also suggest that AChE activity linked with the cell walls and with the external layer of the cytoplasm. The AChE activity in maize also occurred between at the interface between stele and cortex¹⁴⁾ and was detected in a crude membrane fraction¹⁴⁾.

On the other hand, the vacuole of a mature plant cell is the main storage compartment of intracellular Ca^{2+} . The release of vacuolar Ca^{2+} induces an elevation of free cytoplasmic Ca^{2+} concentration from steady-state levels⁷⁾. Further, cytoplasmic Ca^{2+} levels are to mediate several physiological processes in plant cells, such as phototropism, geotropism⁷⁾ and hormone action⁵⁾. In fact, the Ca^{2+} in maize node increased in the endodermal cells and more in the cortex cells by heat stress. Both of AChE and Ca^{2+} increased especially in the endodermal cells between stele and cortex. Appearance of Ca^{2+} in endodermal cells after heat stress can be correlated with the function as a trigger for ACh release. Thus, ACh can be a candidate for a regulator of open/close channels in plasma membrane between the cell and cell wall in maize.

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