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Biochemical and Biophysical Research Communications 307 (2003) 15–22

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An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*

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Received 8 May 2003

Abstract

cDNAs encoding two acetylcholinesterases (AChEs) were isolated from the peach potato aphid, *Myzus persicae*. MpAChE1 was orthologous and MpAChE2 was paralogous with the *ace* of *Drosophila melanogaster*. The deduced amino acid sequence of MpAChE1 cDNA was identical between the pirimicarb susceptible and resistant strains. However, a single amino acid substitution of Ser431Phe on MpAChE2 was found in the pirimicarb resistant strains. This substitution was located in the acyl pocket of the enzyme and was thought to alter the ligand specificity.

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Keywords: Peach potato aphid; Acetylcholinesterase; Acyl pocket; Insecticide resistance

AChE (EC 3.1.1.7) is a target for organophosphate and carbamate insecticides at the post synaptic membrane hydrolyzing a neuro-transmitter, acetylcholine (ACh), thus terminating neurotransmission. Inhibition of AChE by insecticides causes excessive excitement in nerves, a blockage of neurotransmission and death of insects. Insensitivity of AChE to organophosphates and carbamates is one important mechanism for insecticide resistance.

Certain amino acid substitutions make the AChEs of *Drosophila melanogaster* (GenBank: AJ310134) [1–3] and *Musca domestica* (AJ310134) [4,5] insensitive to insecticides. However, no amino acid substitutions existed in the propoxur resistant strain of *Nephrotettix cincticeps* (AF145235) [6]. Recently, the AChEs paralogous to the *ace* of *D. melanogaster* were found in several species [7–9]. In addition, linkage of insecticide resistance and the partially sequenced *Culex pipiens ace-1*

implies that the second AChEs are a target for insecticides in this insect [9].

The peach potato aphid, *Myzus persicae*, is a cosmopolitan pest of fruits and vegetables. Frequent applications of organophosphates and carbamates resulted in the development of insecticide resistance [10]. Pirimicarb insensitive AChE of this aphid was first reported by Moores et al. [11,12]. Inhibitory curves of the AChEs exhibited three phenotypes, the pirimicarb sensitive, insensitive, and their intermediate. As the intermediate had a plateau between the sensitive and insensitive phenotypes (heterogeneous), the intermediate was regarded as a heterozygote of the sensitive and insensitive AChEs [11–13].

Eight strains of *M. persicae* were established from the Japanese populations [14]. Three were pirimicarb resistant and the others were susceptible. The sensitivity of AChE to pirimicarb at I_{50} was 30–50 times lower in the resistant strains than in the susceptible strains. Their inhibitory curves showed similar plateaus as observed in prior studies [11–13]. In this study, we identified the cDNAs of MpAChE1 and MpAChE2, compared them between pirimicarb susceptible and resistant strains, and speculated on a factor that modifies the AChE activity in the aphid.

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Materials and methods

Insects. Pirimicarb susceptible (S14, H36, K1-20, D3-4, and B5) and resistant (R, M2, and K2-0) strains of *M. persicae* were used. The susceptible strains had pirimicarb sensitive AChEs. The resistant strains, exhibiting a 300–2000 times higher LD₅₀ than S14 in the topical application of pirimicarb, possessed 30–50 times more insensitive AChEs. The resistant aphids showed plateaus in the inhibitory curves of pirimicarb between 10⁻⁶ and 10⁻⁵ M [14].

RNA purification, RT-PCR. Total RNA was isolated from the whole bodies of 10 apterous adult females by Isogen (Nippon Gene). Reverse transcription was conducted with total RNA and a primer, C2PT, by Superscript II (Life Technologies) under the condition at 42 °C 60 min, 51 °C 60 min, and 70 °C 15 min. This cDNA was used in the following PCR and 3'-RACE.

Isolation of MpAChE1 cDNA. First PCR was performed by *Taq* polymerase (Takara) with degenerate S11ACE and AS17ACE and then with S1iACE and AS17ACE (Fig. 1, Table 1). Gene specific primers were designed based on the 145 bp product. Downstream region to 3' end was amplified by *ExTaq* polymerase (Takara) with MpS1 and degenerate AS14ACE. This product was nested with MpS2 and AS10ACE.

The cDNA was amplified with MpS3 and C2 and nested with MpS4 and C2 for 3'-RACE. 5'-RACE was performed by a modified method [15] with 5'-full RACE Core Set (Takara). Total RNAs of each strain were isolated from 10 apterous adult females. Poly(A) RNAs were isolated by PolyATtract mRNA Isolation System (Promega). cDNAs were synthesized with Mp5'RTS. First PCR was with Mp5'S1 and Mp5'AS1 and the second was with Mp5'S2 and Mp5'AS2. Because this product did not contain the initiation codon, reverse transcription was repeated with Mp5'RT2. First PCR was with Mp5'S1 and Mp5'AS3 and the second with Mp5'S2 and Mp5'AS4. The open reading frame of AChE1 was amplified for individual genotyping with Mp5'S8 and Mp3'AS1 (see Fig. 2).

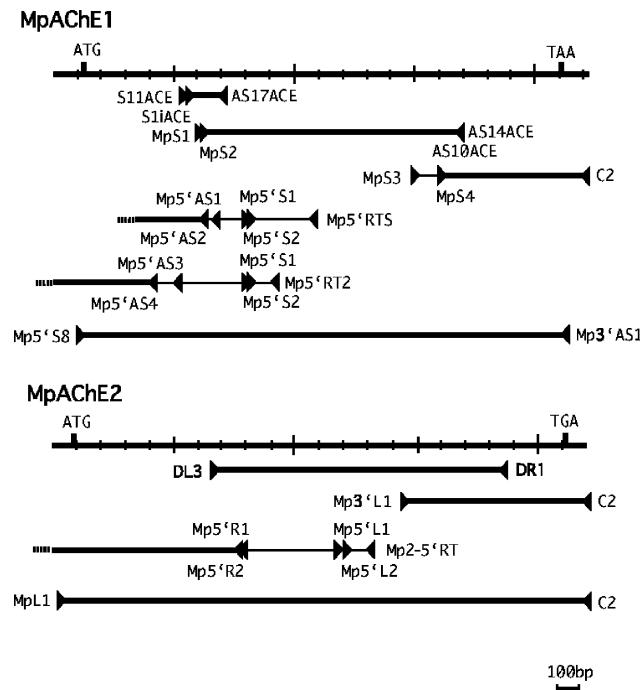


Fig. 1. Strategies for isolating MpAChE1 and MpAChE2 cDNAs by PCR. Triangles indicate annealing points of the primers. The PCR products of the thick lines were amplified and sequenced.

Isolation of MpAChE2 cDNA. At first, 1024 bp of MpAChE2 cDNA was amplified with degenerate DL3 and DR1 and the 3'-end of the cDNA with Mp3'L1 and C2. The 5'-end of cDNA was amplified by the same method with MpAChE1 cDNA. cDNA was synthesized with Mp5'RT and then the first PCR performed with Mp5'L1 and Mp5'R1 and the second with Mp5'L2 and Mp5'R2. The cDNA containing the open reading frame of MpAChE2 was obtained with MpL1 and C2.

DNA sequencing. PCR products were separated by agarose gel electrophoresis and purified by Ultrafree-DA (Millipore). The sequences were analyzed on a Genetic Analyzer 377 system (PE Applied Biosystems) following the instructions of BigDye Terminator (PE Applied Biosystems).

Individual genotyping. Although the aphids were maintained in the apomictic parthenogenetic generation, individual genotypes of MpAChE1 and MpAChE2 cDNAs were investigated. Total RNAs were purified from five individuals of the apterous adult females of each strain. The cDNAs were synthesized with C2PT for each aphid and their sequences determined.

Subcloning. Individual genotyping indicated that MpAChE2 cDNAs of the resistant strains were heterozygous and so MpAChE2 cDNAs were subcloned in order to clarify a series of SNPs on one cDNA. The ORF was amplified with MpL1 and C2. The PCR products of the 2179 bp were ligated into a pGEM-T easy vector (Promega) and competent JM109 transformed with this vector. Plasmids were purified by Concert Rapid Plasmid Miniprep System (Invitrogen).

Computer programs. A phylogenetic tree was generated from an alignment of neighbour-joining method by ClustalW program 1.83 [16]. EMBOSS package 2.6.0 [17] was used with infoalign program and sigcleave program [18,19] to calculate the similarities between the amino acid sequences of AChEs and to predict the sites of signal cleavage.

Results

cDNAs of 2070 and 2120 bp encoding MpAChE1 and MpAChE2 were isolated (GenBank: AF287291 and AY147797) from *M. persicae*. The deduced precursors were composed of 665 and 667 amino acids (Fig. 2). The phylogenetic tree indicated that insect AChEs are assorbed into two clusters (Fig. 3). MpAChE1 belongs to the cluster containing the AChEs of *D. melanogaster* (X05893), *L. decemlineata* (L41180), *N. cincticeps* (AF145235), and *P. xylostella* (AY061975) with 52.78%, 60.00%, 60.07%, and 57.87% identities. MpAChE2 was in the other cluster of aphid and mosquito AChEs. MpAChE2 was more similar to AChE1 of *A. gambiae* (AJ488492) and AChE1 of *A. gossypii* (AF502081) with 67.54% and 96.42% identities than AChE2 of *A. gambiae* (AAAB01008846), AChE2 of *A. gossypii* (AF502082), and *D. melanogaster* with 42.03%, 38.84%, and 40.75% identities.

The MpAChE1 residues contained intramolecular disulphide bonds (Cys128, Cys155, Cys328, Cys345, Cys483, and Cys599), catalytic triad (Ser276, Glu405, and His521), anionic subsite (Trp161), oxianion-hole (Gly189, Gly190, and Ala277), and acyl pocket (Trp309, Phe368, and Phe409). Cleavage of the signal peptide was predicted between Gly22/Arg23 following a glycine rich region (Gly28–Gly59). Its function was unclear, though a glycine rich region existed in the AChE2 of *A. gossypii*.

Table 1
Primers used in this study

Forward primers		Reverse primers		
RT		C2PT	TAATACGACTCACTATAGGGAGA(T)16	
MpAChE1				
	S1iACE	GARATGTGGAAYCCiAAYAC	AS17ACE	CCiCCiCCRTAiAYCCA
	S11ACE	TAYGARTAYTTYCCiGGiTT	AS17ACE	CCiCCiCCRTAiAYCCA
	MpS1	GCCTGTACCTGAACATCTG	AS14ACE	ACiCCCACATCCAYTCiCC
	MpS2	TACCGAAGAAGCAACGCA	AS10ACE	COATCCAYTCNCCCCA
3'-RACE				
	MpS3	ATCAGTATTGGGATGGGA	C2	TAATACGACTCACTATAGGGAGA
	MpS4	ATCAAGTCGTGGAGCTCGG	C2	TAATACGACTCACTATAGGGAGA
RT for 5'-RACE			Mp5'RTS	CGCCAGTCATGTA
5'-RACE				
	Mp5'S1	GGGCATTTGGTTCGTTGTA	Mp5'AS1	AGCACCGGTATCTTGGCGT
	Mp5'S2	TATTTAACGCCGAGCTGC	Mp5'AS2	GCCTTGCTTCTCGGTATC
RT for 5'-RACE			Mp5'RT2	CGAAAGCCGCCGC
5'-RACE				
	Mp5'S1	GGGCATTTGGTTCGTTGTA	Mp5'AS3	TCCTGGTAACAAGTGGTCGG
	Mp5'S2	TATTTAACGCCGAGCTGC	Mp5'AS4	GAACGGTATGCCGTGTACA
Fulllength				
MpAChE2	Mp5'S8	CCAACGTGACCACAATAAAA	Mp3'AS1	TTATAACAATCCATGTGCCG
3'-RACE	DL3	GCIACiATGTGGAAYCCiAA	DR1	GGRTTiCCiGTYTTiGCRAA
	Mp3'LL	GAGGAAAACGTGGTGGTGT	C2	TAATACGACTCACTATAGGGAGA
RT for 5'-RACE			Mp5'RT	AAGGCCATCCACCA
5'-RACE				
	Mp5'L1	TGGGTTGTCCAGATGACAGA	Mp5'R1	ATCCTCCTCCGAAAATCCAT
	Mp5'L2	CGGTCGAGTGCTTAAGGA	Mp5'R2	TCCTCCGAAAATCCATACCA
Fulllength	MpLL	ATTAGCAGTGGTGGTCGA	C2	TAATACGACTCACTATAGGGAGA

also [8]. The cDNA sequences of MpAChE1 were homozygous and identical among the strains and individuals analyzed in this study.

Cleavage of the signal peptide in MpAChE2 was predicted between Gly17/Leu18 followed by the residues for the intramolecular disulphide bonds (Cys169, Cys196, Cys355, Cys368, Cys503, and Cys625), catalytic triad (Ser301, Glu427, and His541), anionic subsite (Trp186), oxianion-hole (Gly220, Gly221, and Ala302), and acyl pocket (Trp334, Phe392, and Ser431). MpAChE2 was polymorphic in contrast to MpAChE1. The sequences of MpAChE2 cDNA from the five pirimicarb susceptible strains were homozygous, whereas the resistant strains (R, K2-0, and M2) were polymorphic at bases 1296, 1390 and 1391. These single nucleotide polymorphisms were separated by subcloning the cDNAs into the two different haplotypes. One had the same sequence as the susceptible strains (G1296, C1400, and A1401) and the other sequence was unique to the resistant strains (A1296, T1400, and T1401). A mutation at base 1296 was synonymous, gtg to gta. Bases 1400 and 1401 were nonsynonymous, tca to ttt, which resulted in an amino acid substitution of Ser431Phe.

Discussion

We demonstrated a nonsynonymous point mutation associated with the pirimicarb resistance of *M. persicae* by determining the genotypes of MpAChE1 and MpAChE2. No substitution existed on MpAChE1. However, a single substitution of Ser431Phe in MpAChE2 was found in this study. Preceding studies on aphid AChE sensitivity to pirimicarb described the existence of at least two AChEs in the pirimicarb resistant strains according to a plateau in the inhibitory curve of AChE activity and suggesting, the genotype of insensitive AChE was heterozygous in the strains investigated [11–13]. This plateau was coincident with the pirimicarb resistant strains of this study and their genotypes of AChE sensitivity were thought to be heterozygous [14]. Considering that MpAChE1 was homozygous and that MpAChE2 cDNA was heterozygous, the substitution, Ser431Phe, on MpAChE2 would explain the phenotypes of AChE heterogeneity in the pirimicarb resistant strains. We suggest, therefore, that MpAChE2 is important as a target of pirimicarb.

This substitution is located at the acyl pocket which determined the orientation of ligands in the active center

MpAChE1

Fig. 2. The cDNAs and deduced amino acid sequences of MpAChE1 and MpAChE2 from a pirimicarb susceptible strain, S14 (GenBank: AF287291 and AY147797). The residues of precursors are numbered from the first methionine. Cleavage sites of signal peptides are indicated by slashes. The cysteines which form intramolecular disulphide bonds are boxed. The residues of the catalytic triads are inverted. Acyl pocket residues are encircled. Anionic subsites are indicated by triangles. The residues with dots shape the oxianion holes.

by hydrophobic bonds attracting and trapping the ligands during the hydrolyzing process (Fig. 4) [20–24]. The 3D-structure of the AChEs by X-ray crystallography showed that the acyl pocket was surrounded by side chains of hydrophobic aromatic amino acids [24–28].

When some amino acid substitutions disturb the space and hydrophobicity of this site, the enzymatic properties of AChE will become modified in ligand specificity. The substitution of Ser431Phe is considered to make the acyl pocket more hydrophobic and narrower, and conse-

M₁AChE2

Fig. 2. (continued)

quently to alter the accessibility of the alkoxy group of the substrates and inhibitors to this site.

The electrostatic field of the acyl pocket is also important for ligand specificity. The acyl pocket locates at the bottom of the aromatic gorge adjacent to the active center. The pocket forming side chain of Phe331 attracted the catalytic His440 by an π -cation interaction in *T. carolinensis* [25-27]. Computer simulation and X-

ray crystallography showed that the side chain of His440 dynamically changed its orientation by transporting a positive charge during the catalytic process [28–34]. Therefore, conformation of the side chain of His is important in the hydrolyzing step. X-ray crystallography exhibited that the orientation of His443 differed between the wild human AChE and its recombinants when Phe334 in the acyl pocket was exchanged with

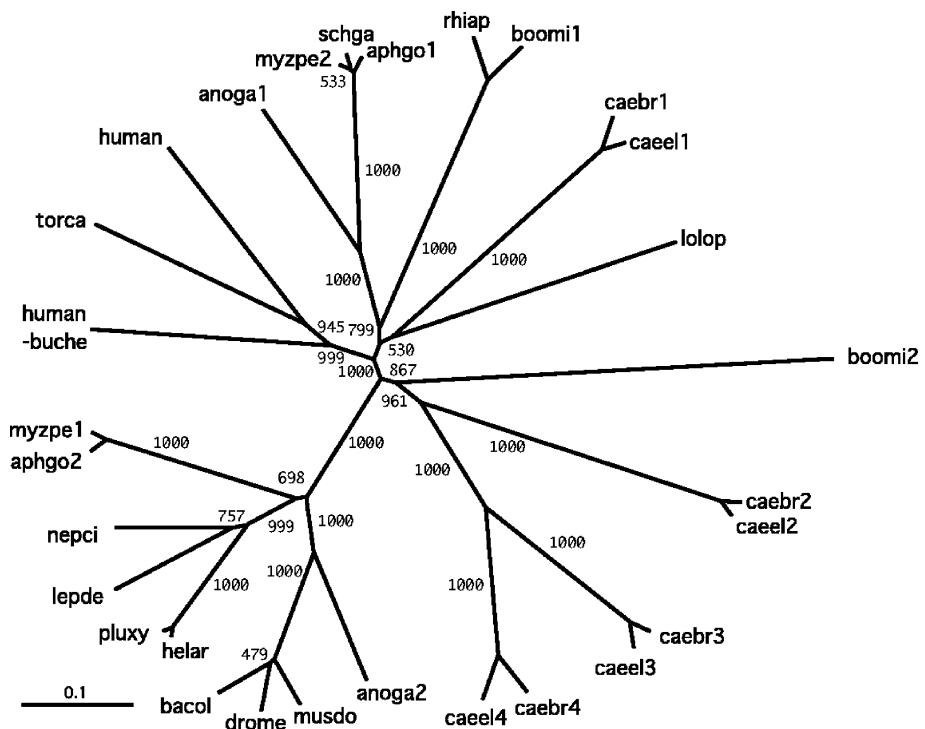


Fig. 3. Unrooted phylogenetic tree of the AChEs constructed by the neighbour joining method. Bootstrap values of 1000 trials are indicated on branches. The scale bar represents 10 percent divergence. drome, *Drosophila melanogaster* (X05893); musdo, *Musca domestica* (AJ310134); bacol, *Bactrocera oleae* (AF452052); anoga1, *Anopheles gambiae* (AJ488492); anoga2, *Anopheles gambiae* (AAAB01008846); helar, *Helicoverpa armigera* (AF369793); lepte, *Leptinotarsa decemlineata* (L41180); pluxy, *Plutella xylostella* (AY061975); nepci, *Nephrotettix cincticeps* (AF145235); aphgo1, *Aphis gossypii* (AF502081); aphgo2, *Aphis gossypii* (AF502082); myzpe1, MpAChE1, *Myzus persicae* (AF287291); myzpe2, MpAChE2, *Myzus persicae* (AY147797); schga, *Schizaphis graminum* (AF321574); rhiap, *Rhipicephalus appendiculatus* (AJ006338); boomi1, *Boophilus microplus* (AJ223965); boomi2, *Boophilus microplus* (AF067771); lolop, *Loligo opalescens* (AF065384); caebr1, *Caenorhabditis briggsae* (U41846); caebr2, *Caenorhabditis briggsae* (AF030037); caebr3, *Caenorhabditis briggsae* (AF159504); caebr4, *Caenorhabditis briggsae* (AF159505); caeel1, *Caenorhabditis elegans* (X75331); caeel2, *Caenorhabditis elegans* (AF025378); caeel3, *Caenorhabditis elegans* (AF039650); caeel4, *Caenorhabditis elegans* (AF025379); torca, *Torpedo californica* (X56517); human, *Homo sapiens* (M55040); and human-buche, *Homo sapiens* (M16541).

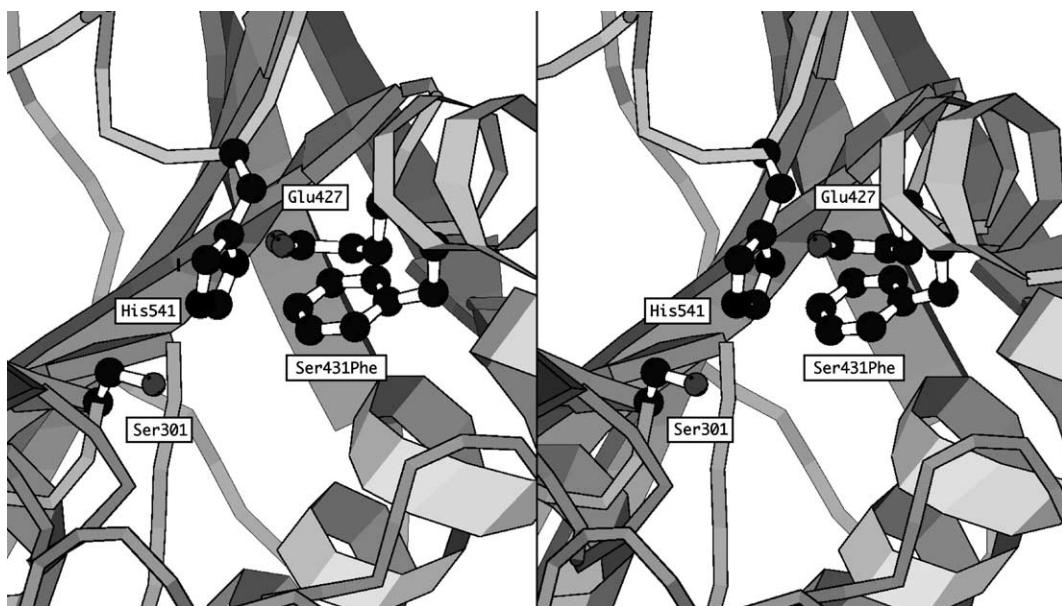


Fig. 4. MpAChE 2 was super-imposed on the AChE of *Torpedo californica* (PDB:1EA5). Stereogram was drawn by MOLSCRIPT program 2.1 [37]. Ball-and-stick model indicates the side chains of the catalytic triad and the polymorphic residue, Ser431Phe, on MpAChE2.

aliphatic amino acids [33–35]. Phe431Ser on MpAChE2, losing an aromatic ring, apparently disturbs the π -cation interaction between the acyl pocket and the catalytic His541. This would cause a conformational change of the side chain of His541 to alter the ligand specificity.

The structural significance of Ser431Phe on MpAChE2 in the pirimicarb resistance of the aphid proposes the possibility that MpAChE2 plays a more important role as a target of organophosphates and carbamates than MpAChE1. The AChE was taken as a single locus in the genome of insects [35,36] and mutations on the AChE gene explained the insecticide insensitivity of AChEs in flies [1–5]. However, some individuals of *N. cincticeps* with propoxur insensitive AChEs did not have nonsynonymous mutations in their AChE cDNAs [6]. Further interpretation was required to explain the mechanism of insensitive AChE in this species. In addition, the insecticide insensitivity of AChE in *C. pipiens* was inherited with certain point mutations on the second locus of the AChE gene [9]. Both *M. persicae* and *C. pipiens* suggest that the AChEs paralogous to the *ace* of *D. melanogaster* are more common as a target for insecticides in some species.

Although correspondence between genotypes of MpAChE2 and phenotypes of pirimicarb insensitivity was confirmed in this study, it was still uncertain whether MpAChE2 was a potential target of insecticides. Subsequent experiments such as expression of mutant AChEs, in situ hybridization, and immunoblotting are needed to confirm the conformational change by substitution according to the pirimicarb insensitivity and the localization of MpAChE1 and MpAChE2. The substitution of Ser431Phe may be a crucial cue to investigate the actual function of the two AChEs and the selective toxicity of organophosphates and carbamates in the aphid.

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