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The *Arabidopsis thaliana* ortholog of a purported maize cholinesterase gene encodes a GDSL-lipase

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Abstract

Acetylcholinesterase is an enzyme that is intimately associated with regulation of synaptic transmission in the cholinergic nervous system and in neuromuscular junctions of animals. However the presence of cholinesterase activity has been described also in non-metazoan organisms such as slime molds, fungi and plants. More recently, a gene purportedly encoding for acetylcholinesterase was cloned from maize. We have cloned the *Arabidopsis thaliana* homolog of the *Zea mays* gene, At3g26430, and studied its biochemical properties. Our results indicate that the protein encoded by the gene exhibited lipase activity with preference to long chain substrates but did not hydrolyze choline esters. The At3g26430 protein belongs to the SGNH clan of serine hydrolases, and more specifically to the GDS(L) lipase family.

Keywords

Cholinesterase; GDS(L)lipase; Serine hydrolase

Introduction

The evolutionary history of acetylcholine (ACh) as a neurotransmitter can be traced as far back as primitive bilaterians (e.g. flatworms), but the recruitment of this signaling molecule for other, non-neuronal, functions predates the evolution of the bilaterian lineage (Le Novere and Changeux 1995; Walker et al. 1996; Dent 2006). ACh and enzymes associated with its metabolism have been found not only in cnidrians that lack cholinergic neurons (Denker et al. 2008) and in organisms that altogether lack an organized nervous system (e.g. sponges, Horiuchi et al. 2003) but also in organisms outside of the fungi/metazoan group such as slime molds (Earle and Barclay 1986), ciliates (Delmonte Corrado et al. 2001), algae (Raineri and Modenesi 1986; Gupta et al. 1998), archaea (Yamada et al. 2005) and bacteria (Domenech et al. 1991). In fact, it seems that ACh metabolism is ubiquitous (reviewed in Horiuchi et al. 2003; Kawashima et al. 2007; Wessler and Kirkpatrick 2008).

Extensive literature describes the involvement of ACh in several processes in plants (reviewed by Hartmann and Gupta 1989; Tretyn and Kendrick 1991; Wessler et al. 1999; Roshchina 2001). Others and we demonstrated the presence of ACh hydrolytic activity in

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many plants, and especially in the families Leguminosae and Solanaceae (Fluck and Jaffe 1974; Gupta and Gupta 1997; Fletcher et al. 2004; Muralidharan et al. 2005). Research suggested that cholinesterase (ChE) activity in plants may be involved in a myriad of physiological processes including phytochrome signal transduction (e.g. Jaffe 1970), regulation of the stomatal aperture (Madhavan et al. 1995), gravitropism (Momonoki 1997; Momonoki and Bandurski 1994; Momonoki et al. 2000), pollen tube elongation (Tezuka et al. 2007) germination (Beri and Gupta 2007), and root development (Bamel et al. 2007).

Despite this wealth of physiological and biochemical studies, the identity of the proteins/ enzymes involved in ACh metabolism in plants and the genes encoding them are not known. Nonetheless, an intriguing report by Sagane and co-workers described the purification of a maize (*Zea mays*) protein with very weak ACh hydrolyzing activity and low sensitivity to the anticholinesterase neostigmine bromide (NB). The researchers determined the amino acid sequence of peptide fragments associated with that preparation, and cloned a gene based on that sequence (Sagane et al. 2005).

To allow for a more detailed study of the putative plant ChE gene, we turned to the model plant *Arabidopsis thaliana* and cloned At3g26430, the *A. thaliana* ortholog of the *Z. mays* gene. Further, by over-expressing the gene in bacteria and in plants, we demonstrate that while the gene's protein product was devoid of ACh hydrolyzing activity, it hydrolyzed esters of fatty acids with a preference for long chain esters. A close examination of the primary structure of the enzyme revealed the distinct motifs of GDS(L) lipases, a group of serine hydrolases with no apparent evolutionary kinship to the α/β fold protein family to which all known ChEs belong. Based on these results we conclude that the At3g26430 gene *of A. thaliana* is a lipase.

Material and methods

Bioinformatics analyses

To identify homologs to the putative ChE gene from *Z. mays* in other plant species, we used its amino acid sequence (GenBank: NP_001105800) as a query in *blastp* and *tblastn* searches of, respectively, the non-redundant protein sequences (nr) and nucleotide collections (nr/nt) available through the National Center for Biotechnology Information (NCBI). A phylogenetic analysis on the first 98 hits from the *blastp* search was conducted on the Phylogeny.fr platform (http://www.phylogeny.fr/version2_cgi/index.cgi, (Dereeper et al. 2008). First, sequences were aligned with MUSCLE (v3.7) configured for highest accuracy (MUSCLE with default settings, http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=muscle, (Edgar 2004)). Next, a phylogenetic tree was constructed using the neighbor joining method implemented in the BioNJ program (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=bionj, (Gascuel 1997)). Number of bootstraps was set to 100 and distances were calculated using ProtDist (Felsenstein 1989). The JTT substitution model was selected for the analysis (Jones et al. 1992) and the TreeDyn online tool was used to draw the tree (Chevenet et al. 2006). Highly similar trees were constructed on the Phylogeny.fr platform using the minimum parsimony method implemented in the TNT program (v1.1, http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=tnt, (Goloboff et al. 2000), using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT, http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=phyml, (Anisimova and Gascuel 2006), or using the Cobalt multiple alignment tool available through NCBI (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeAd, (Papadopoulos and Agarwala 2007)), followed by tree generation using the Fast Minimum Evolution algorithm (Desper and Gascuel 2004).

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The following protein sequences were used for multiple-sequence alignment with the T-Coffee tool (v6.85, http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=tcoffee, (Notredame et al. 2000): *A. thaliana* (NP_189274); *Daucus carota* (carrot, BAF80349); *Glycine max* (soybean ACU20252); *Hevea brasiliensis* (para rubber, Q7Y1X1); Macroptilium atropurpureum (siratro, BAG09557); *Medicago sativa* (alfalfa, AAB41547); *Oryza sativa* (rice, NP_001060129); *Populus trichocarpa* (poplar, XP_002314590); *Ricinus communis* (castor bean, XP_002530043); *Salicornia europaea* (BAI23204); *Sorghum bicolor* (sorghum, XP_002463099); *Vitis vinifera* (grape vine, XP_002282372); *Zea mays* (maize, NP_001105800). The T-Coffee program was also used for other multiple sequence alignments that are presented. Presence of conserved sequence motifs was verified using the Conserved Domain Database from NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The gene structures of the following Cluster A (see "Results") sequences were examined. Maize: AC212002 (genomic, region: 165375–168619), AB093208 (mRNA); Sorghum: NC_012871 (genomic, region: 72074044–72077805), XM_002463054 (mRNA); Rice: NC_008400 (genomic, region: 23766814–23770549), NM_001066664 (mRNA); *A. thaliana*: NC_003074 (genomic, region: 9671517–9675927), BX824162 (mRNA); Poplar: NC_008474.1 (genomic, region: 12595763-12598118), XM_002311724.1 (mRNA); castor bean: NW_002994674.1 (genomic, region: 126749–129456), XM_002529997.1 (mRNA); NW_002994674.1 (genomic, region: 126749–129456), XM_002529997.1 (mRNA); *Medicago truncatula*: AC163897.4 (genomic, region: 106353–109295), Medtr7g104050.1 (predicted mRNA, http://www.medicago.org/genome/show_bac_genecall.php?bac_acc=AC163897); grape: NC_012007.2 (genomic, region: 7386126–7388180), XM_002282336.1 (mRNA).

The gene structures of the following cluster B and cluster C sequences were examined. Rice: NC_008398 (genomic, region: 19358735–19363012), NM_001062020.1 (mRNA); *A. thaliana*: NC_003074 (genomic, region: 1468291–1470658), NM_111391.3 (mRNA); *A. thaliana*: NC_003070 (genomic, region: 3031085–3033700), NM_100809.4 (mRNA); *A. thaliana*: NC_003075 (genomic, region: 485725–488115), NM_116343.3 (mRNA); *A. thaliana*: NC_003070 (genomic, region: 20440907–20444177), NM_104354.3 (mRNA); grape: NC_ NC_012013 (genomic, region: 2183271–2185879), XM_002271434.1 (mRNA).

Cloning the *A. thaliana* gene At3g26430

Total RNA was extracted from mature *A. thaliana* leaves (100 mg fresh weight) using the RNAeasy Plant Mini Kit (Invitrogen), and cDNA was then prepared using the Ambion kit with oligo dT primers. The At3g26430 gene was amplified from the cDNA preparation (100 ng) using gene specific primers 1F and 1R (see Table 1 for all oligonucleotides used in this work) and the amplified product was cloned into a TOPO-TA vector (Invitrogen) and the insert's sequence was verified (pTM359).

To construct an *Escherichia coli* expression vector for At3g26430, the gene was PCR-amplified from pTM359 with primers 1F and 2R (to introduce a *Xho*I site) and cloned into a TOPO-TA cloning vector which was then digested with *Pci*I and *Xho*I releasing a 1.1 kb fragment that was then gel-purified and inserted into a pET22b vector (Invitrogen) replacing an *Nco*I-*Xho*I fragment (*Pci*I and *Nco*I have compatible sticky ends) to yield pTM381, the insert of which was sequence-verified.

To create a plant-expression cassette, the *PciI–KpnI* fragment from pTM359 containing the coding region of At3g26430 was cloned into the backbone of pTM209 (identical to pTM034 described by Mor et al. 2001) by replacing a corresponding *NcoI–KpnI* fragment. The plant-expression cassette consisted of the 35S CaMV promoter, the 5′ UTR of tobacco etch virus

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(TEV leader) and the 3′ UTR of soybean's *vspB* gene (VSP terminator) yielding the intermediate vector pTM366. A *HindIII—EcoRI* fragment containing the expression cassette was then cloned into the pGPTV-Bar plant expression vector (Becker et al. 1992) to yield pTM395. The plasmid DNA was then introduced into *Agrobacterium tumefaciens* transformed LBA4404.

Bacterial expression of At3g26430

Escherichia coli cells harboring pTM381 were grown overnight in 100 ml of 2 × YT (1.6 g tryptone, 1.0 g yeast extract, 0.5 g NaCl pH 7.0) in the presence of ampicillin (100 mg/L) and 1 % glucose to prevent induction of the protein. The starter culture was centrifuged at 5,000 ×*g* for 10 min and the pellet was washed twice with 2 × YT to remove the glucose, resuspended in 1 L of the 2 × YT and grown to OD_{600} of 0.7–0.8. The culture was then induced with 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) for 2 h after which the culture was centrifuged and the pellet was weighed and kept at −80 °C until further use. An *E. coli* strain harboring a plasmid with an unrelated insert served as the control and was treated as described above.

Generating transgenic *A. thaliana* lines over-expressing At3g26430

Six-week old *A. thaliana* plants were transformed using the floral dip method (Clough and Bent 1998) with the *A. tumefaciens* strain harboring pTM395. The seeds obtained from the transformed plant were surface sterilized by soaking for 4 min first with 70 % (v/v) ethanol and then with 50 % (v/v) commercial bleach plus 0.1 % triton X-100 (v/v) followed by rinsing three times with sterile water. Seeds were plated on basal MS medium containing 1% (w/v) sucrose and Gamborg's vitamins supplemented with 7.5 mg/L of the herbicide Basta (glufosinate ammonium, Sigma). The plates were vernalized for 2 days at 4 °C followed by incubation in a growth chamber. Three independently transformed lines were obtained from this transformation. Wild type (WT) Col-0 and transgenic *A. thaliana* T_2 or T_3 lines were used for the experiments.

Determining total and At3g26430 RNA and protein levels

Semi quantitative PCR was used to determine the level of At3g26430 mRNA in WT and transgenic *A. thaliana plants* using cDNA prepared as described above. Fragments of At3g26430 (with primers 4F and 4R) and actin (control, with primers 5F and 5R) were simultaneously amplified under the following PCR conditions: 94 °C (10 min); 33 cycles of 94 °C (30 s), 53 °C (45 s) and 72 °C (30 s); and a final extension step at 72 °C (7 min). Samples were removed after the 27 and 30th cycle were transferred to a second PCR machine to complete the final extension cycle.

Total soluble protein extracts from bacteria and plants were prepared as follows. Frozen *E. coli* cell pellet was thawed and sonicated in the presence of lysis buffer (50 mM Na_2HPO_4/ NaH_2PO_4 [NaP_i] pH 7.5, 150 mM NaCl, 1 mg/mL lysozyme [Sigma] and 10 μg/ml leupeptin [Sigma], 5 ml buffer per 1 g cell pellet). The lysate was spun at 30,000×*g* for 15 min at 4 °C to separate the soluble and the insoluble fractions. Leaf tissue from WT and transgenic plants were homogenized in 100 mM NaPi buffer pH 7.5 (three volumes × mass). The homogenates were clarified by centrifugation (4 °C, 14,000×*g*, 15 min).

At3g26430 protein preparations were resolved by SDS-PAGE on 12 % gels that were then either stained with Coomassie brilliant blue, or transferred to a nitrocellulose membrane and immuno-decorated with a rabbit anti-His antibody (1:1000, Santa Cruz Biotechnology). HRP-conjugated goat-anti rabbit IgGs (1:10000, Santa Cruz Biotechnology) and the ECL-plus kit (Amersham) were used for detection. Total protein concentration was determined using Bradford assay (Biorad) as previously described (Mor et al. 2001).

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Enzymatic assays

Cholinesterase activity was determined using the Ellman assay with acetylthiocholine iodide (ATCh) or propionylthiocholine iodide as the substrates essentially as described before (Mor et al. 2001) except that the final concentration of the Ellman reagent (5,5′-dithiobis-(2-nitrobenzoic acid), DTNB) was 1 mM. Reactions were started by addition of the soluble fractions from either *E. coli* or *A. thaliana* leaf homogenates (containing 150 or 100 ×g of total protein, respectively), carried out at 25 °C and their progression monitored by measuring A_{412} in a Molecular Devices Spectamax 340PC 96-well plate reader.

Esterase activity against *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB) and *p*-nitrophenyl palmitate (PNPP) was assayed as described before (Baudouin et al. 1997). Stock solutions (20 mM) of PNPA and PNPB were prepared by dissolving the substrate in Buffer 1 (100 mM NaP_i, pH 7.5, 150 mM NaCl, 10 % v/v isopropanol and 10 % v/v triton X-100). Similarly, a PNPP stock solution (10 mM) was prepared by dissolving the substrate in Buffer 2 (Buffer 1 supplemented with 20 % sodium deoxycholate and 10 % gum arabica). For the assays substrates were diluted to the indicated final concentrations with Assay Buffer (100 mM NaP_i, pH 7.5, 150 mM NaCl). The final concentrations of the additive was kept under 1 % (v/v) for isopropanol and triton X-100, and under 2 % (v/v) for sodium deoxycholate and gum arabica. In inhibitor studies, either neostigmine bromide (NB) or phenylmethylsulfonyl fluoride (PMSF) were added to 0.1 mM and 1 mM, respectively. Steady-state reaction rates were determined by monitoring A_{412} (at 30 °C) afforded by the protein preparations as described above. Kinetic parameters were determined using Prism (Prism v 4.0, GraphPad Software).

Results

The *A. thaliana* ChE ortholog of the putative maize '*ache*' gene

Plant homologs of the maize gene encoding for hypothetical protein LOC606473 (also called 'ache', NP_001105800) were identified through both *blastp* and *tblastn* similarity searches, which yielded, respectively, 1,361 and 2,138 hits (with the expect value set at $<10^{-6}$). The first 98 hits of the *blastp* search (i.e. those with the lowest expect values) were used to construct a phylogenetic tree using the neighbor joining-based program BioNJ (Fig. 1a). Represented in this group were accessions from both monocotyledonous (monocots) and eudicotyledonous (dicots) plants, 9 families, 14 genera and 17 species (Table 2). We could identify within this group at least three potential homology clusters (A–C), which included both monocots and dicots accessions (Fig. 1). Other phylogenetic analysis tools based on maximum likelihood and parsimony, yielded trees that were largely congruent with the one presented here (data not shown).

The maize gene query could be found within cluster A, the largest group among the homologous proteins examined here. Alignment of several accessions represented in this cluster revealed a high degree of sequence conservation along the length of the proteins, with the exception of their N-terminal domains (Fig. 2). The cluster A accessions could be further segregated into three sub-clusters. Sub-cluster A1 was the only one among the three to have monocot and dicot members (including the maize gene query). The homology between the maize gene and the other monocot members of A1 ranged from 81 to 99 (69–99 % identity, Fig. 1, 2). As could be expected, the homology between query and the A1 dicot members was more modest at 73–79 % (59–63 % identity, Fig. 1, 2), but well within the expected sequence conservation among orthologous genes across the monocot/dicot divide (e.g. Yamaguchi et al. 2004; Woo et al. 2007). Sequences within sub-clusters A2 and A3 are more closely related to each other than to members of sub-cluster A1, but their independence as orthologous groups cannot be determined.

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The genes' exon/intron arrangement was very similar among members of the A1 sub-cluster each with five exons and 4 introns (Fig. 3). In particular, the length of exons 2–4 are practically identical among sampled members of this group at, respectively, 212 ± 2, 170 ± 0 and 271 ± 0 bp (mean ± SD, n = 8). Among members of clusters B and C that posses the 5 exons/4 introns gene structure, the length of exon 4 is also conserved (270 ± 3 bp, n = 6), but the lengths of exon 2 and 3 diverge in a subtle, but statistically significant, manner at 200 ± 8 ($p = 0.0136$, $t(12) = 2.9$) and 166 ± 4 ($p = 0.0094$, $t(13) = 3.1$). We therefore conclude that clusters A, B and C represent orthologous gene clusters and that members of each may be considered as orthologs with common ancestry which predates the monocot/dicot split.

Subcluster A1 contained two *A. thaliana* sequences. The placement of one of these *A. thaliana* genes (NP_176949) in the A1 subcluster is questionable because, while highly similar to the putative maize '*ache*' gene, its exon/intron organization (three and two, respectively) markedly deviated from that of the maize gene (and others in the A1 group). Furthermore, its branch in most models was only weakly supported and in some models it was placed in an outgroup of A1. In contrast, At3g26430 (NP_189274_1), the *A. thaliana* gene most closely related to the maize gene (57 % identity and 78 % similarity, expect value of 2×10^{-120}) was placed by all models within subcluster A1 (Figs. 1, 2, 3). Therefore, we chose At3g26430 for further study as the prospective ChE gene of *A. thaliana*. The gene is located on the third chromosome and while it is annotated as forming a bicistronic expression unit with an upstream gene (At3g26420), there is evidence for independent transcription in the form of full-length mRNA accessions in the database (e.g. BX824162). In addition, the region upstream of the translation start site contains potential core promoter elements (e.g. TATA-box and initiator elements). The coding region of At3g26430 was amplified by PCR from a cDNA preparation and was cloned into bacterial and plant expression vectors.

At3g26430 lacked cholinesterase activity

At3g26430 encodes a 380-residue long protein with a predicted molecular mass of 42 kDa. The first 23 residues are predicted to form a cleavable signal peptide [http://www.cbs.dtu.dk/services/SignalP/, (Emanuelsson et al. 2007)]. In order to determine the biochemical characteristics of the At3g26430 protein, we expressed the protein in *E. coli* using a periplasm-targeting expression vector. We confirmed the expression of a protein with the appropriate molecular mass by SDS PAGE followed by immunoblotting (Fig. 4, insert). Upon disruption of the cells by sonication followed by centrifugation, most of the protein fractionated with the insoluble fraction, presumably in the form of inclusion bodies. However, a substantial portion of the At3g26430 protein remained soluble and therefore allowed us to directly test its enzymatic activity (Fig. 4). Neither the soluble fraction from At3g26430-expressing cells nor the equivalent fraction from an *E. coli* control strain (harboring a non-related plasmid) were able hydrolyze the ACh analog acetylthiocholine (ATCh, Fig. 4). Similarly, the At3g26430 protein in the soluble fraction was not able to hydrolyze the bulkier substrate butyrylthiocholine (BtCh, data not shown). However, rapid hydrolysis of ATCh was observed when transgenic plant-derived human butyrylcholinesterase (Geyer et al. 2010) was added to the soluble fractions, precluding the possibility of the presence of significant interfering activity (e.g. anticholinesterase inhibitors) in these extracts.

Proteins of eukaryotic origin, especially those targeted to the secretory pathway, can sometimes incorrectly fold when expressed in bacteria, even when directed to the periplasm as is the case here (Sahdev et al. 2008). To overcome this potential limitation, we chose to over-express the protein in a homologous expression system—i.e. in transgenic *A. thaliana*. Three independent transgenic lines were obtained by selection with BASTA and confirmed by genomic PCR. Over-expression was verified by semi-quantitative RT-PCR, and the

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analysis suggested about five to eightfold increase in transcript accumulation in transgenic line 1 and even bigger increases in lines 2 and 3 as compared to untransformed wild type (WT) plants.

Soluble proteins were separated by centrifugation of plant homogenates and ChE activity was tested. Extremely low rates of similar magnitude of ATCh or PTCh hydrolysis were supported by both WT and transgenic plant homogenates (Fig. 5). When whole mount WT, At3g26430 over-expressing or human-AChE expressing (Muralidharan, Soreq and Mor, unpublished) seedlings are stained for ChE activities, only the human-AChE transgenic plants show specific staining (Fig. 6). Our results indicate that even when expressed in a homologous expression system, the protein product of At3g26430 was devoid of ChE activity.

At3g26430 and the GDS(L) lipase family within the SGNH hydrolases clan

All known ChEs belong to the α/β fold protein family, to which many other serine hydrolases belong. These hydrolases are characterized by a catalytic triad consisting of serine (within an invariant GXSXL context), glutamate (or aspartate) and histidine residues located far apart within the primary structure of the protein. Alignment of the At3g26430 and the maize '*ache*' gene sequences against a compilation of ChE and other α/β fold proteins (the Esther database http://bioweb.ensam.inra.fr/ESTHER/general?what=index) yielded no significant homologies. The annotation of the gene in the various databases pointed to a different direction. Genbank referred to At3g26430 as a "GDSL-motif lipase/ hydrolase family protein" and identified its central region as an "SGNH_plant_lipase_like" domain. In fact, of the 22 accessions belonging to subcluster A1, twenty, including the product of the putative maize *ache* gene, fell under this latter category and one under "SGNH hydrolase" (one accession lacked designation). To firmly establish this annotation, we compared the sequences of At3g26430 and the putative maize *ache* gene with representative members of the GDS(L) lipase family within the SGNH superfamily (Fig. 7). The alignment revealed excellent conservation of the signature "blocks" centering around the name-sake residues (Ser, Gly, Asn and His), as well as the catalytic triad residues (Ser, Asp, and His) positioned within the primary sequence according to the GDS(L) family consensus (that is very different from that of the α/β fold family, Fig. 7) .

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At3g26430's lipase activity

After we identified GDS(L) lipase motifs within the sequence of At3g26430, we next tested for lipase activity. *E. coli*-derived At3g26430 protein hydrolyzed known lipase substrates with preference toward longer chain substrates. Thus, the affinity of At3g26430 toward substrates increased with substrates' chain size: the K_M for *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB) and *p*-nitrophenyl palmitate (PNPP) were, respectively, 4.6 mM, 2.0 mM and 1.2 mM (Fig 8). Moreover, the hydrolysis was not inhibited by neostigmine bromide (NB), a ChE-specific carbamate inhibitor, but was negatively affected by phenylmethylsulfonyl fluoride (PMSF) a general serine hydrolase inhibitor (Fig. 8). Similarly to the bacterial-produced enzyme, plant-derived At3g26430 exhibited lipase activity with the same substrate preference (PNPA < PNPB < PNPP) confirming lipase activity (Fig. 5).

Discussion

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In this work we have identified an Arabidopsis ortholog of the maize gene encoding for hypothetical protein LOC606473 (also called '*ache*', NP_001105800), expressed it ectopically in bacteria and in *A. thaliana* plants, and characterized its enzymatic activity. Based on our results and on thorough genomic consideration also presented here, we

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conclude that the gene, At3g26430, encodes an enzyme belonging to the GDS(L) lipase family, which in turn belongs to the SGNH hydrolase superfamily.

Similar to other bona fide lipases, the At3g26430 enzyme shows preference to long carbon chain substrates and is not reactive toward acetylthiocholine, propionylthiocholine or butyrylthiocholine, typical substrates of metazoans' ChEs. The enzyme is susceptible to inhibition by serine-hydrolase inhibitors like PMSF, but is not affected by high concentrations of the carbamate NB, a potent ChE inhibitor that inhibits all known ChEs. Although the work presented here conclusively shows that At3g26430 is not a cholinesterase, it does not preclude the possibility that other members of its cluster of homology may exhibit a more promiscuous substrate selectivity, and may potentially be able to hydrolyze choline esters. Indeed, Momonoki and co-workers have shown that an enriched protein preparation from maize seedlings containing the protein product of LOC606473, the probable maize ortholog of At3g26430, had very weak cholinesterase activity (Sagane et al. 2005). The activity was 4,318 fold less reactive toward ATCh then eel AChE and was 2,903 fold less sensitive to neostigmine. As compared to previously characterized plant ChE activities (Muralidharan et al. 2005; Riov and Jaffe 1973), the enzyme encoded by LOC606473, dubbed ‘ache’ by Sagane et al. (2005), can be characterized as a serine hydrolase with marginal reactivity toward choline-esters. Because it is yet to be demonstrated that over-expression of this maize gene would result in ectopic accumulation of a choline-ester hydrolyzing enzyme, alternative explanations to results of Sagane et al. (2005) cannot be ruled out. It is important to stress that our previous work, as well as the work of others, clearly indicated that bona fide cholinesterase activity can be demonstrated in many plant species. The identity of the protein(s) and the genes that encode them is yet to be determined.

The SGNH hydrolase superfamily (CL0264, http://pfam.sanger.ac.uk/clan/SGNH_hydrolase) consists of members displaying “a diversity of hydrolytic enzyme activities” that fall into one of three families: (1) *DUF459* (PF04311, all known members are eubacterial proteins of unknown function), (2) *Hema_esterase* (PF03996, all members come from ssRNA(−) viruses and typified by the influenza hemagglutinin esterase), and (3) *Lipase_GDSL* (PF00657). The GDS(L) family with members belonging to all domains of life, is the largest in the SGNH clan (5119 of 5517 members) and both names are often used interchangeably to describe both family and clan (Akoh et al. 2004; Lo et al. 2003; Upton and Buckley 1995). It is interesting to note that there are more plant accessions than accessions from all other eukaryotic taxa combined. For example, there are 117 genes identified as encoding for GDS(L) lipases in *A. thaliana* as compared to only 14 in humans and 2 in fruit flies.

Yet very little is known about the function(s) and physiological role(s) of any plant GDS(L) lipases, despite the prominent presence of the family in plant genomes. Nonetheless, already several leitmotifs can be recognized in the emerging picture arising from the few studies conducted on plant GDS(L) lipases. Some of the proteins were shown to be involved in pathogen-related responses. For example the *A. thaliana* protein GLIP1, in concert with ethylene-signaling, was shown to elicit local and systemic resistance to several pathogenic bacteria and fungi (Oh et al. 2005; Kwon et al. 2009) and two closely related proteins from *Capsicum annuum*, CaGL1 and CaGLIP1 were shown to have roles in both biotic and abiotic stress responses (Hong et al. 2008; Kim et al. 2008). Another leitmotif is the involvement of other plant GDS(L) lipases in developmental processes, especially connected to sexual reproduction (Takahashi et al. 2010; Kram et al. 2008), embryogenesis and seed development (Kondou et al. 2008), and germination and young seedling development (Naranjo et al. 2006; Katavic et al. 2006; Clauss et al. 2008).

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Supplementary Material

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Acknowledgments

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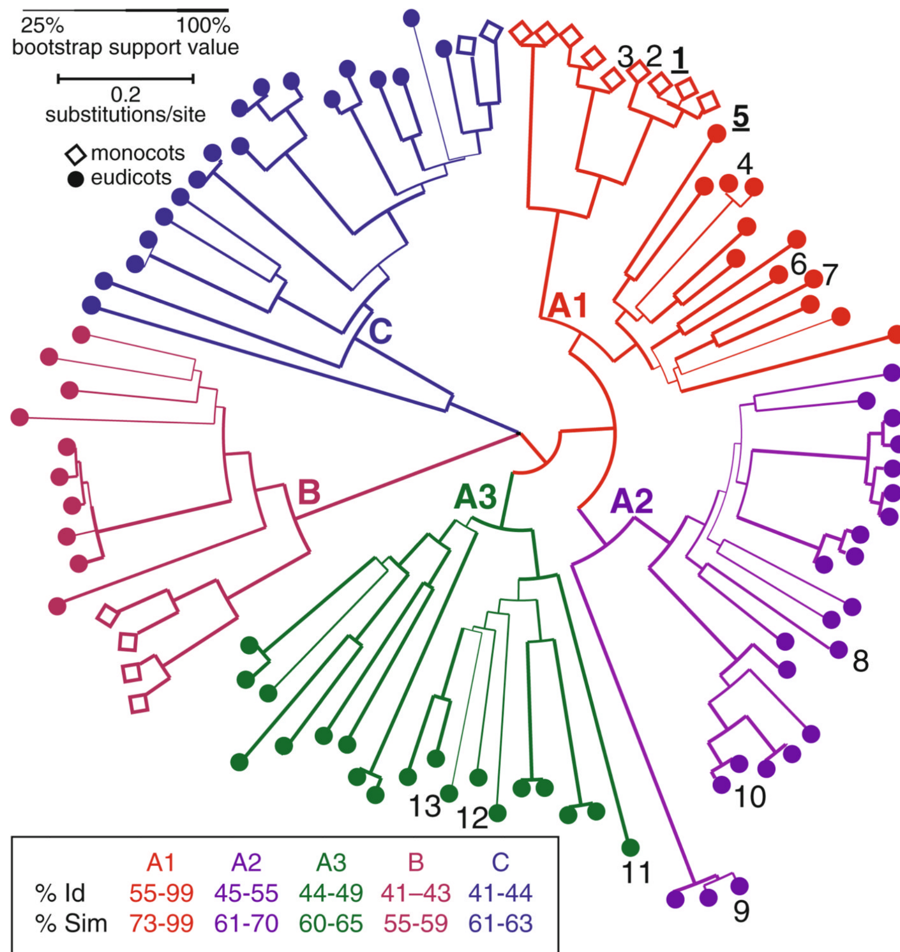

Fig. 1.

Phylogenetic analysis of homologs of the maize gene encoding for hypothetical protein LOC606473 ('ache'). Analysis of the first 98 hits of a *blastp* search was performed on the Phylogeny.fr platform by aligning the sequences with MUSCLE (v3.7), reconstructing the phylogenetic tree with the BioNJ program, and drawing the circular phylogram with TreeDyn (v198.3) as detailed in the "Materials and Methods" section. Five clusters of orthologs can be distinguished with the first three more closely grouped: *A1* (*red*), *A2* (*purple*), *A3* (*green*), *B* (*magenta*), and *C* (*blue*). All three major clusters included monocot (diamonds) and eudicot (circles) members. Numbers refer to the following accessions: *1*, *Z. mays* (maize, NP_001105800); *2*, *S. bicolor* (sorghum, XP_002463099); *3*, *O. sativa* (rice, NP_001060129); *4*, *V. vinifera* (grape, XP_002282372); *5*, *A. thaliana* (NP_189274); *6*, *P. trichocarpa* (poplar, XP_002314590); *7*, *R.* communis (castor bean, XP_002530043); *8*, *H.* brasiliensis (Para rubber, Q7Y1X1); *9*, *D.* carota (carrot, BAF80349); *10*, *M. sativa* (alfalfa,AAB41547); *11*, *G. max* (soybean, ACU20252); *12*, *S.* europaea (BAI23204); *13*, *Macroptilium atropurpureum* (siratro, BAG09557). *Insert*: Homology of accessions constituting the five clusters to the maize 'ache' gene. Ranges of percent identity (I) and similarity (S) as determined by *blastp* are shown

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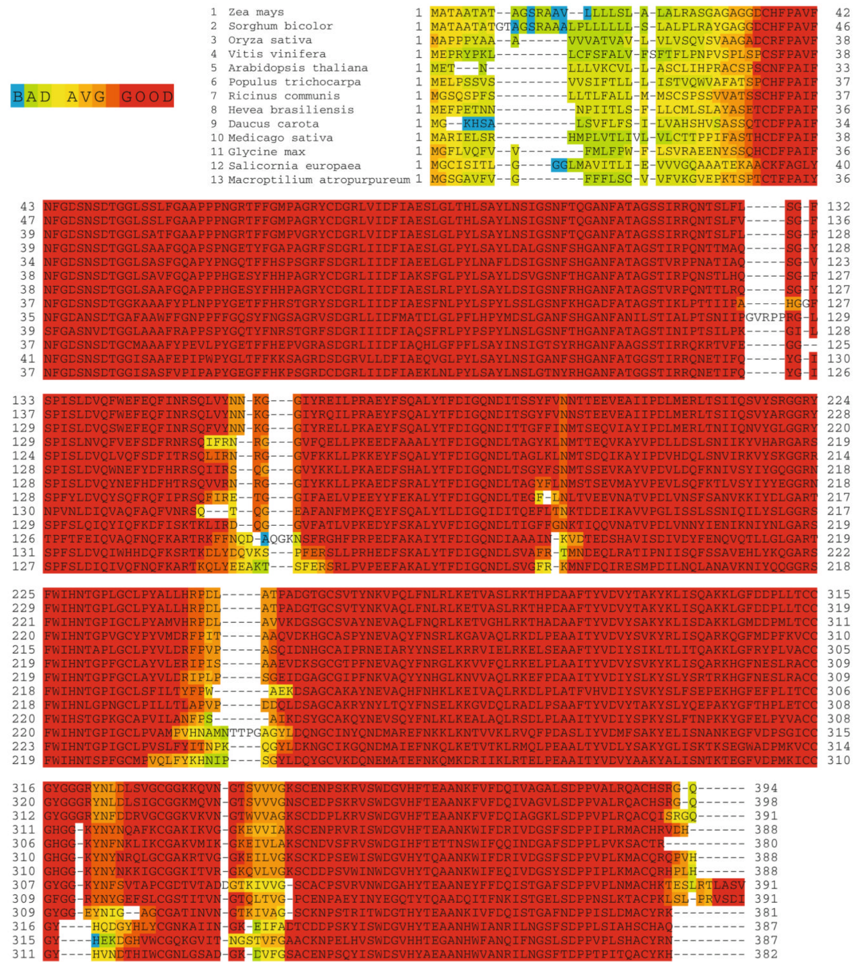

Fig. 2. Accessions belonging to clusters A1-A3 are highly homologous to each other. The thirteen accessions indicated in Fig. 1 were aligned using the T-Coffee program, and the degree of similarity is shown as a heat diagram superimposed on the sequences

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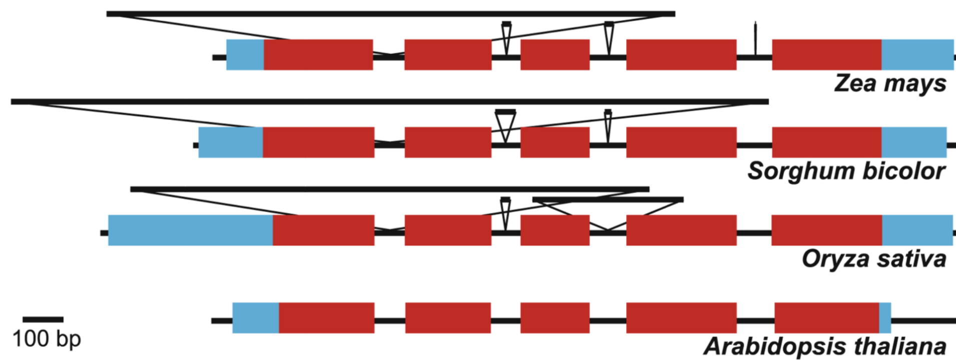

Fig. 3.

Comparison of the intron–exon architecture of orthologs of the maize 'ache' gene, Intron, exon, 5′-UTR and 3′-UTR lengths were deduced by comparing genomic sequences to those of the mRNA sequences. Four cluster A sequences from maize, sorghum, rice and *A. thaliana* are shown. See "Materials and methods" for the accession numbers of these and additional sequences whose gene structure was analyzed. UTRs—*blue*, exons—*red*, introns —*thin black lines*

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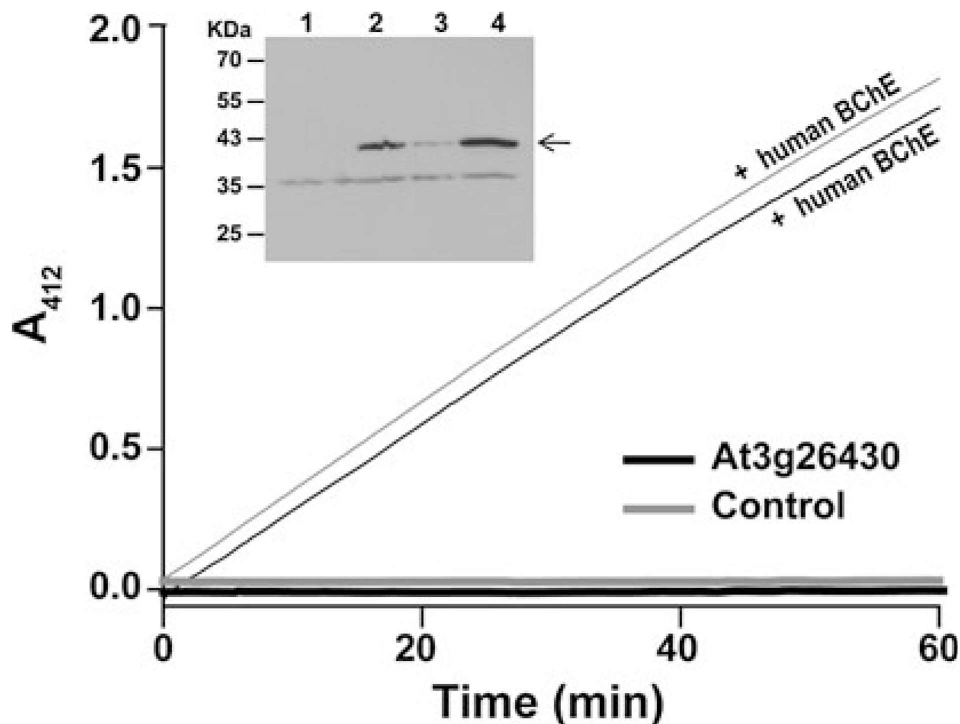

Fig. 4.

E. coli –expressed At3g26430 protein cannot hydrolyze ACh. Expression of At3g26430 was induced by 0.3 mM IPTG for 2 h. Cells were homogenized by sonication and proteins were separated by centrifugation into soluble and insoluble fractions. Control *E. coli* cells harboring a non-related plasmid were similarly treated. The potential of protein(s) in the soluble fractions obtained from At3g26430- and control cell extracts to hydrolyze AtCh was assayed (see “Materials and Methods”). As a positive control, the soluble fractions were “spiked” with purified plant-derived human BChE and AtCh hydrolyzing activity similarly assayed. *Insert*: Proteins were extracted from At3g26430-*E. coli* cells prior to induction (*lane 1*), or following induction (*lane 2*), and then separated into soluble (*lane 3*) or insoluble (*lane 4*) fractions, resolved by SDS-PAGE and subjected to immunoblot analysis using anti-His tag Abs. The *arrow* points to the band corresponding to the At3g26430 protein

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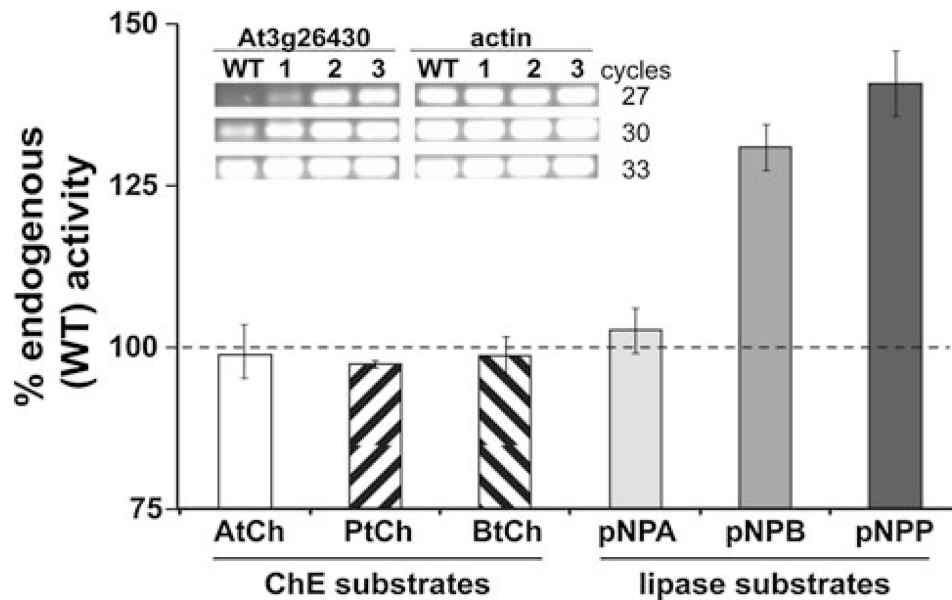

Fig. 5.

Esterase and lipase activities of At3g26430 over-expressed in *A. thaliana*. Over expression of At3g26430 in transgenic *A. thaliana* plants as compared to WT plants was determined using semi quantitative PCR with actin serving as a housekeeping gene control (*Insert*). ChE, general esterase and lipase activities were assayed and results are expressed as percent of the activity levels in WT plants (*dashed horizontal line*). Results reflect at least three repeats (mean ± SEM)

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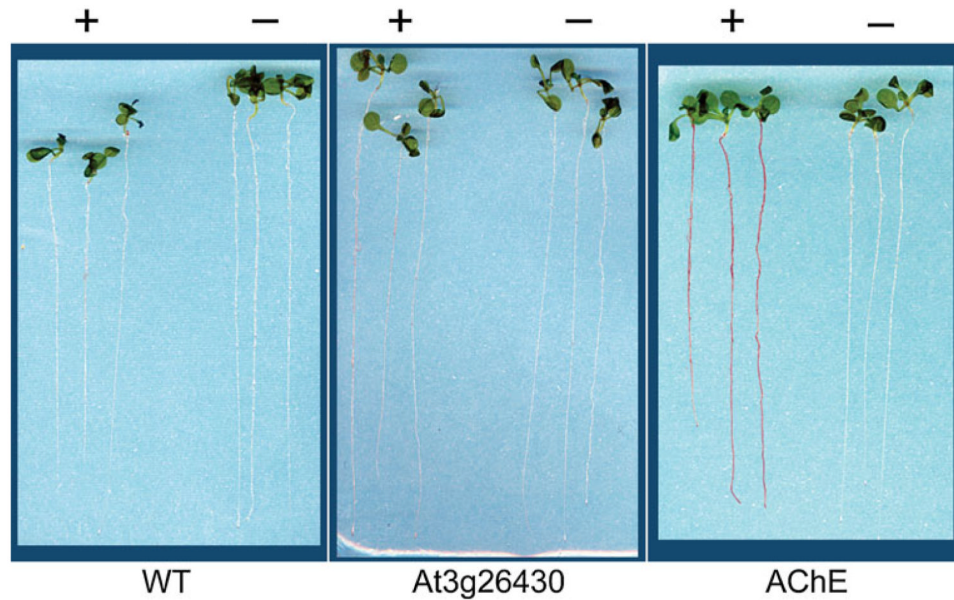

Fig. 6.

Histochemical staining to detect cholinesterase activity. Tissue distribution of ChE activity was determined by the Karnovsky and Roots in situ activity staining procedure. 9-day old seedlings of WT plants, and plants that overexpress At3g26430 or human AChE (Muralidharan and Mor, unpublished and Mor and Soreq 2004) were incubated in solutions containing the substrate ATCh (+) or solutions lacking the substrate (–). Rust-colored cupric ferrocyanide precipitate is deposited following the reduction of ferricyanide by the ChE-generated thiocholine. Only plants that express the bona fide ChE, human AChE, showed significant staining in the presence of the substrate, whereas only very slight staining was visible in WT and At3g26430 plants

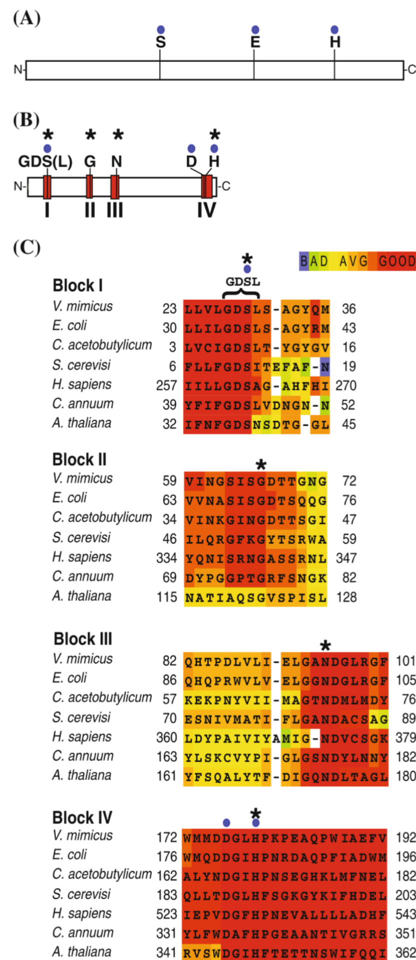

Fig. 7.

At3g26430 is likely a GDS(L) lipases belonging to the SGNH hydrolase clan. True cholinesterases (**a**, represented by human AChE) bear no homology to the At3g26430 protein (**b**, drawn to scale with **a**) with which they share only minimal superficial similarity protein in the form of analogous catalytic triads (*blue dots*). In contrast the At3g26430 protein is highly homologous to the members of the SGNH hydrolase clan and specifically to members of the GDS(L) lipase family (**b**, namesake residues are marked by *asterisks*). Particularly well-conserved are the four SGNH blocks that characterize the clan, which is represent here by several esterases belonging to the "seed" set of sequences for the GDS(L) lipase family (**c**, Pfam PF00657). The degree of sequence homology in these conserved blocks is shown as a heat diagram superimposed on the sequences. The UniProt accession numbers of the sequences compared to At3g26430 (*A. thaliana*) are: Q07792 (*Vibrio mimicus*); P0ADA1 (*Escherichia coli*), Q97DM5 (*Clostridium acetobutylicum*); P41734 (*Saccharomyces cerevisiae*); P28039 (*Homo sapiens*); Q08ET5 (*Capsicum annuum*). The latter is not on the Pfam seed list but was recently shown to be a lipase (Hong et al. 2008)

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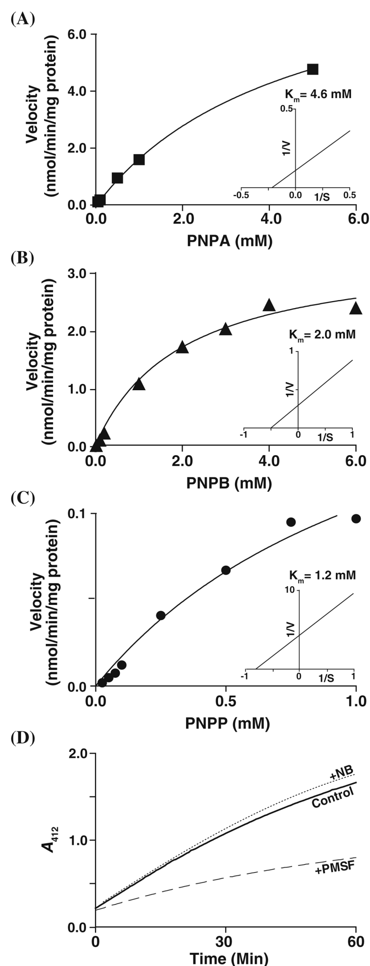

Fig. 8.

Enzyme kinetics of At3g26430's serine hydrolase activity. At3g26430 (overexpressed in *E. coli*) was assayed for its ability to hydrolyze the lipase substrates *p*-nitrophenyl acetate (PNPA) (**a**), *p*-nitrophenyl butyrate (PNPB) (**b**) and *p*-nitrophenyl palmitate (PNPP). Reaction velocities were measured as a function of substrate concentration and the Michaelis constant (K_M) was calculated by non-linear regression using the Prism software. *Inserts*: Lineweaver–Burk analysis. PNPA hydrolysis was greatly inhibited. (**d**) PNPA hydrolysis was inhibited by PMSF (*broken line*), but not by NB (*dotted line*)

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Table 1

Primers used in this study

Primer	Sequence	Purpose
1F	ATACATGTATGGAAACTAATCTCTTGTTGGTAAAGTG	Insert Pci I site
1R	ATGGTACCCTATCTGGTGCAGGCAGAC	Insert Kpn I site
2R	CTCGAGTCTGGTGCAGGCAGACTTC	Insert Xho I site
3F	CACGATCACAACTCATTCGCAA	At3g26430 semiquantitative PCR
3R	CGTTCTGACCGATGTCGAATGT	
4F	CCAGTGGTCGTACAACCGGTAT	Actin semiquantitative PCR
4R	AGACGGAGGATAGCATGTGGAA	

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Table 2

Taxonomy of 98 first hits of *blastp* search of homologs to the maize gene encoding for hypothetical protein LOC606473 (NP_001105800)

Class	Family	Genus	Species	Common name
Liliopsida (monocotyledons)	Poaceae (grass family)	*Zea*	*mays*	Maize
		Sorghum	*bicolor*	Sorghum
		Oryza	*sativa*	Rice
(eudicotyledons)	Vitaceae (grape family)	*Vitis*	*vinifera*	Wine grape
	Salicaceae (willow family)	*Populus*	*trichocarpa*	*Western balsam poplar*
	Euphorbiaceae (spurge family)	*Ricinus*	*communis*	Castor bean
		Hevea	*brasiliensis*	Para rubber tree
	Fabaceae(pea family)	*Medicago*	*truncatula*	Barrel medic
			sativa	Alfalfa
		Macroptilium	*atropurpureum*	
		Glycine	*max*	Soybean
	Brassicaceae (mustard family)	*Arabidops*	*thaliana*	Mouse-ear cress
	Amaranthaceae (amaranth family)	*Salicornia*	*europaea*	Common glasswort
	Apiaceae (carrot family)	*Daucus*	*carota*	Carrot
	Plantaginaceae (speedwell family)	*Digitalis*	*lanata*	Wooly foxglove
			grandiflora	Yellow foxglove
			subalpine	
	9 families	14 genera	17 species	