

Cloning and characterization of a cDNA encoding the endo-1,4- β -D-glucanase gene expressed in rapidly growing tuber and leaf of taro (*Colocasia esculenta* var. *esculenta*)

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ABSTRACT. A cDNA for endo- β -1,4-glucanase (*EGase*) was isolated by RT-PCR, and rapid amplification of cDNA ends reaction from taro leaves (*Colocasia esculenta* var. *esculenta*). This single copy gene has 2,185 bps and an open reading frame (ORF) of 1,854 bps. A polypeptide of 618 residues was deduced from the ORF, with a calculated molecular mass of 68,434 Da and theoretical *pI* of 8.8. The remarked activities of the *EGase* on CMC plate revealed this enzyme to be involved in cellulose metabolism in taro. Taro *EGase* was identified as a kind of membrane-anchored *EGase*. Phylogenetic analysis showed it is a member of the γ subfamily and an ortholog of the *Arabidopsis KORRIGAN* gene (*KOR*), proposed to be involved in cytokinesis, pectin metabolism in the primary cell wall, and cell elongation. Transcripts of taro *EGase* are highly accumulated in 300 g tubers, in the upper part of 1,000 g tubers, and in rolled leaves undergoing rapid growth, indicating that taro *EGase* is involved in the regulation of plant growth. The expression pattern of soluble starch synthase III was similar to that of *EGase* during rapid growth of taro plant tubers. These results suggested that taro *EGase*, an ortholog of *KOR* in *Arabidopsis*, may play an important role in the rapid growth in taro.

Keywords: Cell wall assembly and taro (*Colocasia esculenta* var. *esculenta*); Endo-1,4- β -glucanases; *KORRIGAN*; Rapid elongation.

Abbreviations: **CEL**, cellulase; **CMC**, carboxymethylcellulose; **EGase**, Endo-1,4- β -glucanases; **KOR**, *KORRIGAN*; **ORF**, open reading frame.

INTRODUCTION

Endo-1,4- β -D-glucanases (*EGases*, EC 3.2.1.4) are the enzymes produced in insect, nematodes, crayfish, bacteria, fungi, and plants that hydrolyze polysaccharide containing a 1,4- β -D-glucan backbone (Henrissat et al., 1989; Beguin, 1990; Watanabe et al., 1998; Byrne et al., 1999; Rosso et al., 1999). It also was grouped as cellulase which plays an essential role in the mechanism of cellulose degradation and shows potentiality to modify cellulose-containing materials (Bisaria and Mishra, 1989; Beguin, 1990; Beguin and Aubert, 1994).

Sequence analysis demonstrated that all cloned *EGases*

can be classified into twelve families (Knowles et al., 1987; Henrissat and Bairoch, 1996). All microbial *EGases* are found in all the twelve families. They can degrade crystalline cellulose, and were intensively studied and used in industrial processes. On the other hand, plant *EGases* form a separated subclass, family 9, lack a cellulose-binding domain, and are unable to hydrolyze crystalline cellulose (Beguin, 1990; Levy et al., 2002). Furthermore, higher plants often contain multiple *EGases*. In tomato, for example, seven *EGase* cDNAs with different expression patterns have been characterized (Lashbrook et al., 1994; Del Campillo and Bennett, 1996; Brummell et al., 1997a; Brummell et al., 1997b; Catala et al., 1997). The *Arabidopsis EGase* family includes 25 members (del Campillo, 1999; Levy et al., 2002). It is still not known to what extent different family members vary in their *in vivo* functions.

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The expressions of most plant EGases are tightly regulated, suggesting that they play some critical roles in plant development. Some EGases, i.e. avocado *CELL*, pepper *CX1* and tomato *Cel2*, are expressed specifically during fruit ripening (Christoffersen et al., 1984; Lashbrook et al., 1994; Ferrarese et al., 1995). The EGases, i.e. bean *BAC1*, soybean *SAC1* and elder *JET1*, are found to be associated with abscission (Tucker et al., 1988; Kemmerer and Tucker, 1994; Taylor et al., 1994). Some EGases are proposed to be involved in cell elongation and growth rate, like *Arabidopsis Cell*, which is highly expressed in the elongating stem and root, and tomato *Cel4*, which is highly expressed in the growing etiolated hypocotyls, pistils, and expanding leaves (Milligan and Gasser, 1995; Brummell et al., 1997a; Shani et al., 1997).

Another group of membrane-anchor EGases, located at the plasma membrane, is also required for normal cellulose formation (Brummell et al., 1997a; Nicol et al., 1998; Del Campillo, 1999; Molhoj et al., 2001a). This group includes the *Arabidopsis KOR* gene, which was identified by the dwarf mutant KORRIGAN and found to be involved in cytokinesis and cell elongation (Nicol et al., 1998; Lane et al., 2001). Moreover, KOR has been postulated to trim sterol residues from nascent glucan or out-of-register glucan as a primer for the elongation of microfibril (Peng et al., 2002). However, the KOR protein could not be associated with cellulose synthase complexes during microfibril elongation (Zuo et al., 2000; Szyjanowicz et al., 2004), and *kor* mutants contain normal amounts of sterol glycoside (Robert et al., 2004). This suggested that the action of KOR might be indirect during microfibril elongation, and the role of KOR requires more experimental evidence to be defined.

Although plant EGases were reported to be related to ripening, abscission, and cell wall metabolism, the detailed molecular mechanisms of these enzymes are still unclear. Transformation of *Arabidopsis Cell* conferred on plants an altered structure or morphology, i.e. improved growth rate, a greater biomass, or modified fibers (Shoseyov et al., 2001). Cloning of EGases will not only be critical then for studying these subjects, it is also likely to be useful in the molecular breeding of crops. In our previous work, a partial EGase cDNA of 1.8 kb was obtained unpurposefully from taro (*Colocasia esculenta* var. *esculenta*) during the gene cloning process for soluble starch synthase. To further understand its functions, the full-length cDNA was isolated, characterized, and expressed, and the temporal expression profiles in the tuber and leaf tissues were also described.

MATERIALS AND METHODS

Plant material

Taro (*Colocasia esculenta* var. *esculenta*) tubers were harvested during developmental stages based on their fresh weight (i.e. 100 g, 300 g, 600 g, and 1000 g) and leaf growth (i.e., young [rolled], mature, and senescent). The

collected leaves and tubers were frozen immediately in liquid nitrogen, lyophilized, and then stored at -20°C until required.

RNA isolation

RNA was prepared by the method described previously (Lin and Jeang, 2005). The quality and quantity of RNA were determined by spectrophotometry and agarose gel electrophoresis (Sambrook and Russell, 2001). Finally, RNA was stored at -70°C until required. The mRNA for gene cloning was purified with Oligotex™ (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Taro EGase cDNA cloning

A schematic representation of our cloning strategy is shown in Figure 1A. The full-length cDNA of taro EGase was cloned by combining RT-PCR and 5'-RACE. 3'-RACE using the primers P1 (GCCGTTGGACTTATCGTGCC) and P2 (GACTCGAGTCGACA TCG) using the method described by Frohman et al. (1988). The template used in 5'-RACE was prepared with oligo-dT primer by a SMART™ RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA) following the

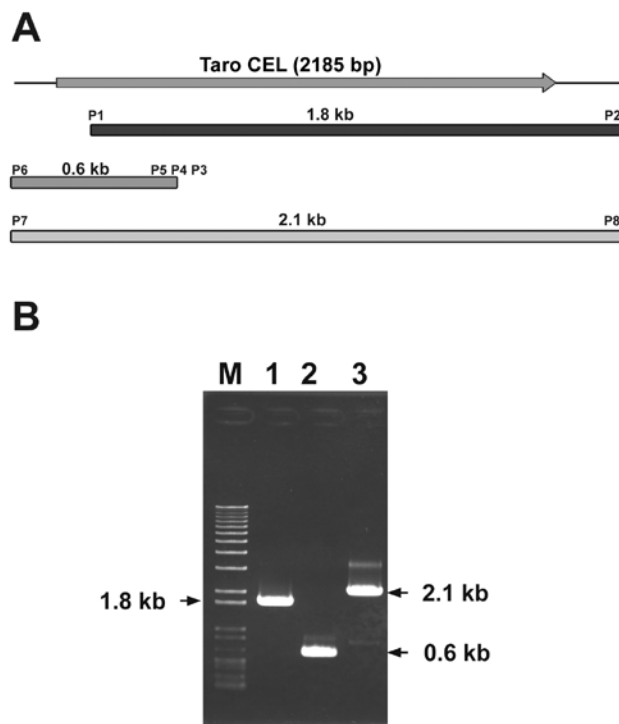


Figure 1. (A) Strategy for taro EGase cDNA cloning. The topmost diagram shows the full length cDNA. The boxes represent cDNAs amplified by RT-PCR and RACE; (B) Agarose gel electrophoresis of RACE and RT-PCR products of EGase. The partial EGase cDNAs were amplified by RT-PCR using P1 and P2 primers (lane 1); The product of 5' SMART- RACE was amplified using P3, P4, P5 and P6 primers (lane 2); The full-length of EGase cDNA was generated by RT-PCR using P7 and P8 primers (lane 3); Lane M: 100 bp ladder (MBI).

manufacturer's procedure. Then it was subjected to PCR with primers P4 (CCCCTCCAGGAGACGTTGTTG T), P5 (CCCAGA GCGCTGGGCGTTGAA), and P6 (AAGCAGTGGTATCAACGCAGAGTA). The final full length cDNA of taro *EGase* was amplified with the primers P7 (GAGGAGGAGCTGGTTGAAGGT) and P8 (CCAGCCTTTGGAATTGGGAACA) using Platinum *Pfx* DNA polymerase (Invitrogen) following the manufacturer's protocol. All PCR products were sequenced after cloning into pGEM-T-Easy vector (Promega, Madison, WI), and pGEGa, pGEG5', and pGEGFL were generated, respectively, in this cloning process. The sequences of taro *EGase* have been deposited in GenBank under Accession No. EF552587.

Expression, production, and purification of recombinant EGase in *E. coli*

The 1.8 kb fragment, encoding the entire coding region of taro *Egase*, was PCR amplified from pGEGFL using primers sets P9 (ATGTTTCGGGCACAACCCCT)-P10 (ATCGGAATTCGGAGGTTTCCAAGCTGCTGGG GGT), with the blunt end / *EcoRI* site introduced. The product was then cloned into pET29a vector (Novagen, San Diego, CA) to generate pETEG. The inserted gene was driven by T7lac promoter, and fused with a His-tag at its 3' end. The entire taro EGase was produced by 2 mM isopropyl- β -D-thiogalactoside (IPTG) induction from *E. coli* BL21 (DE3) harbored with pETEG. The cultured cells expressing pETEG were induced by 2 mM IPTG at 37°C for 5 h before being harvested, sonicated, and centrifuged to remove cell debris and yield a crude enzyme extract. The recombinant EGase protein was further purified by Ni-NTA column (Qiagen) according to the manufacturer's method for the determination of cellulase activities.

Zymogram assay

Zymogram assay for the recombinant EGase was performed by the method of Loopstra et al., 1998. The recombinant EGase was loaded on a 1% agarose plate containing 0.2% carboxymethylcellulose (CMC, Sigma) and stained by Congo red (1 mg/ mL) after incubation at 37°C for 72 h. The protein concentration was determined by the Lowry method (Lowry et al., 1951).

Southern blot analysis

Genomic DNA was prepared by the CTAB based method (Murray and Thompson, 1980). Taro genomic DNAs (10 μ g), digested completely with restriction endonucleases, were electrophoresized on 1.0% agarose gel and blotted onto Hybond-N⁺ membranes (Amersham Biosciences). Nucleic acid hybridization was conducted by the method described previously (Wang et al., 1994). The 1,010 bp fragment of taro *Egase* (1,009-2,018 bps) labeled with α -³²P- dATP using the Prime-It II random prime labeling system (Stratagene, La Jolla, CA) was denatured and applied to the hybridization reaction. After stringent washing, the membranes were covered with polyethylene

wrap and imaged by a Typhoon 9200 Imager (Amersham Biosciences).

RESULTS

Cloning and expression of taro *EGase*

A partial *EGase* cDNA of 1.8 kb (Figure 1B) was unpurposely obtained during the gene cloning process for soluble starch synthase. In order to obtain the full-length cDNA, a 5'-terminal cDNA of 0.6 kb was generated by the primers, designed according to the cDNA sequence of the 1.8 kb fragment by SMART-RACE (Figure 1B). Finally, a near full-length *EGase* cDNA (2.2 kb) was generated by the primers, designed according to the RT-PCR and RACE products, with *Pfx* DNA polymerase, which possesses a proofreading function (Figure 1B). All products of RT-PCR and RACE were cloned into pGEM-T Easy vector to generate pGEGa, pGEG5', and pGEGFL. The sequences of all products were also confirmed by sequencing and application of the "BlastX" program. Sequence analysis demonstrated that the related overlap regions of these products matched perfectly, indicating that these products arose from the same transcript.

Direct evidence that the clone *EGase* encoded one β -1, 4-glucanase was obtained by expressing the gene in *E. coli* BL21. After Congo red staining, the activities of β -1, 4-glucanase were detected on a CMC plate, where the clear halo could be observed around the loading site of the purified recombinant EGase (Figure 2). This indicated that the taro EGase is an active one and can participate in

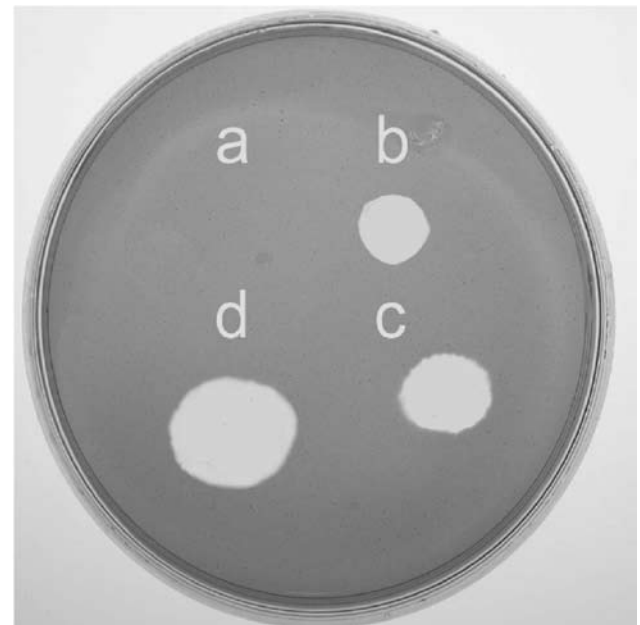


Figure 2. Different amounts of Ni-NTA purified protein were applied onto 1% agarose plates containing 0.2% CMC and stained with Congo red. Spot (a) represents 200 μ g of purified enzyme from the cell harbored with pET29a, and spots (b) to (c) represent 50 μ g, 100 μ g and 200 μ g of purified enzyme from the cell harbored with pETEG.

cellulose metabolism in taro. Similar results have been observed in tomato's *Cel3*, which encodes an EGase (Brummell et al., 1997b).

The yield of recombinant protein was too low to allow further determination of its biochemical properties. This low yield might be caused by the codon usage or secondary structure of mRNA (Lee et al., 1987; Sorensen et al., 1989). For our purpose, we subcloned the ORF of EGase into pGEX-4T-1 expression vector (Amersham Biosciences) and expressed in *E. coli* Rosetta (Novagen) to improve the yield. More recombinant protein was observed than in the expression system, *E. coli* BL21/pETEG (data not shown). The GST-EGase fusion protein will be produced and purified, and then a detailed characterization of the expressed proteins can be described.

Characterization of taro EGase cDNA

The taro *EGase* cDNA is 2,185 bps in length and contains a coding region of 1,854 bps flanked by the 5'- and 3'- untranslated regions of 153 and 177 bp, respectively (Figure 3). The open reading frame, beginning with an ATG codon at position 154-157 and ending with the TGA codon at position 2,008-2,010, encodes 618 residues protein with a theoretical molecular mass of 68,434 Da and pI of 8.8.

The critical motifs of the taro EGase were analyzed by the programs "InterPro Scan" and "ScanProsite" on the ExPASy website (Figure 3). The results displayed several unique features of this protein. A "transmembrane region" signature was present between amino acids 75 to 95. This motif has often been found in other membrane-anchored EGases (Brummell et al., 1997b). This suggests that EGase is a membrane-anchored enzyme involved in cellulose biosynthesis during plant cell wall assembly (Libertini et al., 2004). The signatures of "Glycosyl hydrolase family 9" (Pfam Acc. No. PF00759) and "GLYCOSYL_HYDROL_F9_2" were found between amino acids 111 to 586 and 559 to 577, respectively (Figure 3), indicating that the taro EGase is a member of the glycoside hydrolase family 9, which is reported to possess the activity of endoglucanase or cellobiohydrolase (Henrissat and Bairoch, 1993, 1996). A "six-hairpin glycosidase" signature (Pfam Acc. No. SSF48208), located between amino acids 108 to 591, was proposed to be the catalytic domain of EGase (Itoh et al., 2006). In addition, the EGase also harbored 8 putative N-glycosylation sites, located at Asn 136, 152, 218, 324, 345, 408, 425 and 567, and a Pro-rich region located in the C-terminal region.

The taro EGase displays an identity of 76, 78, 78, 78, 79 and 82% and a similarity of 86, 87, 88, 88, 90 and 91% with the membrane-anchored EGase from bread wheat, tomato, pear, barley, aspen tree, and cotton, respectively. This suggests that the EGase is highly conserved in plant species and has an important function in the growth and development of plants.

To date, 25 *Arabidopsis* EGases have been published

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1  ACGCGGGGGGAGGAGGAGGAGGAGGAGCTGGTTGAGGTGTGCGTGGGAAGCGGGCGGG 60
61  CGGGTCTCCCCCTCCACACCCCTTCTCTCTTTTCTTAATGGCACTGTCATCACTGAC 120
121 AGCTGCAGCTCGAGTCTCCACTCAGCGGGAGGATGTTGGGACAAACCCCTGGGGGGT 180
      M F G H N P W G G
181 CCCTGGAGATCTCCAATGCGGACTCCGCCACCGATGACGACCGGAGCGGAACTCCAG 240
P L E I S N A D S A T D D D R S R N L Q
241 GACCTCGACCGGGGGGACCTGTCGCCGACGTGGACGAGACCCAGCAGAGCTGGCTCTG 300
D L D R A A L S R Q L D E T Q Q S W L L
301 GCGGGCCGGGACCGAGGGAAGAAGAAGCCGTACGTGCACCTGGCTGCATGGT 360
A G P G D Q G K K K R Y V T D L G C M V
361 GTCAGCGCAAGCACTTCTGTGGAGCGGTGGGTCGCTGTCGCGCTGGCTCTCTGTG 420
V S R K H F L W T V G C S V L A V G L L V
421 GGCCTATCATGCTCATCAAGACGGTGGCCGACCAACCCGTCCCGCCGCGCCCGC 480
G L I M L I I K T V P H H R P P P P P
481 GACAGCTACACCCAGGCGCTTCAAGGCCCTCAATGTTTCAACCGCCAGGCGCTTGG 540
D S Y T O A L H K A L M F F N A O R S G
541 CCAATTCGGAGGCAACAACGCTCTCTGGAGGGGAATCCGGCATGAGAGGATGGATC 600
P I P R H N N V S W R G N S G M K D G I
601 TCCGATATGCTACGAAGAAGAATCGTGGGGGTTATTAGATGACGGGACCGCATC 660
S D N A T K K N L V G G Y Y D A G D A I
661 AAGTTCACTTCCCATGTCGTTGGCCATGACATGCTCAGCTGGAGGTGATCGAAGTAC 720
K F N F P M S F A M T M L S W S V I E Y
721 AGCGTAAGTATGAGCGCGGGGAGCTGCGACAGTCAAGGAGATCATCAAGTGGGG 780
S A K Y E A A G E L E H V K E I K W G
781 AGGATTAAGTCTCCGACCTTCAACAACCTTCCGACCACTCCGCGACCATCCGCTCTCT 840
T D Y F L K T F N N S A D T I S G L V
841 CAGTGTGGGTTGGTGCACCTCGAAGAAGGTAACCCCAACGACCACTACTGTGGATG 900
Q V G V G D T S K G V N P N D H Y C W M
901 CGCCCGGAGACATGGACTACGAGCGACCTGTGTACAGATGCCACATGTCGCGACTC 960
R P E D M D Y E R P V Y E C H S C S D L
961 GCTCAGAGATGGCCGCTGCGCTGGCTGCGCATCCATGCTTCAAGGACGCAAGACC 1020
A A E M A A A A L A A A S I V F K D S K T
1021 TACTCCGACAGCTCGCCATGGCCGAGGACCTTCAAGTATGCTAGGATAGCCAT 1080
Y S D K L A H G A R T L F F E Y A R D K I
1081 GGGCGGTACAGCGCGGCGGACCGACGATCCATCTTCAACTCTCCAGCTACTGG 1140
G R Y S A G G T D A S I F Y N S S Y W
1141 GATGAGTTCGCTGGGGCGGCGCTGGTGTACTATGCAACAGGAAATGTGACTAOCIT 1200
D E F V W G G A W L Y A T G N V T Y L
1201 CAGTTCGGGACGATCCCACTCTTCCCAAGCATGCGCGTCCCTTCTGGGGTGGCCCGAT 1260
Q L A T H P T L A K H A G G V W G G P D
1261 TATGTTGCTTCAGCTGGGATAGCAAGCTTCTGGGGCTCAAGTCTTCTCAGCAGGTG 1320
G F A S V F S D Y M E A S D T P G W Y C
1321 AGATTATCTCGAGTCCGGGTTATCCATATGAAGAATTCGACGACATCCACACCAA 1380
R L F L S P G Y P Y E E I L T T F H N Q
1381 ACAAGCAATGTCTGTGCTCTCACTTCCAAATGTCACATCTTAAACAGAAACAAGGA 1440
T S N V M C S Y L P M F T S F N R T K G
1441 GGTCTTATCAACTAAATCATGGAAGGCCAACCCCTCAATATGATGTCAGTACGACGA 1500
G L I Q L N H G R P O P L Q Y V V N A A
1501 TTCTTGGCTCACTTTCAGTGTATACATGGAACTTCAGACACTCCAGGCTGGTACTGT 1560
F L A S V F S D Y M E A S D T P G W Y C
1561 GGACCAATTTCTACTCTACTGAAGTCTACTGTAACITTCGACGACGACAGATGACTAC 1620
G P N F Y S T E V L R N F A R T Q I D Y
1621 ATTCGGGCAACAATCTCAGAAGATGAGCTATCTTGTGGCTATGGCAACGTTATCCA 1680
I L G N N P Q K M S Y L V G Y G K R Y P
1681 AAGCATGTTATCATCGTGGAGCATCAATACCAAGAATGGTGTCAAGTATGGTGGCAA 1740
K H V Y H R G A S I P K N G V K Y G C K
1741 GCAAGTGGAAATGAGGACACAACGAAACCAACCTAACACAATTTGTTGGGCAATG 1800
A G K L A D H K L P M F T N I R A M A
1801 ATTCGGGCTTCAAGCATGATGGTTTAAAGATTCGCTGACCAATTTAATTAATACCA 1860
I A G P D K H D G F E K D V R T N Y N Y T
1861 GAGCTCACTTCAGGGAATGCGGAACTTGGAACTTGGAGCACTGTTGTCACITTCAGGTGA 1920
E P T L L A G N A G L V A A L V S L S G V
1921 CAGCAGGATGACGAAGAACAACCATATTTCTGCGACTTCCACCGATGTTTCCACCATC 1980
Q A G I D K N T I F S A V [P M F P T I]
1981 CCACACCGCCAGCAGCTTGGAACTTGGAAAGGAATAAAGCTCCATTTTATCTTGT 2040
[P P L A W P]
2041 ATGTTCCCAATTCAGGCTGACGAGTTTCTTCTCTTTTGTATCATTTATAAGT 2100
2101 AGACAAGAAATCAAGCAAGAACAAGCTCAAGTCAAAATATATAATATCTTAGGAGGGT 2160
2161 GCATTTGTCTTTTCTCATATTGAA 2185

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Figure 3. The nucleotide sequences of taro EGase cDNA. The "transmembrane_region" signature (at amino acids 75-95) and "GLYCOSYL_HYDROL_F9_2" signature (at amino acids 559-577) are underlined. The "Six-hairpin glycosidase" signature (at amino acids 108-591) is shown in italic. The proline rich region was shown in boxed. The potential poly-adenylation sites are under double line.

in the database and grouped into three subfamilies, the α , β and γ subfamilies, respectively, and the putative function of these subfamilies also have been described (Levy et al., 2002). In this work, the phylogenetic analysis shows that the taro EGase belongs to the γ subfamily and that it is closely related to the *KOR* and *KOR3* genes of *Arabidopsis* (Figure 4). The amino acid sequences of taro EGase displayed 76% and 70% identity with *Arabidopsis* *KOR* and *KOR3*, respectively, suggesting that the taro EGase is an ortholog of *Arabidopsis* *KOR* and may have a similar function in plants.

Determination of copy number of EGase in taro

After full-length *EGase* cDNA cloning, Southern hybridization was applied to examine the copy number of *EGase* in the taro genome (Figure 5). Genomic DNAs digested with *Bam*HI-, *Bgl*III-, *Eco*RI- and *Eco*RV- were

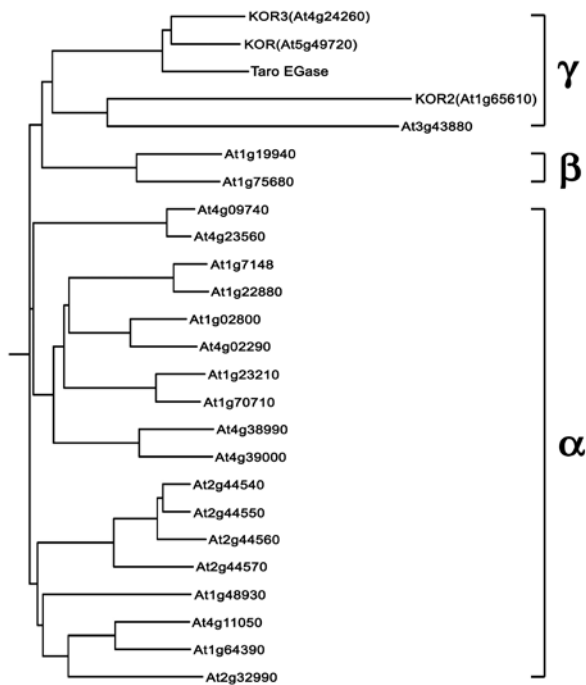


Figure 4. Relationship of the primary amino acid sequences between *Arabidopsis* and taro *EGase*. The dendrogram was generated by the program DRAWTREE in SDSC work bench web site (<http://seqtool.sdsc.edu/CGI/BW.cgi>). All sequences of *EGase* were obtained from GenBank/EMBL/DDBJ.

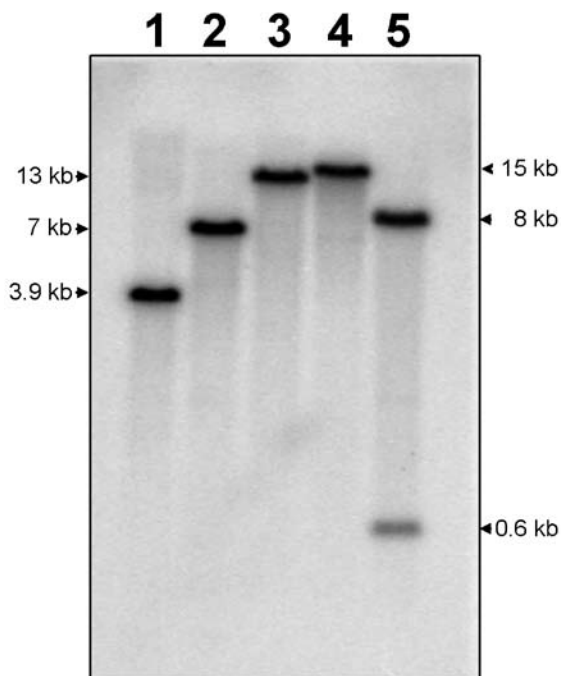


Figure 5. Southern blot analysis of taro genomic DNA. Ten micrograms genomic DNA was digested with restriction enzyme and subjected to Southern blot analysis. Lane 1: *Bam*HI; lane 2: *Bgl* II; lane 3: *Eco*RI; lane 4: *Eco*RV; and lane 5: *Hind*III digested. Hybridization was carried out using the 32 P-labeled 1.0 kb of *cEGase3'* amplified from pGEMTEGFL.

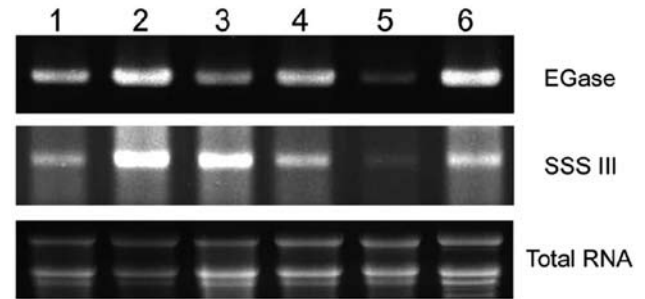


Figure 6. Analysis of the expression of taro *EGase* in tubers and leaves by RT-PCR. Lane 1: 100 g tuber; lane 2: 300 g tuber; lane 3: 600 g tuber; lane 4: the upper part of 1000 g tuber; lane 5: the bottom part of 1000 g tuber; and lane 6: the rolled leaf of the plant with 1000 g tubers.

probed with *cEGase3'*, fragments of 3.9 kb, 7 kb, 13 kb, and 15 kb were observed, respectively, except that two hybridization signals at 8 kb and 0.6 kb were shown in the *Hind*III - digested sample. The 0.6 kb fragment matched well with the predicted size in the restriction map of taro *EGase* cDNA, and this indicates that the *EGase* is a single copy gene in the taro genome.

Expression of *EGase* gene during development in taro

The temporal expression of taro *EGase* during tuber development was monitored by RT-PCR (Figure 6). The *EGase* transcript was expressed in all the developmental stages of tuber with the highest levels in the 300 g tubers, in the upper part of the 1,000 g tubers and in the rolled leaves. Relatively low expression was found in the bottom part of the 1,000 g tubers. In general, the 300 g tubers and the upper portion of larger tubers undergo more rapid elongation than do tubers in other developmental stages. A similar temporal expression pattern also was observed during leaf development (Figure 7). The *EGase* transcript was found in all stages of leaf development, with the highest levels in the rolled young leaves undergoing rapid growth, and lower levels in the mature and senescent leaves. Based on the expression patterns of taro *EGase* in leaf and tuber, it is reasonable to infer that the *EGase* may play an important role in the cell elongation of taro.

DISCUSSION

A taro *EGase* cDNA of 2.2 kb was isolated using a RACE cloning strategy in this work. It encodes an *EGase* which bears a high similarity (86-91%) to the membrane-anchored ones from other species. A transmembrane region was also found to be located between amino acids 75 to 95 by an "InterPro Scan" analysis. Phylogenetic analysis revealed that 25 members of the *Arabidopsis* *EGase* family could be divided into the α , β and γ subfamilies (Libertini et al., 2004). The taro *EGase* was found to belong to the γ subfamily, one group of the membrane-anchor *EGases*. These results indicated that the

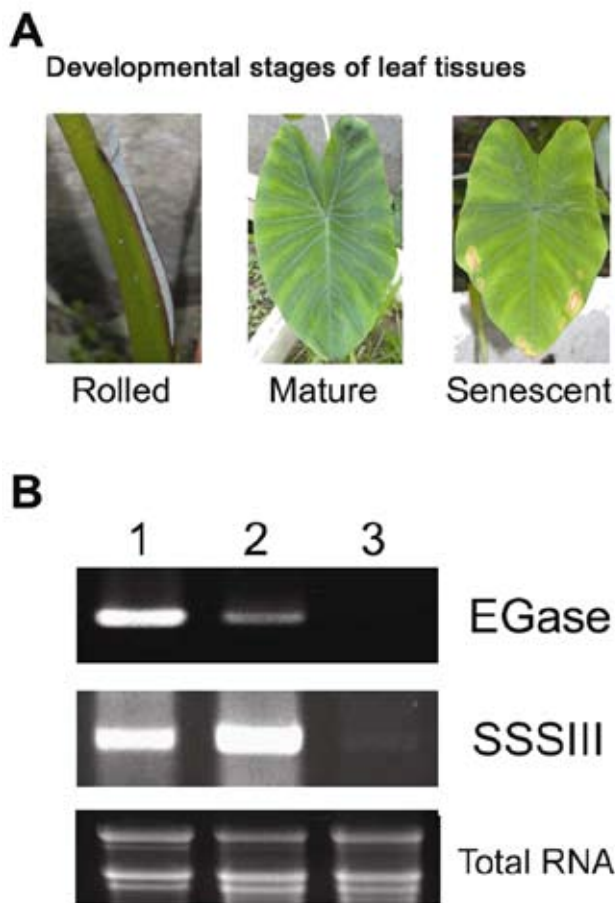


Figure 7. Analysis of the expression of taro *EGase* in leaf of the plant with 1000 g tubers by RT-PCR. Lane 1: the rolled leaf; lane 2: the mature leaf; and lane 3: the senescent leaf.

cloned *EGase* is a membrane-anchored one that exists in taro plants. The specific function of membrane-anchored EGases is currently unknown, but they are located at the plasma membrane and participate in cellulose metabolism at the inner layers of the cell wall (Brummell et al., 1997b; Nicol et al., 1998; Del Campillo, 1999; Molhoj et al., 2001a).

The phylogenetic analysis revealed that, except for one member of the γ subfamily, the taro *EGase* was clustered more closely with *Arabidopsis* KOR and KOR3. A proline rich region was also found in the C-terminal of taro *EGase* and conserved in all KOR-like EGases. These results suggested that the taro *EGase* is a KOR-like enzyme. Furthermore, alignment analysis demonstrated that it shares more identity with KOR (76%) than KOR3 (70%). On the other hand, the temporal expression profiles showed that the transcripts of taro *EGase* could be found in all tissues during various developmental stages. A similar expression pattern was also found in *Arabidopsis* KOR (Molhoj et al., 2001a). These findings strongly suggested that taro *EGase* is an ortholog of *Arabidopsis* KOR and may play some similar roles.

The *Arabidopsis* KOR gene has been identified by the dwarf mutant KORRIGAN and found to be involved in cytokinesis, pectin metabolism in the primary cell wall, and cell elongation (Nicol et al., 1998; His et al., 2001; Lane et al., 2001). On the other hand, the oilseed rape *Cell6*, encodes a KOR-like EGase and has been proposed to be involved in cell division and in cell wall assembly during growth (Molhoj et al., 2001b).

Southern blotting confirmed that only one copy of the *EGase* is present in the taro genome. This observation is unique because several copies of KOR-like EGases have been reported to exist in the *A. thaliana* or *O. sativa* genomes (Nicol et al., 1998; Molhoj et al., 2001a; Yoshida et al., 2006). This finding indicated that the cellulose metabolisms of *Arabidopsis*, rice, and taro may have significant differences.

The transcripts of taro *EGase* were highly accumulated in 300 g tubers, the upper part of 1000 g tubers, and rolled leaves. The 300 g and 1000 g taro plant tubers form about 6 and 8 months after planting, respectively, and tubers displayed a linear growth rate under the planting conditions in Taiwan. At the same time, the rolled leaves of taro have almost reached their maximum leaf area. These findings suggested that taro *EGase* is highly expressed in tissues undergoing rapid growth, indicating that the taro *EGase* is involved in the regulation of plant growth. A similar observation was also made about another non-KOR like EGase, *Arabidopsis* Cell1 (Milligan and Gasser, 1995; Brummell et al., 1997a; Shani et al., 1997). Curiously, the temporal expression profile of the *EGase* resembled that of soluble starch synthase III. This revealed that cellulose and starch metabolisms may undergo the same regulation in taro plants. However, this needs to be confirmed by more experimental evidence.

This is the first report showing that the expression a taro *EGase*, an ortholog of *Arabidopsis* KOR, is involved in the regulation of plant growth and linked with starch metabolism. While multiple EGases have been identified in higher plants, the biochemical and physiological function of each individual EGase remains to be investigated. Identification in this study of the *EGase* gene, which encodes a novel endo-1,4- β -D-glucanase accumulating in tissues undergoing rapid growth, provides an opportunity to fill in the gaps and define its precise functional role in the metabolism of cellulose in taro.

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檳榔心芋內切型纖維素分解酵素基因之選殖及其在快速生長葉片與塊莖之表現分析

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本研究利用 RT-PCR 及 rapid amplification of cDNA ends (RACE) 的方法，自檳榔心芋葉片選殖內切型纖維素分解酵素 (*EGase*) cDNA。*EGase* 以單一拷貝基因存在芋基因體中，其 cDNA 長 1,854 bp，包含 618 個胺基酸之閱讀框架，理論分子量為 68,434 Da、*pI* 值為 8.8。此一芋 *EGase* 重組蛋白質在 CMC 平板培養基上，具有明顯 β -1,4-glucanase 活性，顯示其具纖維素代謝的功能。親緣分析結果顯示，芋 *EGase* 屬 γ 亞家族成員之一，並與阿拉伯芥 *KORRIGAN* 基因 (*KOR*) 互為異種同源基因。推論芋 *EGase* 基因應參與細胞的胞質分裂、主細胞壁的基丁質代謝及細胞的伸展等生理現象。另一方面，此 *EGase* 在 300 公克塊莖、1000 公克塊莖上部及捲葉等快速生長的組織中大量表現，此一表現模式與快速生長的芋塊莖中之可溶性澱粉合成酶 III 基因相似，這些結果顯示，芋 *EGase* 基因應在芋的快速生長機制中扮演重要的角色。

關鍵詞：芋 (*Colocasia esculenta* var. *esculenta*)；內切型纖維素分解酵素；*KORRIGAN*；快速生長；植物細胞壁組裝。

