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Molecular cloning and characterization of a novel mannose-binding lectin cDNA from *Zantedeschia aethiopica*

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ABSTRACT: Using RNA extracted from *Zantedeschia aethiopica* young leaves and primers designed according to the conservative regions of *Araceae* lectins, the full-length cDNA of *Z. aethiopica* agglutinin (ZAA) was cloned by rapid amplification of cDNA ends (RACE). The full-length cDNA of *zaa* was 871 bp and contained a 417 bp open reading frame (ORF) encoding a lectin precursor of 138 amino acids. Through comparative analysis of *zaa* gene and its deduced amino acid sequence with those of other *Araceae* species, it was found that *zaa* encoded a precursor lectin with signal peptide. Secondary and three-dimensional structure analyses showed that ZAA had many common characters of mannose-binding lectin superfamily and ZAA was a mannose-binding lectin with three mannose-binding sites. Southern blot analysis of the genomic DNA revealed that *zaa* belonged to a multi-copy gene family.

Introduction

Lectins are defined as proteins possessing at least one noncatalytic domain, which binds reversibly to specific mono- or oligo- saccharides (Peumans and Van Damme, 1995). Lectins are ubiquitous in the biosphere and several hundreds of these molecules have been isolated so far from plants, viruses, bacteria, invertebrates and vertebrates including mammals (Celia and Maria, 2002). The most thoroughly investigated lectins are those from plant species. There is growing evidence that most plant lectins play a role in the plant's defense against different kinds of plant-eating organisms (Murdock and Shade, 2002). Mannose-binding lectins are widely found in monocot species such as *Alliaceae*, *Amaryllidaceae*, *Araceae*, *Bromeliaceae*, *Liliaceae* and *Orchidaceae* (Van Damme *et al*., 2000). Lectins of many types especially those with mannose-binding capacity were found to be insecticidal to many groups of insects including *Homoptera*, *Coleoptera* and *Lepidoptera* (Sauvion, 1996; Gatehouse *et al*., 1997).

Recently, mannose-binding lectins have been isolated from several *Araceae* species including *Arum maculatum*, *Colocasia esculenta*, *Xanthosoma sagittifolium*,

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Dieffenbachia sequina, *Pinellia ternata* and *Arisaema heterophyllum* (Van Damme *et al*., 1995; Yao *et al*., 2003; Zhao *et al*., 2003). Insect bioassay studies showed that most of the tested mannose-binding lectins purified from *Araceae* species such as *Arisaema* spp. and *Pinellia* spp. had more or less insecticidal activities towards cotton aphids (*Aphis gossypii* Glover) and peach potato aphids (*Myzus persicae* Sulzer) when incorporated into artificial diets (Pan *et al*., 1998; Mao *et al*., 1999; Li *et al*., 2000; Yao *et al.*, 2003). As *Zantedeschia aethiopica*, one of the Chinese horticultural plant species, belongs to *Araceae*, it is speculated that *Z. aethiopica* lectin may also have similar inhibitory effect on sap-sucking insects like lectins from other *Araceae* species and may play a role in controlling sap-sucking insects by genetic engineering. To date, there is no report on the molecular cloning of lectin gene from *Z. aethiopica*. Here, we describe the cloning and molecular characterization of the lectin gene from *Z. aethiopica*, which will enable us to test its insect resistance function by transferring it into tobacco in the future.

Materials and Methods

Plant material and RNA isolation

Z. aethiopica plants, collected from Shanghai Chinese Medical University, China, were grown in pots in the greenhouse under standard conditions. Leaves from *Z. aethiopica* were powdered in liquid nitrogen with mortar and pestle, and the total cellular RNA was extracted using TRIzol Reagent (GIBCO BRL, USA) according to the manufacturer's instruction.

Rapid amplification of cDNA ends (RACE) of Z. aethiopica agglutinin gene

The cDNA synthesis was performed with the 3' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, USA). Essentially, an aliquot of isolated 3 μg RNA was reversely transcribed with a cDNA synthesis primer AP (5'-GGCCACGCGTCGACTAGTAC(T)16-3') provided by the kit (GIBCO BRL, USA). The gene-specific primer ZAA3-1 (5'-ATGCAGGATGACTGCAACCT-3') was designed according to the conserved amino acid sequence (MQDDCNL) possessed by many mannose-binding lectins of *Araceae* species and used as the sense primer for the 3' end cDNA amplification The Abridged Universal Amplification Primer (AUAP, 5'-GGCCACGCGTCGACTAGTAC-3') was used as the antisense primer. The 50 μl PCR reaction mixture contained 2 μl cDNA, 10 pmol each of primer ZAA3-1 and AUAP, 10 μmol dNTPs, and 2.5U *Taq* polymerase. PCR reaction was carried out under the following condition: template was firstly denatured at 94°C for 3 min followed by 35 cycles of amplification (94°C for 50 sec, 55°C for 1 min, 72°C for 1 min) and by 10 min at 72°C. The PCR product was subjected to agarose gel electrophoresis and a distinct DNA band sized about 0.55 kb was amplified and was recovered by gel extraction for ligation with pGEM-T Easy vector (Promega, USA). Competent cells of *Escherichia coli* strain DH5α were prepared and transformed with the ligation mixture in terms of the protocols of Sambrook *et al.* (1989). The amplified fragment was sequenced using T7/SP6 sequencing primers and NCBI BLAST revealed that the fragment had high homology with the 3' ends of lectins from *Gastrodia elata* (GAFP) and *Listera ovata* (LOA).

Subsequently, three specific primers, ZAA5-1 (5'-GATGAGGCGGTAGCTGTTCTG-3'), ZAA5-2 (5'-GAGTTGCTGGACCACACGATGT-3') and ZAA5-3 (5'-CCGTTGTTCTGCAGGGTGG-3'), were designed and synthesized according to the cloned 3' end sequence. The 5' RACE System for Rapid Amplification of cDNA Ends (GIBCO BRL, USA) was used for 5' cDNA cloning. Reverse transcription (RT), dC tailing and PCR amplifications were conducted in terms of the kit protocol. An aliquot of 5 μg total RNA extracted from leaves was reversely transcribed using primer ZAA5-1 with an extra 30 min of RT at 50°C after standard RT at 42°C. Primers ZAA5-2 and kit primer AAP (5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3') were used for primary amplification using 5 μl dC-tailed cDNA as template in a total volume of 50 μl reaction system under the following PCR conditions: template was denatured at 94°C for 3 min followed by 35 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) and by 10 min at 72°C. An aliquot of 0.1 μl primary PCR product was used as template for nested PCR amplification using primers ZAA5-3 and AUAP under following PCR conditions: 94°C for 3 min followed by 35 cycles of amplification (94°C for 50 seconds, 57°C for 1 min, 72°C for 1 min) and by 10 min at 72°C. The PCR product was electrophoresed on 1.0% agarose gel and the amplified 370 bp DNA band was recovered by gel extraction and subcloned as described above for sequencing. NCBI BLAST indicated that the amplified fragment had high similarities to plant lectin genes, especially those of mannose-binding types.

By aligning and assembling the 3' end and 5' end sequences on Vector NTI Suite 6.0, the full-

length cDNA sequence of *zaa* gene was deduced. Subsequently, a pair of PCR primers FZAA (5'-TGAAACCCATCCCCTTGCCTTG-3') and RZAA (5'- CACAATATAGATTGATCTTATTTAGCG-3) were designed according to the deduced *zaa* full-length cDNA and used for the amplification of the full-length cDNA of *zaa*. The amplification was repeated for 3 times and the thermal cycling program was the same as that utilized for 3' RACE.

Southern blot analysis

Total genomic DNA was isolated from 1 g fresh weight of *Z. aethiopica* leaf material according to the procedure described by Dellaporta *et al.* (1983). Aliquots of DNA (10 μg) were digested overnight at 37°C with *Eco*RI, *Eco*RV and *Hin*dIII, respectively, fractionated by 0.8% agarose gel electrophoresis, and transferred to a positively charged Hybond-N^+ nylon membrane (Amersham Pharmacia, Sweden). The *zaa* coding sequence was used as the probe for Southern analysis. Probe labeling (biotin-dUTP), hybridization and detection were performed using *Gene images* random prime labeling module and CDP-Star detection module following manufacturer's instructions (Amersham Pharmacia, Sweden). The hybridized signals were visualized by exposure to Fuji X-ray film at room temperature for 1.5 h.

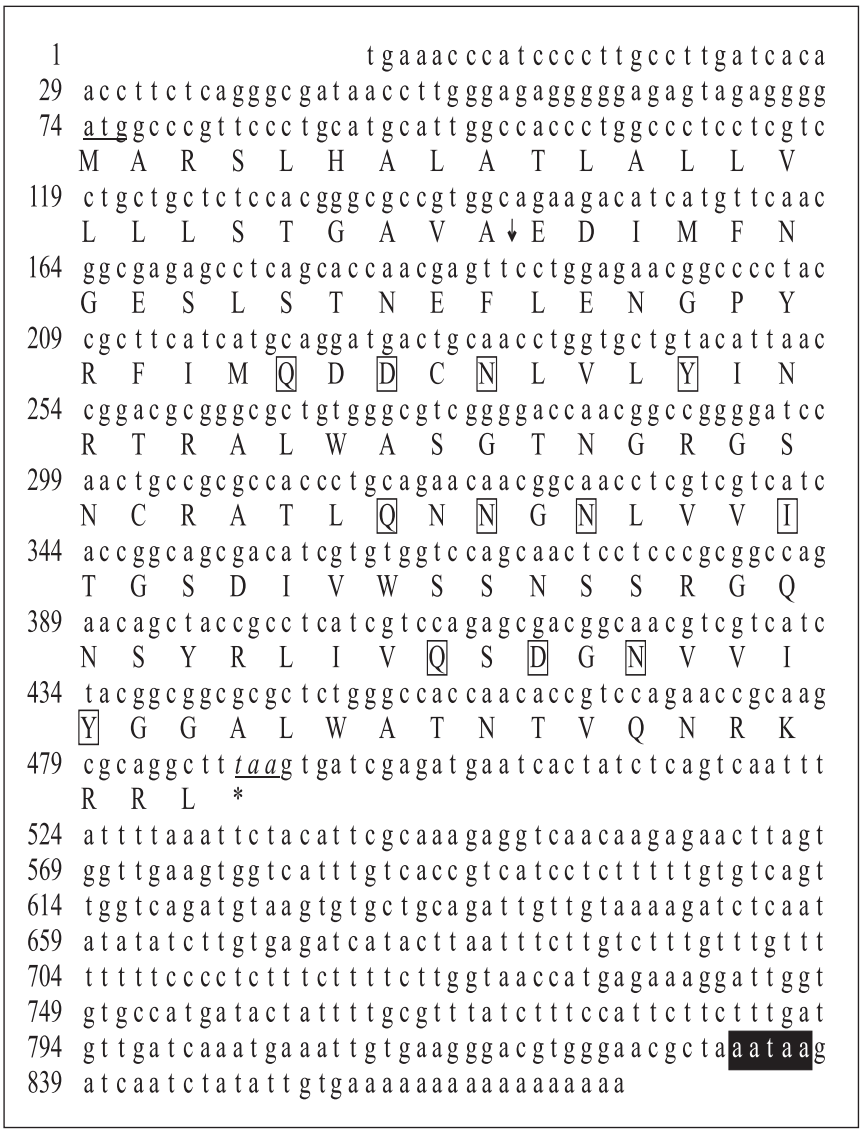

FIGURE 1. The full-length cDNA sequence and deduced amino acid sequence of *Z. aethiopica* agglutinin (ZAA). The start codon (atg) was underlined and the stop codon (taa) was underlined italically. The signal for poly(A) tail-addition AATAA was shown by black background. Mannose-binding sites QDNY (the 1st and the 3rd sites) and variable form QNNI in the 2nd site were boxed. The arrow indicated the cleavage site of signal peptide (between A and E).

Results and Discussion

Isolation and characterization of zaa full-length cDNA

As anticipated, a solely DNA band sized about 870 bp was amplified and sequencing result of this fragment was identical to the speculated *zaa* full-length cDNA. The cloned full-length cDNA of *zaa* was 871 bp (GenBank Accession No. AY308073) and contained a 417 bp open reading frame (ORF) encoding a polypeptide of 138 amino acid residues with an isoelectric point (pI) of 9.24 and a calculated molecular weight of about 15.1 kDa (Fig. 1).

Nucleotide-nucleotide BLAST of the *zaa* full-length cDNA sequence on NCBI website (http://www.ncbi.nlm.nih.gov) indicated that its conserved regions had high similarities to mannose-binding lectin genes from *Ananas comosus* (AY098512.1), *Allium sativum* (S23497) and *Listera ovata* (AAC37423.1). The deduced amino acid sequence of ZAA was 52%, 54%, 49% and 49% identical to GEA (*Gastrodia elata* agglutinin) (CAB94240.1), LOA (*L. ovata* agglutinin) (AAC37423.1), ZGA (*Zephyranthes grandiflora* agglutinin) (AAP37975.1) and GNA (*Galanthus nivalis* agglutinin) (AAL07474.1) respectively, with the corresponding positive percentages being 69%, 68%, 67% and 69% respectively (Fig. 2).

Signal peptide prediction on website (http://www.cbs.dtu.dk/services/singalP) showed that *zaa,* like most other mannose-binding lectins from *Araceae* species (Van Damme *et al*., 1995; Yao *et al*., 2003; Zhao *et al*., 2003), encoded a lectin precursor with a signal peptide of 24 aa, and the most likely cleavage site was predicted between Ala_{24} and Glu_{25}. ZAA is a secretory protein which targets to outside of cells.

Most plant mannose-binding lectins contain 3 sugar-binding sites [Gln (Q), Asp (D), Asn (N) and Tyr (Y)] namely QDNY. Sugar-binding site analysis re-

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GEA         MAASASTAVILFFAVTTVMSLSAIPAFASDRLNSGHQLDTGGSLAQGGYL    50
LOA         -VSFLSMSTILLVSTMALMSLLATPVSASDRLNAGQSLGTGGSLALGGYI    49
ZGA         -----MTKTIFLIGATIFLGVITPSCLSDNILYSGETLSTGESFNYGPYT    45
GNA         -----MAKASLLILATIFLGVITPSCLSENILYSGETLPTGGFLSSGSFV    45
ZAA         ----MARSLHALATLALLVLLLSTGAVAEDIMFNGESLSTNEFLENGPYR    46
Consensus                                            GLT

GEA         FIIQNDCNLVLYDN-NRAVWASGTNGKASNCFLKMQNDGNLVIYSGS--R    97
LOA         FIIQGDCNLVLYDN-NRAVWASGTNGRGSNCILSMQRDGNLVIYSSG--R    96
ZGA         FIMQEDCNLVLYDV-DKPIWASNTGGLARGCHLSMQSDGNLVVYTPSGNR    94
GNA         FIMQEDCNLVLYNV-DKPIWATNTGGLSSDCSLSMQNDGNLVVFTPS-NK    93
ZAA         FIMQDDCNLVLYINRTRALWASGTNGRGSNCRATLQNNGNLVVITGS--D    94
Consensus   FI Q DCNLVLY      WA  T G        Q  GNLV

GEA         AIWASNTNRQNGNYYLILQRDRNVVIYDNSNNAIWATHTNVGNAEITVIP   147
LOA         AIWASNTNRQNGNYYLILQRDRNVVIYDNSNNAIWATGTNVGNAAIAVIP   146
ZGA         AIWASNTQGENGNYVCILQKDRNVVIYGTAR---WATGTNIHGAGIVGVP   141
GNA         PIWASNTDGQNGNYVCILQKDRNVVIYGTNR---WATGTYTGAVGIPESP   140
ZAA         IVWSSNSSRGQNSYRLIVQSDGNVVIYGGAL---WATNTVQNRKRRL---   138
Consensus     W SN        Y  I Q D NVVIY       WAT T           P

GEA         HSNGTAAASGAAQNKVNELYISMY  171
LOA         HNNGTAAASGAKQNKVRELNP---  167
ZGA         GSAPQNTTAEMIKLVRKYLITK--  163
GNA         PSEKYPSAGKIKLVTAK-------  157
ZAA
Consensus
```

FIGURE 2. Multiple alignment of ZAA with other monocot mannose-binding lectins or agglutinins. GEA, *G. elata* agglutinin (CAB94240); LOA, *L. ovata* agglutinin (AAC37423.1); GNA, *G. nivalis* agglutinin (AAA33346); ZGA, *Z. grandiflora* agglutinin (AAP37975). Mannose-binding sites QDNY (the 1st and the 3rd sites) and variable form QNNI in the 2nd site were boxed.

vealed that the first mannose-binding site (QDNY) and the flanking residues of this site in deduced ZAA protein were the same as those of many other plant mannose-binding lectins (Yao *et al*., 2003; Zhao *et al*., 2003). The third sugar-binding site (QDNY) was also conserved in ZAA, however, its flanking residues were slightly variable compared with those of many other plant mannose-binding lectins. The second mannose-binding site in ZAA was more variable in which the QDNY residues were changed to QNNI where the residue I was quite different from Y in amino acid features (Figs. 1 and 2). Sequence alignment also revealed that the sugar-binding sites were normally more variable in *Araceae* lectins than those from other plant families, and this phenomenon was typically characterized by AMA (*A. maculatum* agglutinin) (Van Damme *et al*., 1995), while ZAA was in the middle of the AMA and the typically featured 3-site mannose-binding lectins in view of variation. Whether the residue variation in sugar-binding sites implies a functionally enhancement or basically function shifting deserves further clarification, especially in dealing with lectins from *Araceae*.

Structure and phylogenetic analyses of ZAA

The secondary structure of ZAA was analyzed with SOPMA (Geourjon and Deléage, 1995) (Fig. 3A). The result showed that ZAA consisted of twenty two β-sheets connected by turns and coils. The signal peptide formed α-helix, which was usually helpful for lectin transmembraning and targeting. The C-terminal was composed of α-helix and random coils, which would be cut off when the precursor was post-translationally processed into the mature protein. Molecular homologous modeling of ZAA was carried out by Swiss-Model (Guex and Peitsch, 1997). It was noteworthy that β-sheets occurred predominantly in the structure of ZAA (Fig. 3B). The overall folding of ZAA, which was typically built from β-sheets connected by turns and loops, created very tight structural scaffold. This was very similar to the three-dimensional structure of other plant mannose-binding lectins (Barre, 2001). The ZAA consisted of three tandemly arrayed subdomains. The three-dimensional structure analysis also showed that all the three mannose-binding sites of ZAA (2 QDNY and 1

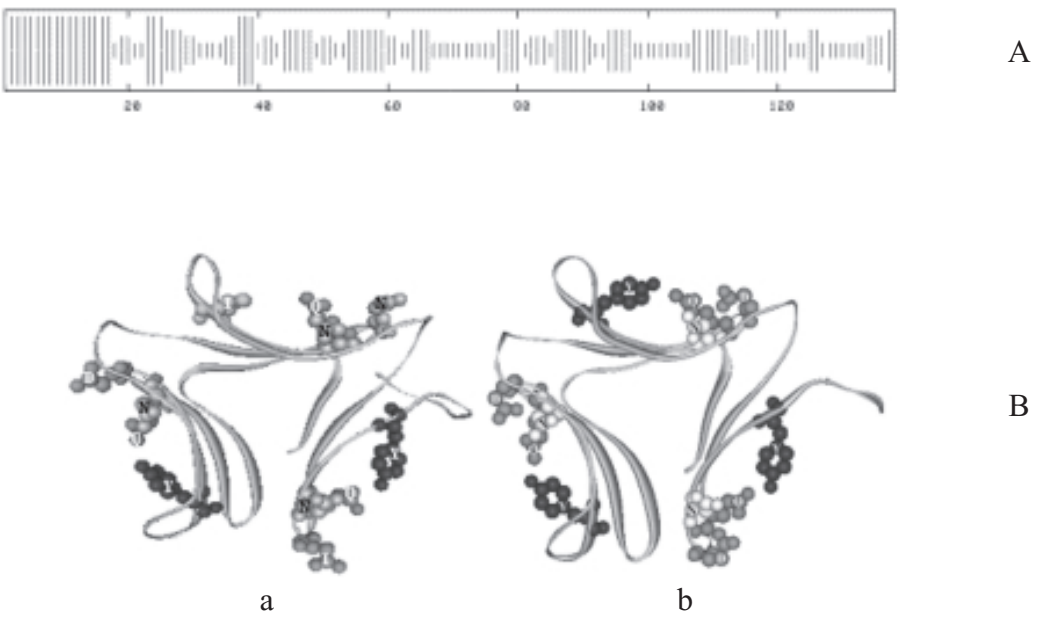

FIGURE 3. The second and three-dimensional structures of ZAA. **A)** The secondary structure of ZAA. Helix, sheet, turn and coil were indicated respectively with the longest, the second longest, the third longest and the shortest vertical lines. **B)** The three-dimensional structures of ZAA (a) and GNA (b). The β-sheets were indicated by patches. Turns and loops were indicated by lines and the amino acids constituting mannose-binding sites were indicated as balls.

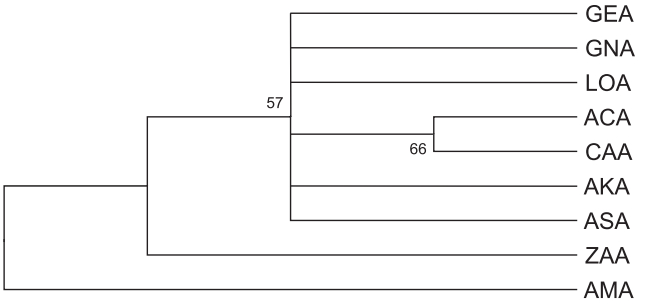

FIGURE 4. Phylogenetic tree built from mannose-binding amino acids of the *Z. aethiopica* agglutinin and other mannose-binding lectins. The dendrogram was constructed using multiple sequence alignment ClustalX (Version 1.81) and phylogeny analysis MEGA (Version 2.1). ZAA, *Z. aethiopica* agglutinin; AMA, *Arum maculatum* agglutinin; ASA, *Allium sativum* agglutinin; GEA, *Gastrodia elata* agglutinin; LOA, *Listera ovata* agglutinin; GNA, *Galanthus nivalis* agglutinin; CAA, *Crinum asiaticum* agglutinin; ACA, *Ananas comocus* agglutinin; AKA, *Amorphophallus konjac* agglutinin.

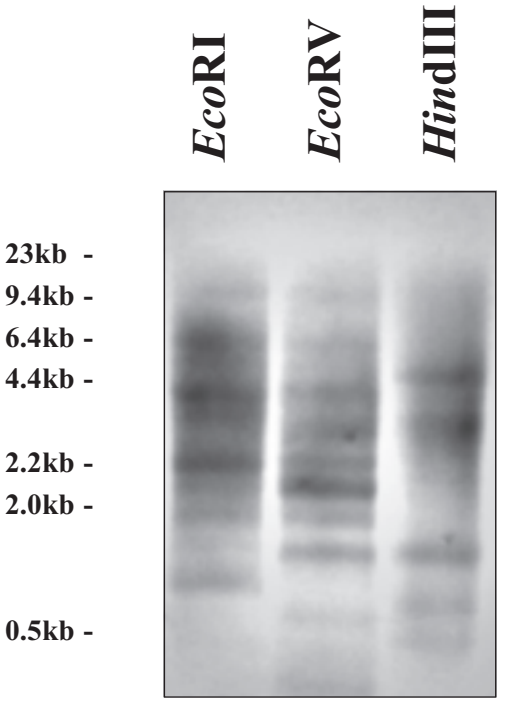

FIGURE 5. Southern blot analysis. Genomic DNA was isolated from leaves of *Zantedeschia aethiopica* and digested with *Eco*RI, *Eco*RV and *Hin*dIII, respectively followed by hybridization with the biotin-labeled *zaa* coding sequence as the probe.

QNNI) were located in the clefts formed by the three bundles of β-sheets. The three-dimensional structure of ZAA strongly resembles that of the GNA (Fig. 3B).

A phylogenetic tree was constructed based on the 27 amino acids at sugar binding sites of mannose-binding lectins including GEA, GNA, LOA, ACA, CAA, AKA, ASA, ZAA and AMA (Fig. 4). The result showed that ACA and CAA were most closely related and as a whole they clustered together with GEA, GNA, LOA, AKA and ASA forming the typical group with 3-site mannose-binding lectins. AMA had far relationship with the typical group with 3-site mannose-binding lectins and ZAA was between them.

Southern blot analysis

The presence of multicopies of mannose-binding lectin gene in the genome has been reported in many plant species, particularly those belonging to *Araceae* families (Barre *et al.,* 1996; Van Damme *et al.*, 1995). In order to investigate if ZAA belongs to a multi-copy gene family, Southern blot analysis was performed by digesting the genomic DNA isolated from *Z. aethiopica* leaves with restriction endonucleases (*Eco*RI, *Eco*RV and *Hin*dIII) respectively and allowed to hybridize with the biotin-labeled *zaa* coding sequence. The result revealed that there were more than 5 hybridization bands in each lane (Fig. 5), indicating the *zaa* belonged to a multi-copy gene family, like those of many other *Araceae* species such as *Arisaema heterophyllum* (Zhao *et al*., 2003).

In summary, we have successfully isolated the lectin gene from *Z. aethiopica* whose deduced amino acid sequence showed similarities to other mannose-binding lectins such as GEA and LOA. Previous studies showed that the lectins from *Araceae* had more or less insecticidal activities towards aphids in feeding experiments (Pan *et al*., 1998; Li *et al*., 2000; Yao *et al.*, 2003, 2004). The cloning of *Z. aethiopica* lectin gene (*zaa*) will enable us to test its potential role in controlling insect pests such as aphids by transferring the gene into tobacco in the future.

Acknowledgements

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