Research Article *Journal of Plantation Crops, 2013, 41(2): 157-162*

Cloning and characterization of metallothionein like protein cDNA in tea using RACE

P. Senthilkumar[#] and A.K.A. Mandal[1]*

Plant Physiology and Biotechnology division, UPASI Tea Research Institute, Valparai, Tamil Nadu, India - 642127

[1]School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India - 632014

(Manuscript Received: 10-02-12, Revised: 03-04-13, Accepted: 02-05-13)

Abstract

Metallothionein (MT) like protein have important role in biotic and abiotic stress tolerance in plants. It also plays vital role in controlling intracellular redox potential and activation of oxygen detoxification in plants after pathogen invasion. In the present study, full length gene encoding MT was amplified using rapid amplification of cDNA ends (RACE) reaction and sequenced (GenBank Acc. No. JN315623). Sequence analysis revealed presence of 276 bp open reading frame with coding capacity of 92 amino acids, flanked by 262 bp and 166 bp 5' and 3' untranslated regions (UTR) respectively. Theoretical pi and molecular weight of the analysed sequence was 5.37 and 9.5 kDa respectively. Phylogenetic analysis of MT with other plant species was discussed.

Keywords: Camellia sinensis, grey blight, metallothionein, *Pestalotiopsis*, RACE

Introduction

Tea is the oldest, medically important, non-alcoholic beverage across the world manufactured from two leaves and a bud of tea plant (*Camellia sinensis* (L.) O. Kuntze). Survival of the plants is challenged by various biotic and abiotic factors (*Chen et al.*, 2002). Among the biotic factors, *Pestalotiopsis spp.* causing grey blight disease is a destructive pathogen, which leads to severe crop loss (Keith *et al*., 2006; Koh *et al*., 2001). Recent advances in agricultural biotechnology suggest genetic engineering for substantial contribution in controlling diseases. Therefore, genes for grey blight disease resistance need to be identified and introduced into elite germplasm, creating novel pathogen resistant lines (Shah, 1997; Staskawicz *et al*, 1995).

Metallothioneins (MTs) are low molecular weight, cysteine rich proteins found in almost all eukaryotic organisms (Chen *et al*, 2006; Margoshes and Valee, 1957; Choi *et al.*, 1996). In tobacco, expression of MT was induced by stresses such as wounding and virus infection (Choi *et al*., 1996). MT plays a major role in wide range of diseases (Bowles, 1990; Carpene *et al*., 2007). Chen *et al* (2003) suggested that the MTs in plants are different in structures and they also play diverse roles in order to cope with complex developmental and environmental conditions. MTs are known to be induced in leaves by wounding, H_2O_2 treatment, and the bacterial pathogen *Xanthomonas campestris* (Obertello *et al*., 2007). MT gene was found to be differentially regulated in *Persea americana* infected with *Phytophthora cinnamomi* (Mahomed and Berg, 2011). MT also plays vital role in controlling the intracellular redox potential and activation of oxygen detoxification in plants after pathogen invasion. Many unknown roles for MT has been suggested in plants but it is mostly induced because of mechanical wounding and pathogen infection.

**Corresponding Author: akamandal@rediffmail.com*

[#]Present Address: Department of Genetic Engineering, SRM University, Kattankulathur - 603 203

Rapid amplification of cDNA ends (RACE) technique is employed in isolation of full length gene of target transcript (Ren *et al.*, 2011; Wang *et al.*, 2011). RACE can potentially be utilized in cloning of full length genes involved in host-pathogen interaction. Zhou *et al*. (1999) isolated a PAD3 cDNA clone from wild-type plants by RACE-PCR from the genomic sequence of *Arabidopsis*. A complete cDNA of *Phytophthora infestans* induced hypersensitive response related protein (POTHR-1) in potato was cloned using RACE by Zhen-Dong *et al,* (2003). In addition to the above reports, cDNAs encoding phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and monocot mannose-binding lectins were cloned from garlic (*Allium sativum*) and *Zingiber officinale* were cloned by RACE-PCR (Chen *et al.*, 2005 and Tuan *et al.*, 2010). The present study report the cloning of full length cDNA encoding differentially expressed metallothionein (MT) like protein from *Pestalotiopsis theae* infected tea plant.

Materials and Methods

UPASI-10, a prevalent chinary tea clone (Balasaravanan *et al*., 2003), growing well in the tea garden of the UPASI- Tea Research Institute (latitude 10° 30´N, longitude 27° 0'S and altitude 1,050 m) was selected in the present study. Field grown tea bush of *P. theae* tolerant cultivar, UPASI-10 was pruned to generate disease free shoots. Pruned bush was inoculated with *P. theae* following the method described by Sanjay (2004), covered with polythene sheet and relative humidity was maintained between 85-90 per cent throughout the experiment. The infected leaves from zero hour onwards at interval of 6 hours up to 7 days were collected, stored immediately in liquid nitrogen and later transferred to -80 °C.

Preparation of total RNA and cDNA synthesis

Total RNA was isolated from leaf tissue of *P. theae* infected shoot using the RNeasy Plant Mini Kit (Qiagen) as per the manufacturer's instructions. The integrity and quantity of the RNA was measured by both electrophoresis and optical absorbance. Synthesis of first strand cDNA of the transcripts was carried out in 25 µl reaction volume containing 5x first strand reverse transcription buffer, 10 mM DTT, 50 µM dNTP, 200 U of Power-script reverse transcriptase (Clontech), 2.0 µg of total RNA and 50 pmol Oligo $T_{(15)}$ for 60 min at 42 °C and a final denaturation step at 72 °C for 7 min.

Amplification of full-length cDNA of metallothionine like protein

Isolation of 5´ and 3´ ends of metallothionine like protein was performed using SMART™ RACE cDNA amplification kit (Clontech) following manufacturer´s instructions. The primers P1 (5´-TGAAGCCAGCTTTCCCTAAA-3´) and P2 (5´-AGCCTCATACCCACAAGCAC-3´) were designed based on EST sequence obtained from our earlier studies in tea (GenBank Accession No: JG017612) and was used for 3´ and 5´ RACE reactions respectively. Based on the RACE sequence data, the primers GP1 (5´- TCCAGGGCTGTCA GTTTTCT-3´) and GP2 (5´-CCATGAAACCCTG TCCACTT-3´) were designed to amplify the full-length cDNA of metallothionine like protein using the following PCR conditions: 94 °C for 5 min, and 35 cycles of amplification of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 2 min and final extension of 72 °C for 5 min. A portion of PCR product was analysed on 1 per cent Agarose gel, the remaining PCR product was purified using QIAquick PCR purification kit (QIAGEN), cloned into T/A cloning vector (Fermentas), transformed into *E. coli* DH5α strain using TransformAid™ bacterial transformation kit (Fermentas) as per the manufacturer's instruction and sequenced using the BigDye terminator cycle sequencing kit on an automated DNA sequencer (ABI PRISM 3730XL Analyzer).

Sequencing and sequence analysis

The vector and the primer sequences were removed using "VecScreen" of NCBI (http://www.ncbi.nlm.nih.gov/VecScreen.html) and manual checking. Processed sequence was compared with non-redundant public databases, using the BLASTn and BLASTx algorithms of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches were considered significant when e-values and identities were <0.01 and >90 per cent respectively.

Full length cDNA sequence designated as *CsMT* (GenBank Acc.No. JN315623) was used for analysis of primary structural parameters (molecular weight, theoretical pI and amino acid composition)

using Protparam (http://www.expasy.ch/tools/protparam.html). Secondary and structure conformational parameters were computed using SOPMA (Self-optimized prediction method; http://npsa-pbil.ibcp.fr/cgi-bin/npsaautomat.pl?page=/NPSA/npsa_sopma.html). Deduced amino acid sequence of MT from other plant sources were retrieved from NCBI database and alignment was performed using ClustalW. Phylogenetic tree analysis of tea CsMT protein with other related MT genes were performed using the Neighbour-Joining method in MEGA 4 program (Tamura *et al*., 2007).

Results and Discussion

An EST encoding metallothionine like protein (GenBank Accession No: JG017612) was obtained while analysing gene expression during grey blight infection in tea. Further to clone the complete region encoding the metallothionine like protein, RACE reaction was performed. Primers were designed from the sequence information of EST and used to perform 3' and 5' RACE reactions. Based on the sequence of 3' and 5' RACE products, gene specific primers GP1 (5´-TCCAGGGCTGTCAGTTTTCT-3´) and GP2 (5´-CCATGAAACCCTGTCCACTT-3´) were designed to amplify the complete cds of the gene encoding metallothionine like protein. Role of MT in pathogenesis of *Uncinula necator* in *Vitis pseudoreticulata* was analysed by Xu *et al*. (2009**)**. Complete gene encoding metallothionein-like protein induced by tobacco mosaic virus and wounding was cloned from *Nicotiana glutinosa* (Choi *et al*., 1996). Cloning and sequencing of the amplified fragment revealed cDNA encoding metallothionine like protein of length 801 bp after trimming vector driven sequences and designated as *CsMT*. Similarly, 924 bp cDNA encoding a metallothionine like protein from coffee leaves was isolated by Moisyadi and Stile (1995). Amplified gene encoding *CsMT* was confirmed by BLASTx and BLASTn. Further analysis of the sequence revealed the presence of 276 bp open reading frame with coding capacity for a polypeptide of 92 amino acids, flanked by 262 and 166 bp 5' and 3' UTR respectively (Fig. 1). Isolation of various genes with complete cds using RACE has been attempted in

```
1   tccagggctgtcagttttctgacggaaagggccgggggggcattcaattaatagagtagtga

63  gccattagccccccggttggcagttaccttgggctcttaattgtgcgaatttgagcgag

123 agaaaatttcacagaaccatttatgccagattagccagtttaatgggttcttttagggaa

183 agctggcttcatgcctagcagtcgcggtccgcgatccgagtggcagggatcatcgcgcag

243 gggccattagggcaggggacaagtgttcacttttggagagcttttcttgtggtttcattc

303 cttgccggtcactttcattgtttttgtaaccactttttttcccaacttttccctaaaaa

363 atgtcttgttgcggaggaaactgtggcccagcactggctccaagtgcgccaacggctttc
    M  S  C  C  G  G  N  C  G  P  A  L  A  P  S  A  P  T  A  F    20
424 gaggatgcaacatttaccgcccatgagctttcagagaccaccactaccggggccttttatt
    E  D  A  T  F  T  A  H  E  L  S  E  T  T  T  G  A  F  I    40
484 gtttgtgatgcccctcaaaacacccacttgaagggatcttggatgggtgcgggacctgag
    V  C  D  A  P  Q  N  T  H  L  K  G  S  W  M  G  A  G  P  E    60
544 aatggctgcaaatgtggagccaaccatcgttgtgacccttgctcatgcaaaaaaacaaat
    N  G  C  K  C  G  A  N  H  R  C  D  P  C  S  C  K  K  T  N    80
605 gacaacttcctaaccattcctagccttctccaaagctagagatgagaagatgattccctc
    D  N  F  L  T  I  P  S  L  L  Q  S  *                        92
665 tcaacaataacccaaatacatatgtaatatgtacttgtatatgtataacatttctgtgct

726 tgtgggtatgaggcttctttaatttaggaaggtttttttaagtgtttttttttaaaaaaaa

787 agtggacagggtttcatgg
```

Fig. 1. Nucleotide sequence and deduced amino acid sequence of tea metallothionein-like protein (*Cs*MT). Stop codon was marked with symbols*. Sequence data was deposited in the GenBank (Acc.no. JN315623)

many plants (Ruffel *et al*., 2002; Sheng *et al*., 2004; Xiong *et al*., 2007). Chen *et al*. (2003) reported genes of two metallothionine like protein showing differential expression patterns, which are associated with developmental and environmental cues.

The sequence of the cDNA clone encoding metallothioneine like protein was submitted to NCBI database (NCBI.Acc.No. JN315623). Theoretical analysis of the sequence using Protparam (http://web.expasy.org/compute_pi/) revealed that the pI and MW of the *CsMT* (NCBI Acc.No. JN315623) was 5.37 and 9.5 kDa respectively. BLASTp in NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and multiple alignment analysis by ClustalW indicated that our metallothionine like protein is identical to metallothionein like protein reported in other species, sharing 61 per cent identity with *Vitis vinefera* metallothionine like protein (acc. no. ABX79344) and 59 per cent with metallothionine like protein of *Persea americana* (acc. no. CAB77242). Analysis of amino acid composition (Table 4) showed 55.5 per cent hydrophobic amino acids (Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Cys), 44.5 per cent polar amino acids (Ser, Thr, Asp, Glu, Lys, Arg, Asn, Gln, His,) which consists of 13 per cent charged amino acids. Further, SOPMA analysis revealed that metallothionine like protein was predominantly random coil which mainly consisted of random coils (66.30%) and α-helices (11.96%), while β-turn (6.52%) also contributed to the secondary structure conformations (Fig. 2).

Phylogenetic analysis of (Fig. 3A) metallothionine like protein from *Camellia sinensis* and other plant species using Mega 4 revealed four groups of MT. Group I consisted of MT from *Hevea brasiliensis*, *Jatropha curcas*, *Vigna angularis*, *Citrus unshiu* and *Vitis vinifera*. While the second group consisted of *Brassica rapa* and *Zea mays*, whereas MT from *Solanum nigrum* and *Camellia sinensis* formed two separate groups. Comparison of deduced amino acid sequence of MT with sequences from other plant species revealed presence of conserved region in *CsMT* (Fig. 3B). Similar conserved region was noticed in *MT2* from *Colletotrichum coccodes* infected velvetleaf (Dauch and Jabaji-Hare, 2006). Gene encoding putative metallothionine like protein sequence (acc.no ABR92329) was noticed from *Salvia miltiorrhiza*, a Chinese traditional medicinal herb (Cui *et al.*, 2007). Observed variation in amino acid sequence

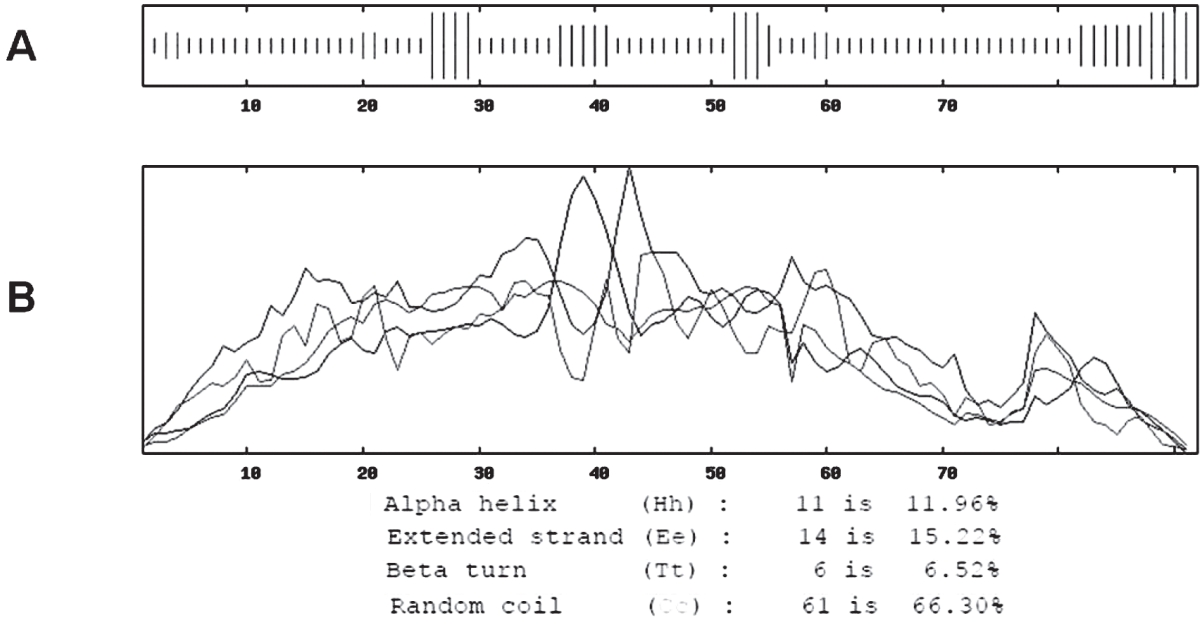

Fig. 2. A. Structural analysis of CsMT. Secondary structure conformation parameters based on SOPMA. Helices, sheets, turns and coils are indicated with the longest, the second longest, the second shortest and the shortest vertical lines respectively. B. Threshold level of predicted secondary structure of CsMT

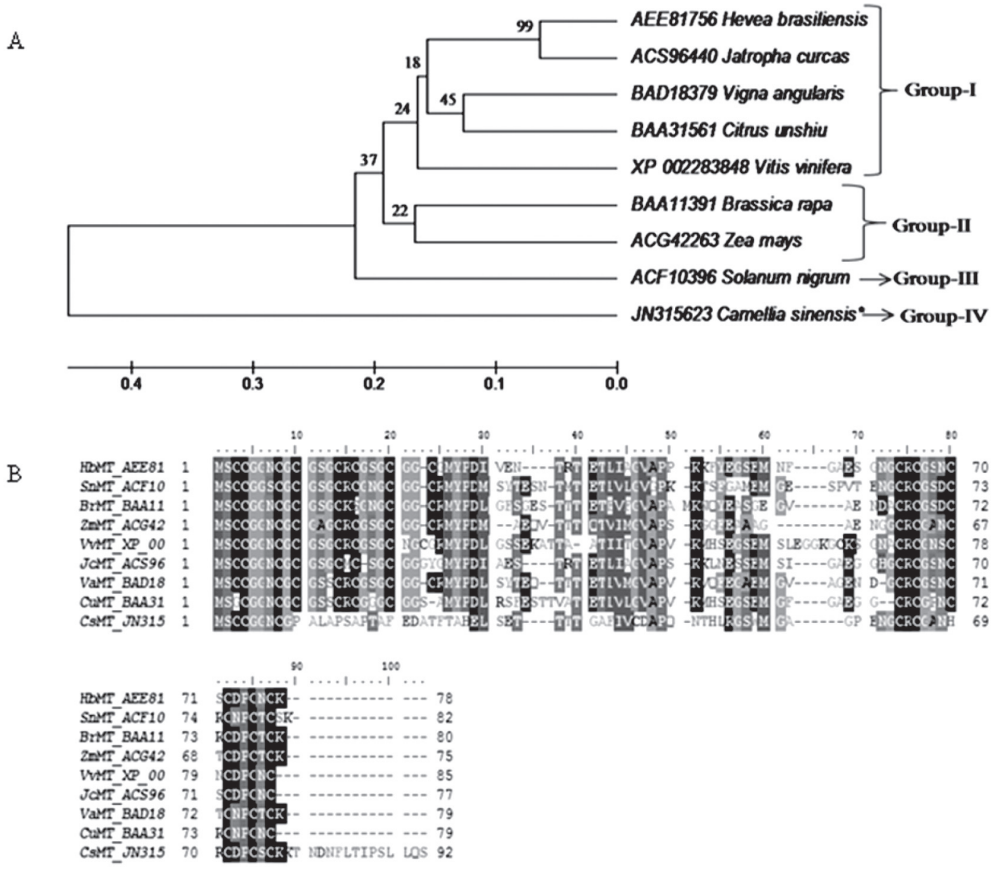

Fig. 3. Phylogenetic analysis of metallothionine like protein (MT). (A) Dendrogram showing the relatedness of amino acid sequence of (MT) from *CsMT* (NCBI.Acc.No. JN315623) with already reported sequences of *MT* in the Genbank. *denotes the amino acid sequence from *CsMT* in the present study. (B) Comparison of the deduced amino acid sequence of *CsMT* with other MT from other plant source

of the *CsMT* from the present study reveals that the cloned *MT* is distinct from other plant species. Further studies on expression pattern of the *CsMT* under different stress conditions will help us to know their function in detail.

Conclusion

In conclusion, the present study provides the information of MT gene sequence, its structural details from tea. Further expression studies on over expression and suppression of MT under different stress condition may help to unravel its potential role in stress response.

Acknowledgement

The authors are thankful to Dr. P. Mohankumar, Director, UPASI TRI for his constant support and encouragement. The authors are also thankful to Tea Board, Kolkata, Govt. of India for financial support under XI-five year plan.

References

Balasaravanan, T., Pius, P.K., Raj Kumar, R., Muraleedharan, N. and Shasany, A.K. 2003. Genetic diversity among south Indian tea germplasm (*Camellia sinensis*, *C. assamica* and *C. assamica spp. lasiocalyx*) using AFLP markers. *Plant Science* **165**: 365-372.

Bowles, D.J. 1990. Defense-related proteins in higher plants. *Annual Review of Biochemistry* **59**: 873-907.

Carpene, E., Andreani, G. and Isani, G. 2007. Metallothionein functions and structural characteristics. *Journal of Trace Elements and Medical Biology* **21**(1): 35-39.

Chen, W.Q., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G.Z., Whitham, S.A. and Budworth, P.R. 2002. Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14**: 559-574.

Chen, H.J., Hou, W.C., Yang, C.Y., Huang, D.J., Liu, J.S and Liu, Y.H. 2003. Molecular cloning of two metallothionein-like protein genes with differential expression patterns from sweet potato (*Ipomoea batatas*) leaves. *Journal of Plant Physiology* **160**: 547-555.

Chen, Z., Kai, G., Liu, X., Lin, J., Sun, X. and Tang, K. 2005. cDNA cloning and characterization of a mannose-binding lectin from *Zingiber officinale* Roscoe (ginger) rhizome. *Journal of Bioscience* **30**: 213-220.

Chen, X., Shang, J., Chen, D., Lei, C., Zou, Y., Zhai, W., Liu, G., Xu, J., Ling, Z., Cao, G., Ma, B., Wang, Y., Zhao, X., Li, S. and Zhu, L. 2006. A B-lectin receptor kinase gene conferring rice blast resistance. *The Plant Journal* **46**: 794-804.

Choi, D., Kim, M., Yun, H.K., Park, J., Kim, W.T. and Hae, S. 1996. Molecular cloning of a metallothionein-like gene from *Nicotiana glufinosa* L. and its induction by wounding and tobacco mosaic virus infection. *Plant Physiology* **112**: 353-359.

Cui, G.H., Mao, Y., Huang, L.Q., Yuan, Y. And Wang, X.Y. 2007. Functional genomics studies of *Salvia miltiorrhiza* III. analyze of *Metallothionein* (*MT-2*) genes. *Zhongguo Zhong Yao Za Zhi*. **32**(14): 1393-1395.

Dauch, A,L and Jabaji-Hare, S,H. 2006. Metallothionein and bZIP transcription factor genes from velvetleaf and their differential expression following *Colletotrichum coccodes* infection. *Phytopathology* **96**: 1116-1123.

Keith, L.M., Velasquez, M.E. and Zee, F.T. 2006. Identification and characterization of Pestalotiopsis spp. causing scab disease of Guava, *Psidium guajava*, in Hawaii. *Plant Disease* **90**(1): 16-23.

Koh, Y.J., Shim, G.H. and Hur, J.S. 2001. Seasonal occurrence and development of gray blight of tea plants in Korea. *Plant Pathology Journal* **17**(1): 40-44.

Margoshes, M. and Vallee, B.L. 1957. A cadmium protein from equine kidney cortex. *Journal of American Chemical Society* **79**: 4813-4814.

Mahomed, W. and Berg, N. 2011. EST sequencing and gene expression profiling of defence-related genes from *Persea americana* infected with *Phytophthora cinnamomi. BMC Plant Biology.* **11**: 167. doi:10.1186/1471-2229-11-167.

Moisyadi, S. and Stile, J. 1995. A cDNA encoding a metallothionein I-like protein from coffee leaves (*Coffea arabica*). *Plant Physiology* **107**: 295-296.

Muraleedharan, N., Hudson, J.B. and Durairaj, J. 2007. *Guiedlines on Tea Culture in South India*. 8th Edn. Coonor: Coonor Printing Press. pp. 13.

Obertello, M., Wall, L., Laplaze, L., Nicole, M., Auguy, F., Gherbi, H., Bogusz, D. and Franche, C. 2007. Functional analysis of the metallothionein gene *CgMT1* isolated from the actinorhizal tree *Casuarina glauca*. *Molecular Plant Microbe Interactions* **20**: 1231-1240.

Ren, W., Zhao, L., Zhang, L., Wang, Y., Cui, L., Tang, Y., Sun, X. and Tang, K. 2011. Molecular cloning and characterization of 4-hydroxyphenylpyruvate dioxygenase gene from *Lactuca sativa*. *Journal of Plant Physiology* **168**: 1076-1083.

Ruffel, S., Dussault, M.H., Palloix, A., Moury, B., Bendahmane, A., Robaglia, C. and Caranta, C. 2002. A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). *The Plant Journal* **32**: 1067-1075.

Sanjay, R. 2004. Studies on *Pestalotiopsis* sp. affecting tea (*Camellia sinensis* (L.) O. Kuntze) in southern India. Ph.D. Thesis submitted to Bharathiar University, Coimbatore, Tamil Nadu.

Shah, D.M. 1997. Genetic engineering for fungal and bacterial diseases. *Current Opinion in Biotechnology* **8**: 208-214.

Sheng, W., Fu-Di, Z., Zu-Jian, W., Qi-Ying, W. and Lian-Hui, X. 2004. Cloning and Sequencing the g subunit of R-phycoerythrin from *Corallina officinalis*. *Acta Botanica Sinica* **46**: 1135-1140.

Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G. and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. *Science* **268**: 661-667.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599.

Tuan, P.A., Park, N.I., Li, X., Xu, H., Kim, H.H., Park, S.U. 2010. Molecular cloning and characterization of phenylalanine ammonia-lyase and cinnamate 4-hydroxylase in the phenylpropanoid biosynthesis pathway in garlic (*Allium sativum*). *Journal of Agricultural Food Chemistry* ***58***: 10911-10917.

Wang, H.Y., Liu, D.Q and Yang, W.X. 2011. A wheat disease resistance gene analog of the NBS-LRR class: identification and analysis. *Journal of Plant Diseases and Protection* **118**: 63-68.

Xiong, Y.H., Xu, Y., Lai, W.H., Li, Y.P. and Wei, H. 2007. Cloning and sequence analysis of the full-length cDNA of a novel yp05 gene associated with citrinin production in *Monascus aurantiacus*. *Biomedical and Environmental Science* **20**: 135-140.

Xu, Y., Zhu, Z., Xiao, Y and Wang, Y. 2009. Construction of a cDNA library of *Vitis pseudoreticulata* native to China inoculated with uncinula necator and the analysis of potential defence-related expressed sequence tags (ESTs). *South African Journal Enology Viticulture* **30**: 65-71.

Zhen-Dong, T., Liu-Jun, Cong-Hua, X.I.E. and Bo-Tao, S. 2003. Cloning of potato POTHR-1 gene and its expression in response to infection by *Phytophthora infestans* and other abiotic stimuli. *Acta Botanica Sinica* **45**: 959-965.

Zhou, N., Tootle, T.L. and Glazebrook, J. 1999. Arabidopsis PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *The Plant Cell* **11**: 2419-2428.