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Role of Hemomucin-Like Genes in *Arabidopsis thaliana*

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Table of Contents

Declaration vi
List of Figures vii
List of Tables ix
Acknowledgment xiii
Abstract xiv
Chapter 1:Literature Review 1
1-1) Pathogenesis Related Genes and Signalling transduction 1
1-1-1) Introduction: 1
1-1-2) Signal transduction and immune response 2
1-1-3) Pathogenesis related genes and proteins 4
1-2) R-gene signalling pathways in plants 6
1-2-1) SA and plant defence 7
1-2-2) Jasmonic acid and plant defence 13
1-2-3) Ethylene and plant defence 15
1-2-4) Cross-talk among different pathways 18
1-2-5) Differential induction of signalling pathways by pathogens 19
1-3) T-DNA tagging and gene function 22
1-3-1) Introduction: 22
1-3-2) Molecular basis of *Agrobacterium*-mediated plant transformation 22
1-3-3) Molecular analysis of T-DNA integration 23
1-3-4) T-DNA tagging vectors 25
1-3-5) Manipulation of *Agrobacterium* for genetic engineering purposes: 25
1-3-6) T-DNA tagged plant populations and isolation of knock-outs: 26
1-4) RNA interference 28
1-4-1) Introduction: 28
1-4-2) RNAi pathway 29
1-4-3) Genetics of RNAi in plants 32
1-4-4) Secondary siRNA 32
1-4-5) Systemic nature of RNA silencing 33
1-4-6) Biological function of RNA silencing 33
1-4-7) Micro RNAs 36
1-4-8) Practical aspects of RNAi/PTGS technique 38
1-5) Conclusion 38

Chapter 2: Materials and Methods 40
2-1) General methods 40
2-1-1) Single-step RNA isolation 40
2-1-2) DNA purification from gel 41
2-1-3) Cloning of PCR Products 41
2-1-4) DNA mini preparation/isolation from Arabidopsis 41
2-1-5) Protein extraction 42
2-1-6) DNA and RNA concentration measurements 43
2-1-7) Preparation of competent *E.coli* cells for heat-shock transformation: 43
2-1-8) Heat shock transformation method 43
2-1-9) Growth of overnight culture from single colonies 44
2-1-10) Preparation of plasmid DNA by alkaline lysis with SDS: mini/midi preparation 44
2-1-11) Bacterial glycerol stock 45
2-1-12) SDS-polyacrylamide gel preparation 45
2-1-13) Staining SDS-polyacrylamide gel with Commasie Brilliant Blue 46
2-1-14) Transfer of proteins from SDS-polyacrylamide gels to solid supports 46
2-1-15) RNA slot blot hybridisation 47
2-1-16) Preparation and ^{32}P labelling of DNA probes 48
2-1-17) Hybridisation and autoradiography 48
2-1-18) Stripping 49
2-1-19) Analysis of RNA by Northern blot 49
2-1-20) Transfer of RNA from gel to membrane 50
2-2) Heterologous expression 51
2-2-1) Cloning strategy in heterologous expression hemomucin like molecules 51
2-2-2) Expression of hemomucin-like molecules 52
2-2-3) Sonication lysis of bacterial cells 53
2-2-4) Purification of insoluble protein under denaturating condition 53
2-2-5) Antibody production 54
2-2-6) Serum preparation 54
2-3) T-DNA knockout line analysis 54
2-3-1) Southern blotting of T-DNA 54
2-3-2) Plant maintenance 55
2-3-3) *Arabidopsis* Crosses 56

2-3-4) Seed sterilisation 57
2-3-5) Analysis of knockout lines by direct PCR 57
2-3-6) Modified Thermal Asymmetric Interlaced (TAIL-) PCR 58
2-3-7) Genetic characterization of mutants 59
2-4) Isolation of RNAi plants 59
2-4-1) Propagating of donor (pDONR 201) and destination vector (pHellsgate 8) 59
2-4-2) Performing the BP recombination reaction 59
2-4-3) Sequencing entry clone 61
2-4-4) Performing the LR recombination reaction 62
2-4-5) Preparation of competent *Agrobacterium* cells for electroporation 62
2-4-6) Electroporation of *Agrobacterium* 63
2-4-7) *In planta* transformation of *Agrobacterium* 63
2-4-8) Selecting transformed plants 64
2-4-9) Non-competitive RT-PCR to study gene expression 65
2-4-10) Quantitative Real Time-PCR analysis of transcripts 66
2-5) Expression analysis 68
2-5-1) Methyl jasmonate treatment 68
2-5-2) Salicylic acid treatment 68
2-5-3) Ethylene treatment 68
2-5-4) Wounding Treatment 69
2-5-5) Phenotypic mapping, root growth 69
2-6) Pathogenicity assays 70
2-6-1) Growth of fungus *Alternaria brassicicola* and plant inoculation 70
2-6-2) Growth of *Botrytis cinerea* fungus and plant inoculation 70
2-6-3) Growth of *Pseudomonas syrigae* and plant inoculation 71
2-6-4) Bacterial pathogen enumeration 72
2-6-5) Growth of *Pythium* and plant inoculation 73
2-6-6) Establishment and inoculation of Cucumber Mosaic Virus (strain K) 73
2-6-7) Maintenance and inoculation of *Fusarium oxysporium* 74
2-6-8) Disease symptom analysis of *Fusarium oxysporium* 74
2-6-9) Trypan Blue Staining 75
2-6-10) Monitoring susceptibility to *Alternaria brassicicola* 75

Chapter 3: Results 77
3-1) Gene Analysis 77
3-1-1) Putative *cis*-acting regulatory DNA elements 77
3-1-2) Expression summary of HML coding genes 79
3-1-3) Phylogenetic Analysis 81
3-1-4) Gene structure 85
3-1-5) Protein Analysis 85
3-1-6) Microarray analysis 88
3-1-7) Conclusion 89
3-2) Analysis of T-DNA knockout lines 90
3-2-1) Obtaining plants with T-DNA inserts in *hml* genes 90
3-2-2) Characterisation of genomic DNA flanking T-DNA 91
3-2-3) Selection of homozygous T-DNA insert in specific T-*hml* knockout loci 93
3-2-4) Estimation of the number of unlinked T-DNA insertions 94
3-2-5) To check the expression of the *hml* genes in related knockout lines 96
3-2-6) Conclusion 96
3-3) RNAi experiment 98
3-3-1) Cloning of a *hml* gene 98
3-3-2) Analysis and selection of RNAi plants 101
3-3-3) Conclusion 103
3-4) Expression analysis of *hml* genes 104
3-4-1) Inducibility of the *hml* genes by salicylic acid 104
3-4-2) Inducibility of the *hml* genes by Methyl jasmonate 104
3-4-3) Inducibility of the *hml* genes by Ethylene 109
3-4-4) Expression profile following inoculation with *A. brassicicola* 110
3-4-5) Wound-induced expression of the *hml* genes 111
3-4-6) Inducibility of the *hml* genes by CMV 112
3-4-7) Conclusion 114
3-5) Pathogenicity assays 116
3-5-1) *Alternaria brassicicola* assay 116
3-5-2) *Botrytis cinerea* assay 119
3-5-3) *Pseudomonas syringae* assay 124
3-5-4) *Fusarium oxysporum* assay 128
3-5-5) *Pythium* assay 129

3-5-6) Conclusion 131

3-6) Heterologous expression of *hml* genes 132

3-6-1) Conclusion 136

Chapter 4: Discussion 137

4-1) Hemomucin-like proteins in animals 137

4-2) Transcription analysis of *hml* genes 139

4-3) *cis*-acting regulatory DNA elements of *hml* genes 144

4-4) Pathogenicity assay of *hml* genes 146

4-5) RNAi technique and responses in *hml* genes 150

4-6) T-DNA knockout lines 151

4-7) Heterologous expression 151

4-8) Conclusion and future directions 151

References 153

Abstract

Hemomucin, a secreted and membrane-bound *Drosophila* immune protein, contains a protein domain, which is conserved among multicellular organisms including plants. The main aim of this project was to test whether related genes in plants thave the same function as their insect orthologs and are involved in plant defence reactions. Bioinformatics and reverse genetics approaches were used to characterise four genes in *Arabidopsis thaliana* that are more similar to animal hemomucin-like genes than to a group of *Arabidopsis* strictosidine synthase genes that are involved in the production of monoterpene indole alkaloids. The four hemomucin-like (*hml*1-4) genes are located on chromosome 3 in tandem repeat structure with similarities of 75-82% among the deduced protein sequences, which suggests that the four genes emerged from gene duplications relatively recently. This is confirmed by inspection of the location and sizes of three intron sequences, which are identical, with one minor exception, in all four genes. The inspection of DNA sequences located upstream of the coding regions suggested similar putative regulatory elements, but with some significant differences among the four gene regions that could indicate independent regulation of the four genes.

The expression of the four individual genes under various conditions was analysed and reverse genetics experiments were performed to experimentally examine whether the four HML-coding genes are regulated separately and to establish their possible functional involvement in plant development and defence against pathogens.

Using PCR primers unique to each of the four genes, real time PCR experiments under conditions, where wild-type plants were treated with elictors, such as salicylic acid, ethylene, methyl jasmonate and pathogens, such as pathogenic fungi and viruses were performed. Expression patterns suggested that the gene coding for the HML1 protein was expressed in the absence of immune induction and did not respond to elicitors and pathogens by up-regulation of transcripts. The other three genes were not expressed in the absence of elicitors, but responded to treatments with various degrees of up-regulation of expression. The genes coding for the HML2 and HML3 proteins showed similar responses to elicitors and pathogens. Both showed intermediate induction of transcription after salicylic acid and methyl-jasmonate treatment and strong induction after treatment with *Alternaria* and virus. The gene coding for HML4 showed intermediate induction after

salicilic acid, ethylene and *Alternaria* treatment but strong induction after methyl-jasmonate, wounding and virus treatment. These observations indicated that the HML1-coding gene has developmental or housekeeping functions, whereas the other three genes are involved in inducible defence functions against pathogens.

To experimentally test mutant lines in a pathogenicity assay, reverse genetic approaches using T-DNA mediated insertion mutants and dsRNA inactivation of HML-coding genes was performed.

Arabidopsis plants from T-DNA insertion mutagenesis experiments were available from various institutions. To use these lines for phenotypic analysis, the insertion sites within the *hml* gene regions and the number of insertion sites in the genome were determined. In addition, homozygous lines for the T-DNA mutations had to be generated. Four mutant lines, each representing T-DNA insertions into one of the *hml* genes, were obtained and were used for phenotypic analysis. The T-DNA insertion sites in the four lines were characterised at the DNA sequence level and offspring of each mutant line were examined for homozygosity of the T-DNA in the HML-coding gene respectively.

Since the four HML proteins show significant sequence similarity, one HML protein may be able to compensate for a mutant protein from another gene. Therefore it was not known whether mutations in individual HML-coding genes would show a phenotype. Since the four genes are closely linked in a tandem repeat gene locus, crossing two or more T-DNA mutations was not an option. We therefore decided to inactivate most, if not all four genes by double-stranded RNA inactivation (RNAi). A transformation vector (pHellsgate 8) was used that contained the *hml* template in an inverse orientation with a spacer in between to allow the RNA to form a stem-loop structure. A 211 bp fragment from the HML1-coding region was used, which had more than 80% sequence similarity among the four genes. *Agrobacterium*-mediated transformation yielded numerous transgenic plants, from which 3 isolates were characterised further. RNAi-1 and RNAi-5 showed reduction of all four transcripts up to four-fold. RNAi-32 showed similar reduction of transcripts in *hml*1-3, but more than two-fold over-expression in the gene coding for HML4. The unexpected induction of *hml*4 transcripts remains to be investigated. It is possible that *hml*4 may respond to double-stranded RNA as an elicitor with immune-induction, in addition to gene-specific degradation of *hml* transcripts. A prediction of this assumption is that *hml*4 transcripts are induced in RNAi-plants producing dsRNA from unrelated genes. Given the possible variation of *hml* transcripts by simultaneous induction and suppression of gene expression, the phenotypes of RNAi plants can be expected to be highly variable.

Both T-DNA insertion mutants (*t-hml1-4)* and RNAi plants (RNAi-1, RNAi-5 and RNAi-32) were examined in pathogenicity assays and compared to wild-type plants and specific mutants affected in defence-related pathways.

When *Alternaria* fungal spores were counted on wild-type and mutant plants, significant differences were observed on the T-DNA insertion mutant *t-hml3*, *coi1-16* and *npr-1,* with the latter two mutants being defective in the methyl-jasmonate and salicylic acid response, respectively. In drop-inoculation of *Arabidopsis* leaves by the fungus *Botrytis,* a significant increase in lesion size and number of spreading symptoms was observed in *t-hml4* plants, together with highly significant size increases in the ethylene pathway mutants *ein2-1* and *etr3*. A difference in lesion size and type of response was observed in RNAi-32 plants, which require further investigation. When plants were inoculated with bacteria from the strain *Pseudomonas syringae,* higher numbers of bacteria were found in RNAi-32 and *npr-1* plants, compared to other mutants and wild-type plants.

When a systemic acquired resistance (SAR) assay was performed with wild-type and mutant plants differences were observed in response to some elicitors. In this assay, plants were inoculated with a compatible strain of *P. syringae* on one side of the plant and three days later on the other side with an incompatible *P. syringae* strain, containing the *Rpm1* gene. Under these conditions, an increased bacterial titre, which represents less acquired resistance, was observed in the *t-hml4* mutant.

Taken together, these data suggest that the four HML-coding genes are regulated individually upon exposure to elicitors. Whereas three of the genes (*hml*2-4) are expressed at low levels in the absence and up-regulated in the presence of elicitors, one of the genes (*hml*1) is expressed in the absence but nor significantly up-regulated by elicitors.