

LIU Qing, FENG Dongxin, WANG Xiaowu, DU Yongchen

Cloning and functional analysis of the genes involved in signal transduction in tomato *Cf-4-Avr4* pathosystem

© Higher Education Press and Springer-Verlag 2007

Abstract Hypersensitive response (HR) is one of the most efficient and common resistance mechanisms in plants. Cloning signaling genes are very important to elucidate the resistance mechanisms. A gene in tomato homologous to several resistance proteins in plant was involved in HR and named as *RGL* (Resistance Gene Like). *RGL* protein was used as a bait to screen interacting protein(s) from tomato cDNA library through the yeast two-hybrid system. Two interacting proteins were found, which were called as *RGLIP-1* and *RGLIP-2* (*RGL* Interacting Protein), respectively. *RGLIP-1* is a protein of 291 amino acids with significant homology with thylakoid lumen protein. *RGLIP-2* is a protein of 248 amino acids with significant homology with transducin protein. Virus-Induced Gene Silencing (VIGS) of the two genes results in a partial and complete suppression of *Avr4*-induced HR, which indicates that both genes are involved in hypersensitive response.

Keywords tomato, *Cladosporium fulvum*, hypersensitive response, yeast two-hybrid system, virus-induced gene silencing

1 Introduction

Leaf mold is one of the most important diseases in tomato production, and the *Cladosporium fulvum*-tomato interaction mechanism is a hypersensitive response (HR). As one of the most efficient and common resistance mechanisms in plants, the HR is an active defense response and is triggered upon recognition of a virulence factor (*AVR*) of *C. fulvum* and a matching resistance *Cf* gene in plant. The interaction between the pathogenic fungus *Cladosporium fulvum* and tomato has served as a model system for studying the mechanism of HR.

Translated from *Acta Horticulturae Sinica*, 2006, 33(1): 52–57 [译自: 园艺学报]

LIU Qing, FENG Dongxin, WANG Xiaowu, DU Yongchen (✉)
Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China
E-mail: yongchen.du@mail.caas.net.cn

Four avirulence genes (*Avr2*, *Avr4*, *Avr4E*, and *Avr9*) in the pathogen and the corresponding resistant genes (*Cf-2*, *Cf-4*, *Cf-4E*, and *Cf-9*) in plants have been cloned (Jones et al., 1994; Cai et al., 2001; Luderer et al., 2002; Westerink, 2003). As reported, the interaction of *Cf* and *AVR* can cause cell death in potato and tobacco and the signaling transduction pathway is conservative in Solanaceae plants (Piedras et al., 1998; De Jong et al., 2000). Therefore, cloning the genes involved in the HR pathway is significant to understand the HR mechanism and improve the resistance of the variety. Because the signal transduction of the HR is still obscure as to how this process is initiated and executed, it is necessary to study the unknown components.

The yeast two-hybrid system is a molecular genetic tool that facilitates the study of protein-protein, protein-nucleic acid, and protein-small molecule ligand interactions, especially in the field of signal transduction. Virus-Induced Gene Silencing (VIGS) has recently been developed as a powerful method for the identification of resistant gene functions in plants (Ratcliff et al., 2001; Holzberg et al., 2002; Jin et al., 2003).

Frank analyzed the functions of differentially expressed genes in the proceeding of HR by VIGS and identified 20 fragments suppressing HR. In this study, five full-length cDNAs of these fragments were isolated, from which one *RGL* (Resistance Gene Like) was screened, and the interaction proteins were analyzed by the yeast two-hybrid system and VIGS.

2 Materials and methods

2.1 Materials

Experimental materials used in this study were eukaryotic expressing vector pGBKT7, yeast strain PJ69-4a, full-length cDNAs tomato library inoculated by aphids, transgenic tobacco with *Cf-4*, *Agrobacterium tumefaciens* GV3101 containing binary vector pRH78:*Avr4*, *Agrobacterium tumefaciens* GV3101 containing different VIGS vector, empty virus vector containing TRV:00, virus vector

containing *RGL* (TRV:*RGL*), which were all afforded by the Laboratory of Phytopathology, Wageningen University and Research Center. High fidelity Taq enzyme was bought from TaKaRa Co. and restriction endonucleases *Nco* I, *Sma* I, and *Sal* I were purchased from Promega Co..

2.2 Methods

2.2.1 Analysis of the gene sequence involved in the hypersensitive response

The five nucleotide sequences involved in the HR were translated into amino acid sequences and submitted to GenBank database, finally, the conservative function domains were found by analyzing the homology with BLASTp.

2.2.2 Construction of recombinant plasmid for yeast two-hybrid system

The primers were designed based on the *RGL* gene sequence, and *Nco* I restriction enzyme cutting site was added to the forward primer to make PCR amplification with *RGL* as a template, and the primer sequence was as follows:

F: 5'- GGGATCCATGGTTGATGTAGGGGTTGA -3'

R: 5'- GGGGCATGTTCAATATGTCT -3'

The PCR product retrieved from the gel was purified, and digested with *Nco* I, and retrieved after electrophoresis. At the same time, pGBKT7 (BD plasmid) was digested with *Nco* I and *Sma* I, respectively, and the product was retrieved after electrophoresis. The bait plasmid pGBKT7/*RGL* was constructed with these two products. The tomato cDNA phage library was transferred into the activation domain (AD) plasmid library.

2.2.3 Yeast two-hybrid analysis

The bait plasmid and AD plasmid were co-transformed into yeast PJ69-4a strain by lithium acetate method as described in the MATCHMAKER library protocol (Clontech). At first, the strains containing AD plasmid and bait plasmid were screened on the medium lacking L-tryptophan and L-leucine, then screened on the medium Try⁻/Leu⁻/Ade⁻/His⁻, cultivated for 2 days at 28°C. The colonies more than 2 mm in diameter were rescreened on the medium Try⁻/Leu⁻/Ade⁻/His⁻ and cultivated for 2 days at 28°C, finally the AD plasmid was isolated from the positive colonies for sequence analysis.

2.2.4 Construction of VIGS vector

The primers were designed based on the gene sequence of the two interacting proteins, with *Bam*H I and *Asp*718 cutting sites added to the forward and backward primers, respectively. The sequences of primers were as follows:

The interacting protein 1:

F: 5'- CTGGATCCGACTGTATACTCTCACTGGA -3',

R: 5'- CTGGTACCAGTTCATACTTACCAACTCCAG -3'

The interacting protein 2:

F: 5'- CTGGATCCATTCCAAGTTATCGAATCCA -3',

R: 5'- CTGGTACCGGTAGGTGAAGGCTTCAACT -3'

The two PCR products and VIGS empty vector were digested with *Bam*H I and *Asp*718, respectively then the digested products of the two PCR products and VIGS empty vector were ligated, respectively with TRV:00 to construct TRV:*RGLIP*-1 and TRV:*RGLIP*-2, which were transformed into *Agrobacterium tumefaciens* GV3101.

2.2.5 Function of the interacting proteins by VIGS analysis

The first and second leaves were inoculated with *Agrobacterium tumefaciens* GV3101 containing different VIGS vectors in *Cf*-4 transgenic tobacco by injection, with TRV:00 as negative control and TRV:*RGL* as positive control. TRV:*RGLIP* was used to detect the function of *RGL* interacting proteins in the proceeding of HR. The third and fourth, the fifth and sixth leaves were inoculated with *Agrobacterium tumefaciens* GV3101 containing pRH78:*Avr*4 after three and five weeks, respectively, and hypersensitive response was investigated 3–5 days later. This experiment was repeated for three times.

3 Results

3.1 Analysis of the gene sequences involved in HR

The sequence analysis indicated that one gene showed homology with many genes such as *Prf*, *Bs2*, and was named *RGL*. The amino acid from 167 to 439 is the NB-ARC conservative domain which is one kind of signal transduction domain in many resistant genes (Fig. 1). It was chosen as the bait protein for yeast two-hybrid.

3.2 Interacting protein analysis

One million yeast colonies containing two plasmids were obtained, 43 positive colonies were further screened, and 3 positive colonies were obtained by excluding the autonomous transcriptional activity (Plate I-A–B).

Analysis of the screening showed that two interacting proteins, *RGLIP*-1 and *RGLIP*-2, were identified from tomato cDNA library. *RGLIP*-1 was a protein of 291 amino acids with 63% homology with thylakoid lumen protein (Fig. 2). *RGLIP*-2 was a protein of 248 amino acids with 60% homology with transducin protein (Fig. 3).

3.3 Function analysis by VIGS

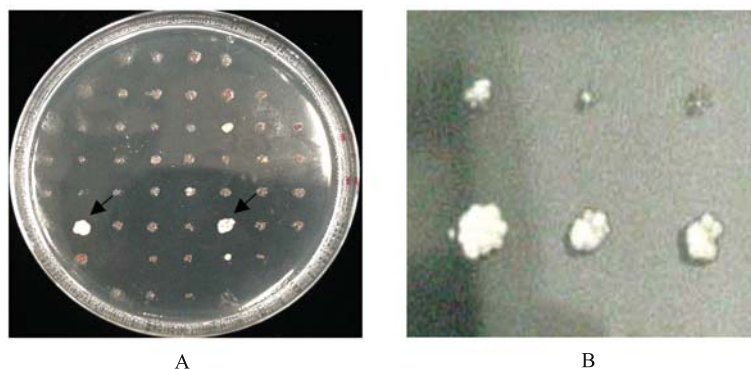
HR was induced by treating with *Avr*4 in *Cf*-4 transgenic tobacco. In the early days, the leaves withered, then died. The empty vector TRV:00 was found unable to affect the origination of HR (Plate II-C). The positive control TRV:*RGL*

```

1  MVDVGVFLEENLKQLVLDNVELIGGAKDEIENLRDDLSEFNAFLKQAAMVRS ENPVLKELVRSIRKVVNRAED
75  AVDKFVIEAKVHKDKGFKGVFDKPGHYRRVRDAAVEIKGIRDKMREIRQNKAHGLQALLQDHDSSIRSGEERQ
149 P PVVEEDDVVGFDDAQTVIDRLL EGGSGDLEVI PVVGMPLGK T T L A T K I F K H P K I E Y E F F T R L W L Y V S Q S Y K T
223 R E L Y L N I I S K F T G N T K H C R D M S E K D L A L K V Q E I L E E G G K Y L I V L D D V W S T D A W D R I K I A F P K N D K G N R V L L T T R
297 D H R V A R Y C N R S P H D L K F L T D E E S W I L L E K R A F H K A K C L P E L E T N G K S I A R K C K G L P L A I V V I A G A L I G K S K T I K
371 E W E Q V D Q S V G E H F I N R D Q P N S C D K L V R M S Y D V L P Y D W K A C F L Y F G T F P R G Y L I P A R K L I R L W I A E G F I Q Y R G D L
445 S P E C K A E E Y L N E L V N R N L V M V M Q R T V D G Q I K T C R V H D M L Y E F C W Q E A T T E E N L F H E V K F G G E Q S V R E V S T H R R L
519 C I H S S V V E F I S K K P S G E H V R S F L C F S P E K I D T P P T V S A N I S K A F P L L R V F D T E S I K I N R F C K E F F Q L Y H L R Y I A
593 F S F D S I K V I P K H V G E L W N V Q T L I V N T Q Q I N L D I Q A D I L N M P R L R H L L T N T S A K L P A L A N P K T S K T T L V N Q S L Q T
667 L S T I A P E S C T E Y V L S R A P N L K K L G I R G K I A K L M E P S Q S V L L N N V K R L Q F L E N L K L I N V G Q I D Q T Q L R L P P A S I F
741 P T K L R K L T L L D T W L E W D D M S V L K Q L E N L Q V L K L K D N A F K G E N W E L N D G G F P F L Q V L C I E R A N L V S W N A S G D H F P
815 R L K H L H I S C D K L E K I P I G L A D I C S L Q V M D L R N P L N Q Q N L P E R Y K P K K T S C N L L N P R S S S F L Y S L L I L M Y R Q L L
889 R K V

```

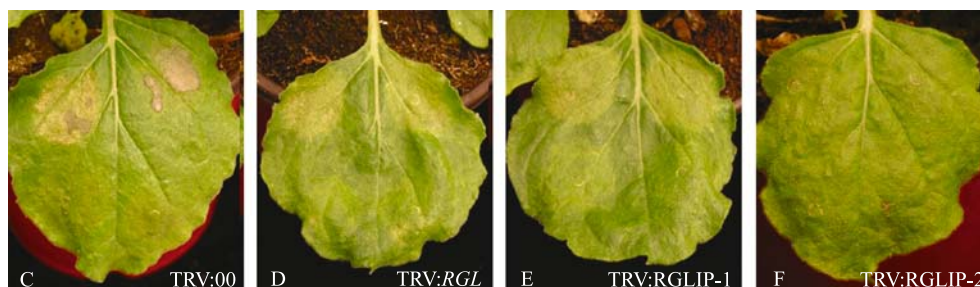
Fig. 1 Sequence and putative conserved domain of *RGL*



A: Screen on Try⁻/Leu⁻/Ade⁻/His⁻ medium (arrows indicate candidate clones)

B: Auto activation test (the upper three clones are the yeast with empty BD plasmid and AD plasmid; the lower three clones are the yeast with bait BD plasmid and corresponding AD plasmid)

Plate I Screening result of yeast two-hybrid



C: Negative control; D: Positive control; E and F: RGLIP-1 and RGLIP-2

Plate II Functional analysis of *RGLIP* genes using VIGS in *Cf-4* transgenic *Nicotiana benthamiana* Plants

resulted in weakening HR (Plate II-D). RGLIP-1 also showed a partial suppression hypersensitive response by VIGS (Plate II-E), RGLIP-2 suppressed hypersensitive response reaction completely (Plate II-F).

4 Discussion

Five genes involved in HR were screened by VIGS in tomato, the analysis of one *RGL* sequence suggested that there was a NB-ARC conservative domain, which was one kind of signal transduction domain in many resistant genes. This result proved that there were some proteins interacting with this

protein in tomato cells. As reported, this function domain was one kind of regulators of cell death in animals (van der Biezen et al., 1998).

RGL protein was used as bait to screen interacting protein(s) from tomato cDNA library through the yeast two-hybrid system. Two interacting proteins named RGLIP-1 and RGLIP-2 (*RGL* Interacting Protein) were found. RGLIP-1 showed 63% homology with thylakoid lumen protein in the chloroplast. It was reported that the decline of the chloroplast could accelerate HR, the interaction of *RGL* and RGLIP-1 resulted in chloroplast decline and accelerating HR (Heldin, 1995). It is suggested that NB-ARC is the key regulator to activate cell death in plants and animals especially. The other interacting

ATAOGATGTTCCAGATTAOGCTAGCTTGGGTGGTCAT

38 ATG GCC ATG GAG GCC OCG GGG ATC CGA ATT CGG CAC GAG GOC AAT ATC TCT GCT TCT
M A M E A P G I R I R H E A N I S A S

95 TAC AAG AAA ACA AAA AAA ATA AAA ACA GAA ATG GCG ACT CTT TCA TCT TCA TCT TCA
S K K T K K I K T E M A T L S S S S S

152 TCT TCA TCT OCA TGT TTG AAC CAG TAC CAG TAT CAA GCT ATT CTT CGC TTG CCA CGT
S S S P C L N Q Y Q Y Q A I L R L P R

209 GTC CCT TTA ATT TCC TCT CAT CTT CTT AAA GTT OCC AAG AAA AAT CGA AAC TCA CTT
V P L I S S H L L K V P K K N R N S L

266 ATT TTC TGC TGC AAC AAC ACT GTG CCT GAT TCA AGA ACA GGT GAG CAA GTT AAA GGA
I F C C N N T V P D S R T G E Q V K G

323 GAA TGC GTA ACC AAG AGA AGA GAG CTC CTG CTA CAG GCA GGC TCT GTT GCA TTT TCT
E C V T K R R E L L L Q A G S V A F S

380 CTG TCC GCC TTT ACA TCG ATT GCA TTG GCA GAG AAG GAT GTC CCG GAG GAG TTT CGT
L S A F T S I A L A E K D V P E E F R

437 GTT TAT TCA GAT GAT GTC AAC AAG TTT AAG ATC ATG ATA CCT AGT GAT TGG CAA ATA
V Y S D D V N K F K I M I P S D W Q I

494 GGC GCG GGA GAA GGT GAT GGA GTA AGG TCA CTC TTA GCT TTC TAT CCT OCA GAA GCT
G A G E G D G V R S L L A F Y P P E A

551 TCT AAC TCA AAT GTC AGC ATA GTA ATC ACA AGC CTT GGT GCT GAT TTC ACC AAG TTG
S N S N V S I V I T S L G A D F T K L

608 GAA TCT TTC GGG AAA GTT GAT GCT TTT GCT GAG AAT CTG GTC AGC GGA TTT GAT AGA
E S F G K V D A F A E N L V S G F D R

665 AGC TGG CAA AGG CCT OCG GGA GTG AAA GCA AAA CTC ATA GAT AGC AAA GCT TCT AAA
S W Q R P P G V K A K L I D S K A S K

722 GGG TTG TAT TAC ATC GAG TAC ACT CTC CAA AAT OCC GGT GAA AGT CTC AGA CAT CTA
G L Y Y I E Y T L Q N P G E S L R H L

779 TTT TCA GTG CTT GGG ATA GCA AAC AAT GGG ATT TAC AAC AGA CTG TAT ACT CTC ACT
F S V L G I A N N G I Y N R L Y T L T

836 GGA CAG TTT GTA GAC GAG GAG GCA GAG AAA TAT GGT GCC AAA ATA CAG AAG GCT GTT
G Q F V D E E A E K Y G A K I Q K A V

893 TCT TCT TTC AGA TTA ATA TGA TGACATGAACAGAGAGOGCGATATCGCAAATTTTGGCTTGAGCTTCT
S S F R L I *

GGTTTTTCTCGTTTGGTGAATGGTAAACATAATTGAGAGOGCGATATCACAGATTCAAGTTCTGGTTAAGGTATATAT
GACGACTOGAGAAAAAACTGGAGTTGTAAGTATGAACTAGCAACTTGATCAATGTTAGAGTTAGTATTTGCATATATOG
TTATAAOCAAAACCTGTATOGATTTTTTGATAAAAAATATGACCTTAGTGCAAATAATTTGAKGCTCAAGTTTTGATTAT
ATATTTGTA CTCTACTOCCGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCTCGAGAGATCTCTAATCG

Fig. 2 Nucleotide sequence and deduced amino-acid sequence of RGLIP-1 isolated from tomato cDNA library through yeast two-hybrid system

protein RGLIP-2 had a 60% homology with transduction protein in arabisopsis, named G protein. There are two types of G proteins in the live cell, one type is made of three subunits (α , β , γ), and the other type has only one subunit. As membrane binding proteins, G proteins participate in many kinds of cell signal transduction by coupling with the receptors on the surface of cells (Freissmuth et al., 1989; Neer and Clapham, 1988; Neubig, 1994). It has been reported that

small G proteins usually transmit the resistant reaction in plants (Ono et al., 2001) , but it is not clear which type the RGLIP-2 protein is, thereby, it is essential to make further study about it.

In this study, *RGL* and the two interacting proteins possibly existed in the same signal transduction branch or at the signal transduction branch point. Besides, the HR was suppressed partially, *RGL* may not be silent effectively and

GAATTCGGCACGAGG

CTATTCAGAATCCTTCCAGAAAGGAGTTAGTGACAAGGCAGTAAACCAACTGGAGAAAACAGTTAGCTCTAAACTTGA
 AGCTTCTGTTGCTAGGCA AATTCAAGCACAATTCCAGAOCTCTGGCAAGCAAGCTCTTCAGGAACTTTGAAATCTATA
 174 ATG GAA GGT TOG GTG ATT OOC GGC TTT GAG ATG TCA TGC AAG GCA ATG TTT GAG CAA
 M E G S V I P G F E M S C K A M F E Q
 231 GTA GAT TTG AOG TTT CAA AAA GGA TTT GCT GAA CAC ACT GGT TOC GCT CTA CAG CAA
 V D L T F Q K G F A E H T G S A L Q Q
 288 TTT GAG TOC ATG CAT TCA OOG TTA GTA CAT GCT TTA AGG GAT GOT ATT AAT TCT GCA
 F E S M H S P L V H A L R D A I N S A
 345 TCA TOG ATG ACT CAA ACA TTG AGT GGA GAG CTA GCT GAT GGT CAA AAG AAC TTG CCT
 S S M T Q T L S G E L A D G Q K K L L
 402 ACA CTT GCA GTT TCA GGA GCA AAT TOC AAG TTA TOG AAT CCA CTG GTT AGC CAC ATG
 T L A V S G A N S K L S N P L V S H M
 459 AGT AAT GGA OCA TTA CTG CAT GAG AAG CTT GAA GCT OCT GTT GAT OCA ATC AAA GAG
 S N G P L L H E K L E A P V D P I K E
 516 TTA TCA AGA TTG TTG GOG GAG OGC AAG TAC GAG GAG GCA TTC ACT ACA GOC TTG CAC
 L S R L L A E R K Y E E A F T T A L H
 573 AGA ACT GAT GTG TCT ATT GTA TCA TGG TTA TGT TTG CAG GTT GAT CTG TOG GGT ATC
 R T D V S I V S W L C L Q V D L S G T
 630 TTG TCA ATG AAT CCT CTC OOG TTG AGT CAA GGT GTA CTT CTT TCA CTT CTT CAG CAG
 L S M N P L P L S Q G V L L S L L Q Q
 687 GTG GOG TGT GAT ATT AOC AAT GAG ACA TCT OGA AAA TTA TOC TGG ATG AGG GAT GTG
 V A C D I T N E T S R K L S W M R D V
 744 GTA TCA GOC ATA AAT CCA ACT GAC COG GTG ATT GTA CTG CAC GTA OGG CCT ATT TTC
 V S A I N P T D P V I V L H V R P I F
 801 GAG CAA GTA TAT CAA ATA CTA AAC CAT CAT OGG ACT CTA CCT AOC ACA AOC OOC GCG
 E Q V Y Q I L N H H R T L P T T T P A
 858 GAA CTT TCA AGC ATT CGC CTT ATA ATG CAT GTT ATC AAC TCT ATG CTG CAT GTT OCA
 E L S S I R L I M H V I N S M L H V P
 915 TGC TAA TGA GAOCTGTAATGATTTTTCAOGAACAACATACTATTOGOCTGGTGAGTTATTAAGTTGTAC
 C *

AAATGTTCTGGATAGACAAAAAGTACTATAGAGTTGAAGOCTTCAOCTACCTTTTATGTTGATTTCTACTGTTGATTT
 TTTTTTCC AAGGTA AATCTTATAGTCGGGTGGGTGGGATTTTCGGGTTTGTGAGTATTAGTCTTTTTTGGTTGGA
 AGTATAGTGTATACAAATTTTTGTACTTTGTACATTGTTATCTTACTGCAATTTCAAATGGTACTGTAAAAA AAAAA
 AAAAAA AACTOGAG

Fig. 3 Nucleotide sequence and deduced amino acid sequence of RGLIP-2 isolated from tomato cDNA library through yeast two-hybrid system

some transcripts may be translated into proteins which expressed the function of *RGL*. Actually, half of the cDNA library was screened by yeast two-hybrid system, and new interacting proteins would be found if the other half library was screened.

References

- Cai X, Takken F L W, Joosten M H A J, De Wit P J G M (2001). Specific recognition of AVR4 and AVR9 results in distinct patterns of hypersensitive cell death in tomato, but similar patterns of defence-related gene expression. *Molecular Plant Pathology*, 2(2): 77–86
- De Jong C, Honée G, Joosten M H A J, De Wit P J G M (2000). Early defence responses induced by AVR9 and mutant analogues in tobacco cell suspensions expressing the *Cf-9* resistance gene. *Physiological and Molecular Plant Pathology*, 56(4): 169–177
- Freissmuth M, Casey P J, Gilman A G (1989). G proteins control diverse pathways of transmembrane signaling. *FASEB Journal*, 3(10): 2125–2132
- Heldin C H (1995). Dimerization of cell surface receptors in signal transduction. *Cell*, 8(2): 213–223

- Holzberg S, Brosio P, Gross C, Pogue G P (2002). Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant Journal*, 30(3): 315–327
- Jin H L, Liu Y D, Yang K Y, Kim C Y, Barbara B, Zhang S Q (2003). Function of a mitogen-activated protein kinase pathway in N gene-mediated resistance in tobacco. *Plant Journal*, 33(4): 719–731
- Jones D A, Thomas C M, Hammond-Kosack K E, Balintkurti P J, Jones J D (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*, 266(5186): 789–793
- Luderer R, Takken F L W, de Wit P J G M, Joosten M H A J (2002). *Cladosporium fulvum* overcomes *Cf-2*-mediated resistance by producing truncated AVR2 elicitor proteins. *Molecular Microbiology*, 45(3): 875–884
- Neer E J, Clapham D E (1988). Roles of G protein subunits in transmembrane signalling. *Nature*, 333(6169): 129–134
- Neubig R R (1994). Membrane organization in G-protein mechanisms. *FASEB Journal*, 8(12): 939–946
- Ono E, Wong H L, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K (2001). Essential role of the small GTPase Rac in disease resistance of rice. *Proceedings of the National Academy of Sciences of the United States of America*, 98(2): 759–764
- Piedras P, Hammond Kosack K E, Harrison K, Jones J D G (1998). Rapid, *Cf-9*- and *Avr9*-dependent production of active oxygen species in tobacco suspension cultures. *Molecular Plant-Microbe Interaction*, 11(12): 1155–1166
- Ratcliff F, Martin-Hernandez A M, Baulcombe D C (2001). Tobacco rattle virus as a vector for analysis gene function by silencing. *Plant Journal*, 25(2): 237–245
- van der Biezen E A, Jones J D (1998). The NB-ARC domain: a novel signaling motif shared by plant resistance gene products and regulators of cell death in animals. *Current Biology*, 8(7): R226–227
- Westerink N (2003). The role of *AVR4* and *AVR4E* proteins in virulence and avirulence of the tomato pathogen *Cladosporium fulvum*. Dissertation for the Doctoral Degree. Wageningen: Wageningen University