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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：Study on function of type III effectors in *Ralstonia solanacearum*
Title of Dissertation（青枯病菌におけるIII型エフェクターの機能に関する研究）

学位論文要約：
Dissertation Summary

Ralstonia solanacearum is a devastating plant pathogen with a global distribution and invades an unusually wide host range, including economically important crops such as tomato, potato, tobacco, and eggplant. Like other gram-negative phytopathogens, *R. solanacearum* possesses a type III secretion system (T3SS) and injects type III effectors (T3Es) directly into the host plant cells. Type III effectors, once internalized in the plant cells, interact with plant substrates either to activate or suppress plant defense systems, resulting in the hypersensitive response (HR) or disease promotion. Bacterial T3Es contribute to disease development, although the mechanisms, by which each T3E functions inside plant cells, are not fully understood. The objective of my study is to investigate the functions of T3Es of *R. solanacearum.*

A well-known family of type III effector, RipG (formerly GALA), consisting of seven genes contains an F-box domain and leucine-rich repeats (LRRs). The F-box domain resembles eukaryotic F-box proteins, which form SCF ubiquitin ligase complex in combination with Skp1 and Cullin1 and control specific protein ubiquitination. RipG effectors are known to interact with Skp1-like protein in *Arabidopsis thaliana* through their F-box domains. Several reports indicate non-eukaryotic F-box proteins act by hijacking host SCF ubiquitin ligase complex for disease development. However, proteins targeted by the complex are still unknown.

In my first study, in order to find target proteins by the hijacked E3 ubiquitin ligase, I investigated the proteins that interact with RipG T3Es of *R. solanacearum*. In my second study, I analysed the binding sites for host proteins

(Skp1 and chloroplastic) in RipG T3Es of *R. solanacearum*. In my final study, I investigated the involvement of two avirulence genes, *avrA* (*ripAA* in unified nomenclature) and *popP1* (*ripP1* in unified nomenclature), of Japanese *R. solanacearum* strains in the pathogenicity to tobacco.

Chapter I: Yeast Two-Hybrid Screening of plant proteins interacting with type III RipG effectors of *Ralstonia solanacearum*

Yeast two-hybrid (Y2H) screening of *Nicotiana benthamiana* and *Nicotiana tabaccum* cDNA library was conducted to find out plant proteins interacting with the RipG effectors of *R. solanacearum* OE1-1 strain.

Materials and Methods:

Total RNA was purified from leaves of *Nicotiana tabacum* or *Nicotiana benthamiana* using RNAiso plus (Takara). After first strand cDNA synthesis, plant cDNA was fused to yeast GAL4 activating domain (AD) in pGADT7-Rec vector in Y187 yeast cells using Make Your Own “Make & Plate” Library System (Clontech). Yeast cells were spread on SD (synthetic defined) agar medium without leucine (SD/-Leu) to construct a prey library.

Seven *ripG* genes were PCR amplified and fused to yeast GAL4 DNA-binding domain (DNA-BD) in pGBKT7 vector (Clontech) with the in-fusion cloning method (Clontech). Y2HGold yeast cells were transformed with the recombinant plasmids and spread on SD agar medium without tryptophan (SD/-Trp).

Y2HGold cells containing bait plasmids grown in SD/-Trp liquid media were mated with 1 ml of prey library cell suspension and then plated on the agar media of SD/-Trp/-Leu, referred as minimal media double dropouts (DDO), with aureobasidin A (DDO/A). Colonies appeared on these agar media were transferred on the agar media of SD/-Trp/-Leu/-adenine/-histidine, referred as minimal media quadruple dropouts (QDO), with

aureobasidin A and X-α-Gal (QDO/X/A) for a higher stringency selection to eliminate false positives. The prey plasmid was rescued from the blue colonies grown on QDO/X/A agar media.

The protein screened by Y2H system was identified from *N. benthamiana* draft genome sequence v1.0.1 and *N. tabacum* BX genome in Sol Genomics Network (https://www.sgn.cornell.edu/). The full length of cDNA was PCR amplified using plant cDNA as a template and cloned into the linearized pGADT7 AD vector with the in-fusion cloning method. Two plasmids containing either the bait plant gene or the prey *ripG* gene were put into Y2HGold cells and plated on the DDO agar media. Transformants were grown in YPDA broth and spotted on the QDO/X/A agar media with series of dilutions, 10^{-1}, 10^{-2} and 10^{-3}.

Results:

Tobacco Skp1s of both *N. benthamiana* and *N. tabacum* were observed to interact with RipG effectors, RipG1, RipG2, RipG5, RipG6, and RipG7. No proteins were screened with RipG3 and RipG4. Full lengths of *skp1* cDNA, *Nbskp1a*, *Nbskp1b*, *Ntskp1a*, and *Ntskp1b*, were amplified from mRNAs of *N. benthamiana* and *N. tabacum* and cloned into the prey expression pGADT7 AD vector. All four Skp1s interacted well with RipG effectors except RipG3 and RipG4.

I found that several tobacco proteins rather than Skp1 interacted with RipG effectors, especially RipG2 and RipG7. Although identified plant proteins were quite diverse, most of them turned out to be chloroplastic proteins, which are encoded in the nucleus. Among the identified plant proteins, I cloned the full lengths of cDNAs of chlorophyll a-b binding protein 13, chaperonin-like RbcX protein, and ribulose bisphosphate carboxylase small chain from *N. benthamiana* mRNA, *Nbcab13*, *NbrbcX*, and *NbrbcS*, respectively, into the prey expression pGADT7 AD vector. All three chloroplastic proteins interacted well with RipG2 and RipG7.

Conclusion:

I found that RipG effectors, especially RipG2 and ripG7, interact with chloroplastic proteins of *N. tabacum* and *N. benthamiana*. I speculate that SCF^{RipG} complex interacting Skp1 with RipG effectors would target the plant chloroplastic proteins for ubiquitination and subsequent degradation.

In order to survive and spread in the plant, *R. solanacearum* might control the photosynthetic activity by degrading the chloroplastic proteins through SCF^{RipG} complex. So, it is tempting to speculate that *R. solanacearum* RipG effectors target the chloroplastic proteins of host plants to inhibit photosynthesis and impair plant immunity for disease development.

Chapter II: Identification of binding sites for Skp 1 and chloroplastic proteins in RipG2 and RipG7 type III effectors of *Ralstonia solanacearum*

From my first study, I reported several chloroplastic proteins, in addition to the Skp1, interact with the RipG effectors, however, the binding sites for those proteins were unknown. In this study, I prepared domain deletion versions of Rip G effectors to find the binding sites for Skp1 and chloroplastic proteins. Yeast-three hybrid assay was conducted to test if Skp1 and chloroplastic protein interact with RipG effector at the same time.

Materials and Methods:

The F-box domain was deleted from RipG2 to construct RipG2dF. The F-box domain, LRR, and N-terminal region were deleted from RipG7 to construct RipG7dF, RipG7dL, and RipG7dN respectively.

The *N. benthamiana skp1* gene *Nbskp1a* was cloned into pGBKT7, to construct pGBKNbskp1a. The *gal4* BD domain was deleted from pGBKT7. The *ripG7* gene was cloned into this modified vector, to construct pGBKC1357dBD. DNA fragment containing *ripG7* on pGBKC1357dBD was inserted into pGBKNbskp1a, to

construct pGBKNbskp1a-ripG7. This plasmid contains both *Nbskp1a* fused to BD domain and *ripG7*.

Results:

NbSkp1 did not interact with RipG2 when the F-box domain was deleted. Instead, the chloroplastic protein NbCab13 did bind to RipG2dF even with higher affinity. These results indicate that Skp1 and chloroplastic proteins do not share the binding site on RipG2.

NbSkp1 did not interact with the F-box deletion derivative of RipG7, as observed with RipG2. All three chloroplastic proteins, NbRbcX, NbCab13, and NbRbcS, interacted with RipG7 even if the F-box domain was deleted.

NbSkp1 interacted with LRR-deletion and N-terminal deletion derivatives of RipG7. On the contrary, NbRbcX lost interaction with RipG7 when LRRs was deleted, indicating that NbRbcX binds to RipG7 through LRRs. While both NbCab13 and NbRbcS interacted with RipG7dL, no bindings were observed with RipG7dN, indicating that binding site of RipG7 to NbCab13 and NbRbcS is the N-terminal region.

In yeast three-hybrid assay, NbSkp1 did not directly interact with NbRbcX. When RipG7 existed together with NbRbcX fused to GAL4-AD and NbSkp1 fused to GAL4-BD, yeast cells turned blue. This result clearly indicates that NbSkp1 and NbRbcX simultaneously bind to RipG7.

Conclusion:

One of the chloroplastic proteins NbRbcX bound to RipG7 through LRRs. Based on the selective evolutionary pressure acting on RipG proteins, it is hypothesized that the convex surface of the LRR domains might be the binding site of ligand protein relevant to the adaptor function of the F-box RipG proteins. NbRbcX could be the ligand for ubiquitination.

Two other proteins, NbCab13 and NbRbcS, bound to the N-terminal region instead of the LRR domain. No significant domain structures are observed in the N-terminal region of RipG7. Nucleus-encoded chloroplastic proteins contain chloroplast transit peptides that act as chloroplast targeting sequences. Although at a primary structural level, transit peptide sequences are highly divergent in length, composition and organization, transit peptides are suggested to contain multiple domains for direct interaction with envelope lipids, chloroplast receptors and the stromal processing peptidase. The N-terminal region of RipG7 could mimic either of domains for binding to chloroplastic proteins.

Among 7 RipG effectors, RipG5 and RipG7 belong to the core T3Es, which are conserved in almost all *R. solanacearum* strains and estimated to probably present in the ancestral *R. solanacearum* strain. A strong likelihood of positive selection is proposed to act on ripG7. Furthermore, RipG7 is an essential host-specificity factor on *Medicago truncatula*. All together, RipG7 could function as a main effector in the RipG family.

Chapter III: Involvement of avirulence genes *avrA* and *popP1* of Japanese *Ralstonia solanacearum* strains in the pathogenicity to tobacco

I investigated the involvement of two avirulence genes, *avrA* (*ripAA* in unified nomenclature) and *popP1* (*ripP1* in unified nomenclature), of Japanese *R. solanacearum* strains in the pathogenicity to tobacco. One virulent strain OE1-1 and four HR-eliciting strains, 8107, MAFF 211471, MAFF 211496, and MAFF 301520, were used. While 8107 and MAFF 211471 contain *popP1*, other three strains do not.

Materials and methods:

The *avrA* deletion mutant of HR-eliciting strains MAFF 211496, and MAFF 301520 were prepared.

The *avrA* or *popP1* single deletion mutant and the *avrA popP1* double mutant of HR-eliciting strains 8107, and

MAFF 211471 were prepared.

The *avrA* deletion mutant of virulent strain OE1-1 was prepared. The *popP1* gene of strain 8107 was transferred into the virulent strain OE1-1 and its *avrA* mutant to prepare transconjugant strains.

The *avrA* gene of OE1-1 strain was transferred into *avrA* mutants of all four HR-eliciting strains to complement *avrA* mutation.

Bacterial suspensions at 10^8 CFU mL^{-1} were infiltrated into leaves of 2 to 3-week-old tobacco (*N. tabacum* cv. Bright Yellow or *N. benthamiana*) with a 1-mL disposable syringe without needle. The virulence assays were made with at least 4 plants for each bacterial inoculation and carried out in 3 replicates.

Bacterial cell suspensions (at 10^8 CFU mL^{-1}) in 10 mM $MgSO_4$ were infiltrated into tobacco leaves. Leaf disks (an area of 0.38 cm^2) were cut from the infiltrated area every 24 h by a borer. Colonies were counted after 2-day incubation at 28°C, and the bacterial populations were calculated as CFU cm^{-2} of leaf area.

Results:

Necrotic lesions appeared faster on the leaves infiltrated with the *avrA* mutant of OE1-1 strain than on the leaves infiltrated with the wild type. When the *popP1* gene of strain 8107 was transferred to the OE1-1 chromosome, the transconjugant strain showed a strong HR-like phenotype at 24 hpi. I concluded that *popP1* is involved in HR induction by *R. solanacearum* OE1-1.

When OE1-1 was infiltrated into tobacco leaves, the tobacco wilted in 2 weeks. The *avrA* mutant retained pathogenicity to tobacco. On the contrary, *popP1* transconjugant strains had significantly impaired virulence on tobacco. Cell growth of the *popP1* transconjugant strains was severely inhibited in the infiltrated leaves. The growth inhibition is in good agreement with a strong HR-like phenotype and severely reduced virulence of the *popP1* transconjugant strains. Taken together, PopP1 was demonstrated to function as the avirulence determinant

in OE1-1.

Appearance of HR phenotype was delayed on the tobacco leaves infiltrated with MAFF 211496 *avrA* mutant and MAFF 301520 *avrA* mutant compared to their corresponding wild types. The *avrA* and/or *popP1* mutants of strain 8107 elicited HR slightly slower than the wild type. MAFF 211471 *avrA* or *popP1* single deletion mutant and its *avrA popP1* double mutant showed typical HR phenotypes on the tobacco leaves, which were almost indistinguishable from the wild type.

The *avrA* mutants of all HR-eliciting strains, complemented with $avrA_{OE1\text{-}1}$ showed the same phenotypes as their wild types.

The cell number of MAFF 211496 continuously decreased in the infiltrated leaf area, whereas that of its *avrA* mutant increased initially and started to decrease at 72 hpi. The cell number of MAFF 301520 dramatically decreased by more than two log orders in the infiltrated leaf area, whereas that of its *avrA* mutant increased on the first day and started to decrease at 48 hpi. While the cell number of strain 8107 decreased by more than 2 log orders in the infiltrated leaf area, that of its *avrA* mutant increased initially and started to decrease at 72 hpi. Although the cell number of strain 8107 *popP1* mutant decreased as an indication of the HR phenotype, its decrease was slower than that of the wild type. The *avrA popP1* double mutant multiplied much faster than the *avrA* single mutant for up to 48 hpi, but then, its cell number dramatically decreased by more than 2 log orders at 72 hpi. In contrast to strain 8107, in MAFF 21147, the contribution of *avrA* and *popP1* on cell multiplication seemed to be small. Cell number of the *avrA* mutant slightly increased at 24 hpi and then decreased in a manner similar to its wild type. Cell numbers of the *popP1* mutant and *avrA popP1* double mutant were lower than that of the wild type at 72 hpi. These trends in cell numbers are all in good agreement with the phenotypes of the mutant-infiltrated leaves.

Among all strains, only MAFF 211496 elicited a HR on eggplant, while the other four wild type strains were

pathogenic on eggplant. All mutants showed the same phenotype as their corresponding wild type. These results indicate that *avrA* and *popP1* of phylotype-I Japanese *R. solanacearum* strains did not contribute to disease development on eggplant.

Conclusion:

Cell numbers of all effector-deleted mutants of HR-eliciting strains eventually decreased, indicating that these strains contain additional avirulence effector genes responsible for HR induction.

Most of of the Japanese virulent strains do not contain *popP1* gene. *popP1*-transconjugants of the virulent OE1-1 strain had severely impaired virulence and showed a HR-like phenotype, which supports the hypothesis that the OE1-1 strain had lost *popP1* to gain virulence to tobacco. The OE1-1 transconjugant with *popP1* somehow retained its virulence and its cell numbers in infiltrates leaves did not decrease dramatically, indicating that OE1-1 could contain other effector(s) that prevent HR induction.

AvrA works as the avirulence determinant in OE1-1 and HR-eliciting strains. There are two types of AvrA sequences, GMI1000-type and RS1000-type, in Japanese strains. No significant difference in HR delay was noted between strains irrespective of the type of AvrA in the strain. RS1000-type *avrA* similarly complemented both RS1000-type and GMI1000-type *avrA* mutations. These results indicate that both types of AvrA function in the same way, at least in Japanese strains.

The *avrA popP1* double mutant of GMI1000 completely abolishes HR induction on tobacco and is virulent to tobacco. However, the *avrA popP1* double mutant of strain 8107 retained the ability to induce HR on tobacco. MAFF 211471 and GMI1000 contain similar alleles for *avrA* and *popP1*. However, the double deletion of these genes had a very little effect on HR induction. These results suggest that there are additional unknown avirulence determinant(s) in Japanese strains, which interfere with host immunity and manipulate the host cellular processes.