Functional characterization of dense granule protein 9 in *Toxoplasma gondii*

2019

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トキソプラズマの GRA9 分子の機能解析

令和元年

(2019)

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博士後期課程　畜産衛生学専攻

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Contents

Contents I

Abbreviations III

General introduction

1. The life cycle of *Toxoplasma gondii* 1

2. *T. gondii* genotypes 4

3. Dense granule proteins of *T. gondii* 5

4. CRISPR/Cas9 technology and its application in *T. gondii* 6

5. Aims of the present study 7

Chapter 1

Molecular characterization of *Toxoplasma gondii* dense granule protein 9

1-1. Introduction 9

1-2. Materials and methods 10

1-3. Results 14

1-4. Discussion 15

1-5. Summary 17

Chapter 2

Functional analysis of dense granule protein 9 in *Toxoplasma gondii* using CRISPR/Cas9 technology

2-1. Introduction 22

2-2. Materials and methods 24

2-3. Results 30

2-4. Discussion 33

2-5. Summary 35

General discussion 48

General summary 51

Acknowledgments 55

References 57

Abbreviations and unit abbreviations

B	BSA	Bovine serum albumin
C	cDNA	Complementary deoxyribonucleic acid
	Cas9	Clustered regularly interspaced short palindromic repeats-associated protein
D	DAPI	4',6-diamidino-2-phenylindole
	DHFR	Dihydrofolate reductase
	DNA	Deoxyribonucleic acid
E	*E. coli*	*Escherichia coli*
	EMEM	Eagle's minimum essential medium
G	GFP	Green fluorescent protein
	GRA	Protein secreted from dense granule organelles
	gRNA	Guide ribonucleic acid
	GST	Glutathione S-transferase
H	HFF	Human foreskin fibroblast
	HIV	Human immunodeficiency virus
	HRP	Horseradish peroxidase
	HXGPRT	Hypoxanthine xanthine guanosine phosphoribosyl transferase
I	IFAT	Immunofluorescent antibody test
	IgG	Immunoglobulin G
	i.p.	Intraperitoneal injection

K	kDa	Kilodalton
M	MNN	Membranous nanotubular network
N	NLS	Nuclear localization signal
	NFAT4	Nuclear factor of activated T cells 4
	NF-κB	Nuclear factor of kappa-light-chain-enhancer of activated B cells
	NHEJ	Nonhomologous-end joining
P	PV	Parasitophorous vacuole
	PLK	Type II low virulent strain of *T. gondii*
	PBS	Phosphate buffered saline
	PAM	Protospacer-adjacent motif
	PCR	Polymerase chain reaction
R	r	Recombinant
	RT	Room temperature
	RNA	Ribonucleic acid
	RH	Type I highly virulent strain of *T. gondii*
	RON	Protein secreted from neck region of rhoptry organelles
	ROP	Protein secreted from bulb region of rhoptry organelles
S	SAG	Surface antigen
	SDS	Sodium dodecyl sulfate
	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	SEM	Standard error of mean

U	UPRT	Uracil phosphoribosyl transferase gene
	UTR	Untranslated region
V	VEG	Type III low virulent strain of *T. gondii*
	Vero	Monkey kidney adherent epithelial cell

Unit abbreviations

bp	Base pair
°C	Degree Celsius
µg	Microgram
mg	Milligram
µm	Micromolar
min	Minute
h	Hour
%	Percentage
ml	Milliliter
mM	Millimole

General introduction

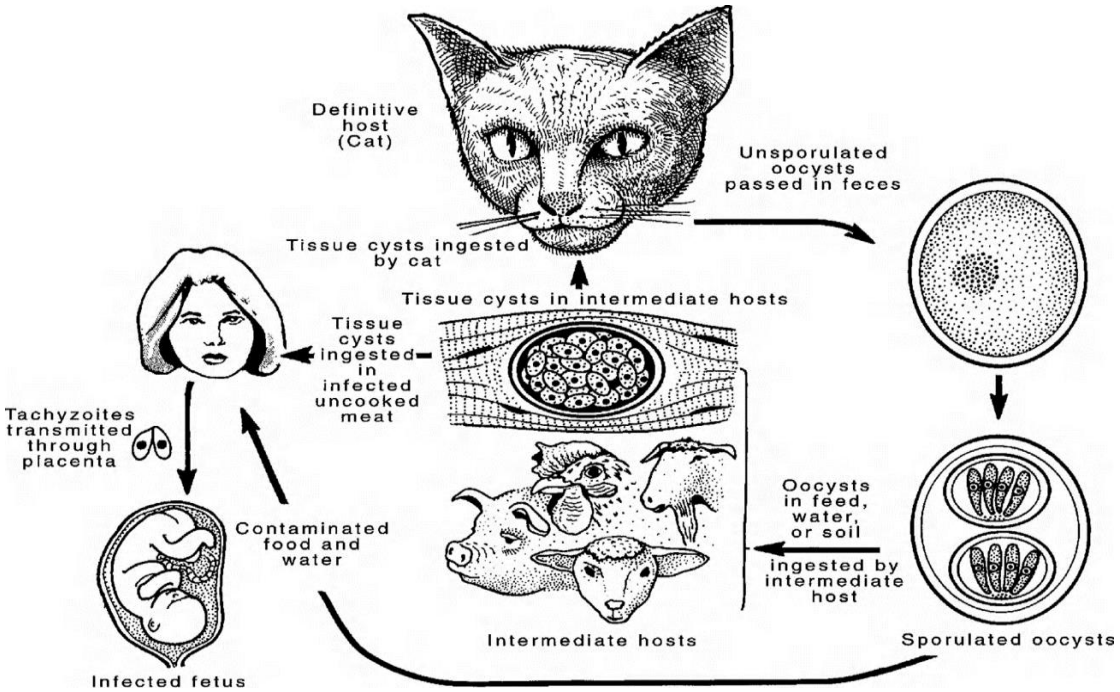
1. The life cycle of *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular apicomplexan parasite which causes toxoplasmosis and is capable of virtually infecting any kind of warm-blooded animal including humans (Dubey 2010). *T. gondii* is acquired orally by ingestion of raw or undercooked meat, fresh water, vegetables that are contaminated with oocysts (Mercier and Cesbron-Delauw, 2015). Infection by *T. gondii* has a worldwide prevalence in animals and humans. Approximately 25-30% of the world's population is infected with *T. gondii* and about 200,000 new cases of toxoplasmosis occur every year (Montoya and Liesenfeld, 2004; Maenz et al., 2014). The healthy adults with acute toxoplasmosis have no clear symptoms (Dupont et al., 2012), however, immunocompromised people, such as those infected with HIV or have recently received an organ transplant, may develop severe toxoplasmosis which can cause encephalitis in the brain (Jones et al., 2001). Furthermore, primary infection with *T. gondii* in pregnant women or animals also leads to congenital diseases such as hydrocephalus and chorioretinitis in newborn children (Goldstein et al., 2008).

The life cycle of *T. gondii*, as shown in Fig. 1, includes a sexual stage that develops only in the definitive host such as cat, and an asexual stage which can develop in both the definitive and intermediate hosts (Dubey, 2010). Development of *T. gondii* occurs in three infectious stages which include bradyzoites, tachyzoites and sporozoites. Both definite and intermediate hosts can be infected by any above infectious stage's parasite. Asexual reproduction occurs in two phases: first, tachyzoites (or endozoites) replicate rapidly by repeated endodyogeny in host cells, then, the next phase starts from the last generation of the tachyzoites that transform

into tissue cysts in which bradyzoites (or cystozoites) replicate slowly by endodyogeny (Dubey and Beattie, 1998; Dubey et al., 1998). The tachyzoite can cause a strong inflammatory response and tissue destruction, therefore is responsible for clinical manifestations of this disease. While bradyzoiyes persist inside cysts for a life time of the host (Maenz et al., 2014), bradyzoites can be released from cysts, transform back into tachyzoites and cause reactivation of the infection (Weiss and Kim, 2000).

The sexual stage of the parasite only develops in the felids. Infectious tissue cysts rapidly multiply in the intestinal epithelial cells by repeated endopolygeny, after which, gametes and oocysts are formed in the intestinal epithelial cells (Dubey et al., 1970; Evans, 1992). Unsporulated oocysts are released into the environment and then converted into infectious oocysts within feces, these are the infectious stage of the parasite (Evans, 1992). Although cats only secrete oocysts in a short period of time, they release large amounts of oocysts that are resistant to environmental conditions, including freezing and drying (Jackson and Hutchison, 1989; Dubey, 1993; Bhopale, 2003). All hosts can become infected by ingestion of food or drinking water that is contaminated with oocysts. In other words, *T. gondii* can be transmitted between the intermediate host, the definitive host or from intermediate to final host and vice versa (Hill and Dubey, 2000; Tenter et al., 2000; Flegr et al., 2014).



(Dubey JP, J Am Vet Med Assoc 189:166, 1986）

Fig. 1. Life cycles of *Toxoplasma gondii*.

2. *T. gondii* genotypes

According to its virulence in mice, *T. gondii* can be divided into three genotypes: Types I, II and III (Grigg et al., 2001; Khan et al., 2005). Usually, in mice, one parasite of type I (such as RH strain) is lethal while the lethal doses of types II (such as PLK strain) and III (such as VEG strain) are about three to four orders of magnitude (Boothroyd and Grigg, 2002). Inoculation of type I can led to a widespread parasite dissemination and death of mice within 10 days; in contrast, mice survival of type II or III infection and tachyzoite dissemination is much less extensive (Dardé et al., 1988). Generally, type III is also considered as avirulent in mice, although a few weeks or months after inoculation, neurological symptoms can occur, even death of mice (Dardé et al., 1988). Due to the genetic difference among them, infection with different genotypes elicit a different immune response in the host which could partly explain the different patterns of virulence (Gavrilescu and Denkers, 2001; Nguyen et al., 2003; Robben et al., 2004; Rosowski et al., 2011; Dubremetz and Lebrun, 2012; Niedelman et al., 2012). For example, previous studies showed that polymorphic rhoptry secreted kinase (ROP) 5 and ROP18 as virulence factors were involved in evading innate immune mechanisms of intermediate hosts (Hunter and Sibley, 2012; Behnke et al., 2015a).

Type I grows faster than types II and III and has a lower interconversion rate from tachyzoites to bradyzoites than type II genotype *in vitro* (Soete et al., 1993). Due to a higher reinvasion rate or a shorter doubling time, the higher growth rate of virulent strains may also explain the higher tissue burden observed in mice infected with type I parasites (Saeij et al., 2005). Experimental crosses among three genotypes proved useful in identifying genes which determine their virulence in mouse (Saeij et al., 2006; Behnke et al., 2011; Reese et al., 2011). When defining virulence factors, the definition

of *T. gondii* virulence with respect to mice infection remains vague since other hosts may behave quite differently from mice (Dubremetz and Lebrun, 2012).

3. Dense granule proteins of *T. gondii*

Although *T. gondii* is a single celled-organism, it has well-structured organelles which made suitable as a model for investigating parasite-host interactions and immunomodulatory responses. Apical secretory organelle dense granules are known to secrete dense granule proteins (so called GRA proteins) at the end of the parasite invasion process on formation of the parasitophorous vacuole (PV) (Carruthers and Sibley, 1997). The GRA proteins are secreted abundantly in both the tachyzoite and bradyzoite stages. They localize at the PV and PV-derived cyst wall, and are important for the maturation of the PV (Cesbron-Delauw et al., 2008; Mercier and Delauw, 2012). Up to now, although the full proteome of dense granules is unknown, more than 20 GRA genes were considered as 'canonical' GRA genes (Mercier and Cesbron-Delauw, 2015). Most GRA proteins were shown to be partly soluble and partly membrane-associated within the PV whereas GRA1 was a soluble protein (Mercier and Cesbron-Delauw, 2015).

Although, GRA proteins usually contributed to the PV maturation within the dense granules or the PV (Mercier et al., 2002; Travier et al., 2008), some GRA proteins were proved to be not essential in the type Ⅰ RH strain. Knockout of GRA2, GRA6, GRA7 or GRA14 gene did not affect parasite growth *in vitro* (Mercier and Delauw, 2012). However, GRA2 or GRA6-knockout mutant showed decreased virulence phenotypes in mice (Mercier and Delauw, 2012; Ma et al., 2014). Moreover, in association with their importance in the PV maturation, phenotypic analyses showed

that both GRA2 and GRA6 were involved in the formation of the membranous tubules (Mercier et al., 2002; Travier et al., 2008; Gendrin et al., 2010). Furthermore, a previous study showed that GRA10 played a significant role in the growth and propagation of intracellular *T. gondii in vitro* (Witola et al., 2014). In addition to contributing to the PV formation, recent studies indicated that GRA proteins could be involved in manipulation of host response (Alaganan et al., 2014; Ma et al., 2014).

4. CRISPR/Cas9 technology and its application in *T. gondii*

Clustered regularly interspaced short palindromic repeats (CRISPR) are the feature of a naturally occurring adaptive immune system found in bacteria and archaea where it uses short RNA to direct degradation of foreign nucleic acids (Horvath and Barrangou, 2010; Bhaya et al., 2011, Wiedenheft et al., 2012). The CRISPR/Cas9 (CRISPR associated gene 9) demonstrated utility in facilitating genome editing and adapted in a variety of organisms for targeted genome editing (Mali et al., 2013a). The type II CRISPR system is most commonly used for its well-known mechanism of action (Jinek et al., 2012). The key point of the system is that the endonuclease Cas9 introduces double-strand DNA breaks in a target sequence which is homologous to CRISPR RNA, and these breaks can be repaired by recombination or nonhomologous-end joining (NHEJ) (Jinek et al., 2012). The Cas9 can use a hybrid single-guide RNA (gRNA) made of the fusion of 20-nucleotide CRISPR RNA to the trans-activating RNA scaffold to generate double-strand breaks in the target sequence (Jinek et al., 2012). The CRISPR/Cas9 technology combined with gRNA has been used for gene manipulation in many model organisms due to its high efficiency (Friedland et al., 2013; Jiang et al., 2013; Mali et al., 2013b; Ren et al., 2013; Wang et al., 2013).

This technology first successfully facilitated the site-specific genome editing in *T. gondii* (Shen et al., 2014; Sidik et al., 2014). In addition, the CRISPR/Cas9 technology also improved the genetic complementation efficiency based on negative selection such as uracil phosphoribosyl transferase (Behnke et al., 2015b). Thus, the CRISPR/Cas9 has been widely used to perform genome editing such as gene deletion, gene insertion, or gene modification in *T. gondii* (Shen et al., 2014; Sidik et al., 2014; Wang et al., 2017; Zheng et al., 2018; Gas-Pascual et al., 2019).

5. Aims of the present study

T. gondii is a protozoan parasite that infects up to one-third of the world's human population causing toxoplasmosis (Montoya and Liesenfeld, 2004). Toxoplasmosis can induce health problems in human beings and cause economic losses in animals, and is considered as the third most common food-borne parasitic infection requiring hospitalization (Vaillant et al., 2005). Identification of the growth and virulence factors of this parasite is important for the development of control strategies.

Dense granule proteins play major structural functions within the PV and the cyst wall of *T. gondii*. Moreover, their particular location within the PV allows them to be involved in various interactions between parasites and the host cells. However, the precise role of GRA9 in different *T. gondii* strains is still unclear. In this study, I sought to investigate the role of GRA9 protein on the growth and virulence of *T. gondii* type I RH and type II PLK strains. First, the nucleotide sequence of GRA9 and its expression in the type I RH and type II PLK strains were analyzed to confirm the characterization of GRA9 protein in different strains. Second, basing on the difference of the GRA9 amino acid sequence and expression level in RH and PLK strains, the roles of GRA9

in the lytic cycle in both strains were evaluated using CRISPR/Cas9 technology. Third, the virulence of RHΔGRA9 and PLKΔGRA9 strains in mice was investigated.

Chapter 1

Molecular characterization of *Toxoplasma gondii* dense granule protein 9

1-1. Introduction

The protozoan parasite *Toxoplasma gondii* is an obligate intracellular pathogen belonging to the phylum Apicomplexa and is capable of virtually infecting any kind of warm-blooded animal, including humans. *T. gondii* is acquired orally by ingestion of raw or undercooked meat containing cysts, or after ingestion of fresh water or vegetables contaminated with oocysts (Mercier and Cesbron-Delauw, 2015). The other coccidian parasites, such as *Plasmodium*, *Cryptosporidum*, and *Theileria*, possess a limited host range and their infection patterns depend on the physical interactions with the hosts.

Similar to other apicomplexan parasites, *T. gondii* possesses main three morphological distinct secretory organelles, micronemes, rhoptries, and dense granules. The contents of these organelles are coordinately secreted during the invasion process of the parasite, ensuring proper recognition, attachment, and entry to the host cell, as well as survival and further development in the parasitophorous vacuole (PV) (Gubbels and Duraisingh, 2012). Dense granule proteins (GRA) are parasitic molecules secreted to the PV during host invasion (Sibley et al., 1991). More than 20 GRAs have been

identified in *T. gondii* (Charif et al., 1990; Mercier et al., 2005; Mercier and Cesbron-Delauw, 2015). Among these proteins, GRA1, 16 and 24 can be detected within the vacuolar space; GRA2, 4, 6, 9 and 12 have been located at the intravacuolar membranous nanotubular network (MNN); GRA3, 5, 7, 8, 10, 15, 22 and 24 localize to the PV membranes; and GRA16 and GRA24 target the host cell nucleus (Mercier and Cesbron-Delauw, 2015; Witola et al., 2014).

These proteins are likely to be involved in intracellular survival and manipulation of host immune response (Alaganan et al., 2014; Ma et al., 2014; Witola et al., 2014). GRA9, a new dense granule protein, was identified (Adjogble et al., 2014). Like most of the other GRA proteins, GRA9 exists in both a soluble and an insoluble state, but is only secreted as a soluble form. However, the molecular characterization of GRA9 protein is less clear.

In this study, amino acid sequence of GRA9 protein from *T. gondii* RH and PLK strains was analyzed. Furthermore, its expression level also was evaluated.

1-2. Materials and methods

Ethics statement

The recommendations in the Guide for the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan were strictly followed. The protocol of this chapter was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine, Japan (permission numbers: 29-134, 201711).

Animals

Six-week-old, female ICR mice and Japanese white rabbits were obtained from Clea Japan for the preparation of polyclonal antibodies against *T. gondii*. All the mice and rabbits were housed in the animal facility of National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. All animal experiments started one week after habituation.

Cell and parasite cultures

T. gondii type I RH strain with hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) deficiency and type II PLK strain were maintained in monkey kidney adherent epithelial (Vero) cells in Eagle's minimum essential medium (EMEM) (Sigma, USA) supplemented with 8% fetal bovine serum (Biowest, Japan), 100U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin (Sigma, USA) at 37 ℃ and 5% CO_2. To purify tachyzoites, the parasites and host cells were washed in cold PBS, and the final pellet was re-suspended in cold PBS and passed through a 27-gauge needle syringe three times. Then the parasites were filtered through a 5.0-μm pore filter (Millipore, USA) and were counted.

Sequencing of GRA9

Total RNA of *T. gondii* was extracted using Trizol (Life Technologies, Germany) from purified parasites and then cDNA was transcribed using SuperScript III First Strand Synthesis Kit (Invitrogen, USA) according to the manufacturer's protocol. The full length of the open reading frame of GRA9, was amplified from *T. gondii* RH and PLK strain cDNA using the primers: forward 5'-AAGAAGCTTGATGGG GATATCATGCGGTCACTCAAGTCAAT-3' and reverse 5'-GTCGTACGGATACATGATATCTCAGAGTCCTCGGTCTTCCT-3'. These primers were designed based on the predicted GRA9 sequences (*Toxoplasma* Genomics

Resource TGGT1_251540 and TGME49_251540). The fragments were cloned into pGEM-T Easy vector (Promega, USA) and sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and the ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA).

Production of *T. gondii*-infected sera

Six-week-old ICR mice were infected intraperitoneally with purified live tachyzoites of PLK strain (1,000/mouse). The sera were collected at weeks two, four and eight after infection, respectively.

Production of recombinant proteins and polyclonal antibodies

To generate mouse polyclonal antibodies against GRA9, the gene fragment corresponding to residues 21-318 of GRA9 (TGGT1_251540) was amplified from *T. gondii* tachyzoite cDNA using the following primers: GRA9Fwd (5'-AAAGAATTCCTCGACCTTTTCCTCGGTG-3') and GRA9Rev (5'-ATAAGAATGCGGCCGCTCAGAGTCCTCGGTCTTCC-3'). The amplified product was cloned into *Eco*RI and *Not*I sites of pGEX-4T-1. pGEX-4T-3-SAG1 (Kimbita et al., 2001) and pGEX-4T-3-GRA7 (Masatani et al., 2013) plasmids from previous studies were used to produce recombinant SAG1-GST and GRA7-GST proteins. Recombinant proteins were expressed in *Escherichia coli* BL21 DE3 cells. Purified recombinant protein GRA9-GST (rGRA9) (100 μg) and SAG1-GST (100 μg) emulsified in Freund's complete adjuvant (Sigma, USA) were intraperitoneally injected into ICR mice on day 0. Then, the same proteins mixed with Freund's incomplete adjuvant (Sigma, USA) were injected into the mice on days 14 and 28 after the first immunization. Serum was collected 14 days after the last immunization. Specificity of the serum was analyzed by Western blot analysis. Purified recombinant proteins GRA7-

GST (1 mg) was subcutaneously injected into female Japanese white rabbits to produce rabbit anti-GRA7 polyclonal antibody.

Western blot analysis

To assess GRA9 expression, recombinant protein or lysates from purified parasites tachyzoites mixed with 5 × SDS gel loading buffer (250 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 0.5% (w/v) bromophenol bule, 50% (w/v) glycerol and 5% (w/v) β-mercaptoethanol) were used for Western blot analysis as previously described (Parussini et al 2012). Mouse anti-GRA9 serum was used to identify GRA9. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Amersham Pharmacia Biotech, USA) was used as secondary antibody. The blot was scanned using VersaDoc™ imaging system (Nippon Bio-Rad Laboratories, Japan) according to the manufacturer's recommendations.

Immunofluorescence

Immunofluorescence was carried out as described previously (Zheng et al., 2018). Vero cells were plated at 1×10^5 cells/well in 12-well plates and incubated for 24 hs. Parasites were inoculated onto Vero cell monolayers in 12-well plates (2×10^5 parasites per well). Then, 24 hs post infection, they were fixed with 4% paraformaldehyde. Then, the coverslips were blocked with 3% bovine serum albumin (BSA) for 30 min at RT. Followed, the coverslips were incubated with mouse anti-GRA9 polyclonal antibody diluted 1:100 in 3% BSA in PBS for 1 h at RT. After washing 5 times with PBS, the coverslips were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Sigma, USA) diluted 1:1,000. Samples were examined using an All-in-one Fluorescence Microscope (BZ-900, Keyence, Japan).

Phylogenetic analysis

Phylogenetic analyses were done using MEGA version 7.0 program and the neighbor-joining distance method (Kimura 2-parameter model). Bootstrap analysis was performed with 1,000 replicates to estimate the confidence of branching patterns of the tree.

Statistical analysis

To analyze the data, GraphPad Prism 5 software (GraphPad Software Inc., USA) was used. Statistical analysis was performed using an unpaired Student's *t*-test. Data represent the mean $\pm$ standard error of mean. A *P*-value < 0.05 was considered statistically significant.

1-3. Results

Characterization of the GRA9 gene

Only one GRA9 gene (TGGT1_251540) was found among all the *T. gondii* genome sequence annotated in the ToxoDB database. At the same time, the amino acid sequence of GRA9 from different *T. gondii* strains could be also found in the ToxoDB. Thus, phylogenetic analysis was performed with GRA9 protein from different *T. gondii* strains and other species. *T. gondii* GRA9 shares 82% sequence identity with the *Hammondia hammondi* protein. Phylogenetic analysis showed GRA9 to be relatively conserved in the different strains of *T. gondii* (Fig. 2). Furthermore, sequence analysis showed that the nucleotide sequences of GRA9 in the type I RH and type II PLK strains were both 957 bp, which were consistent with the predicted sequences of type I (TGGT1_251540) and type II (TGME49_251540), respectively. Alignment of predicted amino acid sequence showed that RH GRA9 contains one amino acid

substitution compared with that of PLK GRA9 (Fig. 3). This indicates that the sequence of GRA9 gene is polymorphic.

Characterization of the GRA9 protein

In order to characterize the antigenicity of GRA9 protein, partial sequence of GRA9 fused with GST-tag was expressed in *E. coli* and purified (Fig. 4A). A ~60 kDa band was obtained on SDS-PAGE, consistent with the predicted size (~33 kDa GRA9 fused with the ~27 kDa GST tag). The rGRA9 was reactive with the sera from mice experimentally infected with live PLK tachyzoites (Fig. 4B).

To determine the native expression of GRA9 in RH and PLK strains, Western blot analysis was carried out. The mouse anti-GRA9 sera recognized specific protein bands in the lysates of RH and PLK tachyzoites (Fig. 5A). However, there was a higher expression level of GRA9 in PLK tachyzoites. The relative quantification of the bands indicated that the intensity of PLK GRA9 band (set to 100%) was significantly higher than that of RH strain (~35%) (Fig. 5B). This maybe mean that the roles of GRA9 in RH and PLK strains were different. Confocal laser-scanning microscope detection of GRA9 protein with fluorescently-labeled anti-GRA9 mouse polyclonal antibody indicated that GRA9 localized to the PV of the RH and PLK strains (Fig. 5C).

1-4. Discussion

Like the other GRA genes, GRA9 gene was found as a single copy gene from the ToxoDB database consistent with previous report which was initially identified as a member of excreted-secreted antigen family (Nockemann et al., 1998). This secreted antigen was proved as a new dense granule protein, named GRA9, by immunofluorescence and cryo-electron microscopy (Adjogble 2004). Phylogenetic

analysis showed GRA9 to be relatively conserved in the different strains of *T. gondii*. *T. gondii* GRA9 shares 82% sequence identity with the *Hammondia hammondi* protein. Furthermore, alignment of *T. gondii* GRA9 and *Neospora caninum* GRA9 protein sequences also showed a high sequence identity of 60%, and with similar alpha helices, hydrophobic and hydrophilicity domains (Leineweber et al., 2017). The high homology of the two GRA9 sequences could indicate that this protein is involved in a functional activity that is important for these species, and may be similar.

The full GRA9 gene sequences of different *T. gondii* strains has been reported in ToxoDB database except RH and PLK strains. Therefore, GRA9 gene of two strains was cloned and sequenced in this study. Sequence analysis of the cloned gene showed 99.8% identity between RH and PLK strains, implying GRA9 gene is conserved in the two strains. Furthermore, predicted amino acid sequence alignment indicated that there was only one polymorphic amino acid in the sequence of GRA9 of the two strains. Polymorphisms in the amino acid sequences of GRA6 and GAR15 have been reported, which is associated with strain-specific function (Rosowski et al., 2011; Ma et al., 2014). Thus, it is possible that GRA9 is involved in a functional activity in a strain-specific manner. In immunoblot analysis, the rGRA9 was reactive with the sera from mice infected with *T. gondii*, indicating the immunogenicity of this protein. Furthermore, the polyclonal antisera raised against *T. gondii* rGRA9 also recognized specific protein bands in the lysates of RH and PLK tachyzoites. It is notable that the expression level of GRA9 is significantly higher in the PLK strain than in the type I RH strain. This might suggest different roles of GRA9 in RH and PLK strains.

Consistent with the same pattern of secretion into PV as that described for the GRA proteins, GRA9 protein could be detected also in PV in *T. gondii* RH and PLK strains by immunofluorescence (Mercier and Cesbron-Delauw, 2015). GRA protein are

important for the formation of the parasitophorous vacuolar membrane (PVM) that protects the parasite from the hostile host cell environment (Dubey et al., 1998; Plattner and Soldati-Favre, 2008; Blader and Saeij, 2009). Therefore, roles of GRA9 in parasite growth and virulence need to be evaluated.

1-5. Summary

T. gondii is an obligate intracellular apicomplexan parasite. The dense granules are important secretory organelles existing in all apicomplexan parasites. GRA proteins are released into PV at the end of host cell invasion and are known to play important roles in intracellular survival and manipulation of host immune response. In this chapter, a detailed characterization of the *T. gondii* GRA9 was provided. Phylogenetic analysis was performed with GRA9 protein from different *T. gondii* strains and other species indicating GRA9 to be relatively conserved in the different strains of *T. gondii*. Sequence analysis showed that the nucleotide sequences of GRA9 in the type I RH and type II PLK strains were both 957 bp, which were consistent with the predicted sequences of type I (TGGT1_251540) and type II (TGME49_251540), respectively. Furthermore, alignment of predicted amino acid sequence showed that RH GRA9 contains one amino acid substitution compared with that of PLK GRA9. All these information are useful for further elucidating the function of GRA9 protein in *T. gondii*.

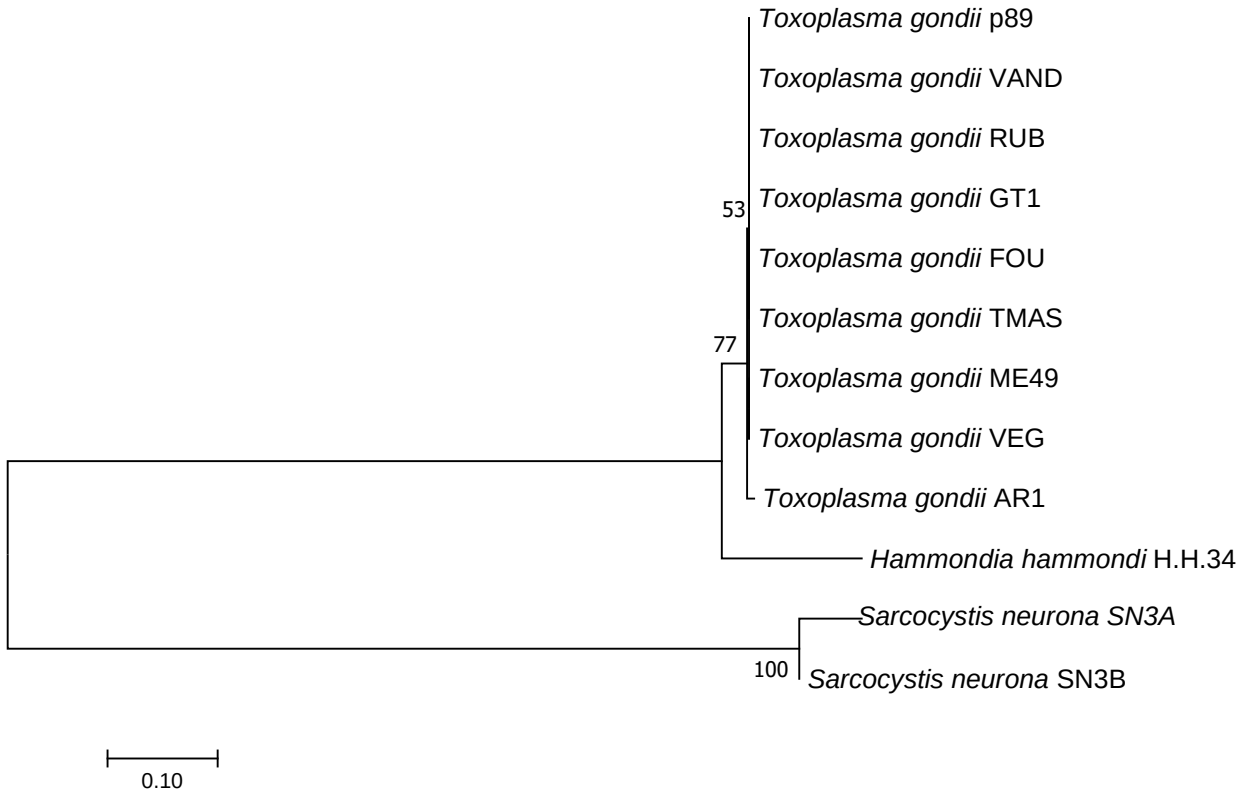


Fig. 2. Phylogenetic analysis of GRA9 with *Hammondia hammondi*, *Sarcocystis neurona* and multiple *T. gondii* strains annotated in the ToxoDB database generated using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed.

Fig. 3. The predicted amino acid sequences of the primary translated *T. gondii* GRA9 from RH and PLK strains.

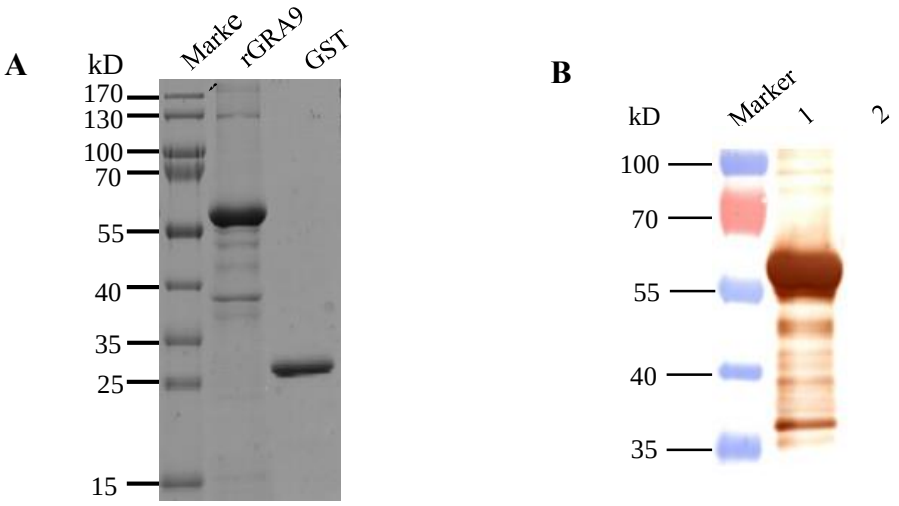


Fig. 4. Expression of recombinant GRA9 protein. (A) SDS-PAGE analysis of purified recombinant GRA9 protein and GST. Marker, prestained protein standard marker (10-170 kDa). (B) Western blot analysis of rTgGRA9. Lanes 1, rTgGRA9 incubated with serum from mice infected with PLK strain. Lines 2, rTgGRA9 incubated with serum from mice with no treatment.

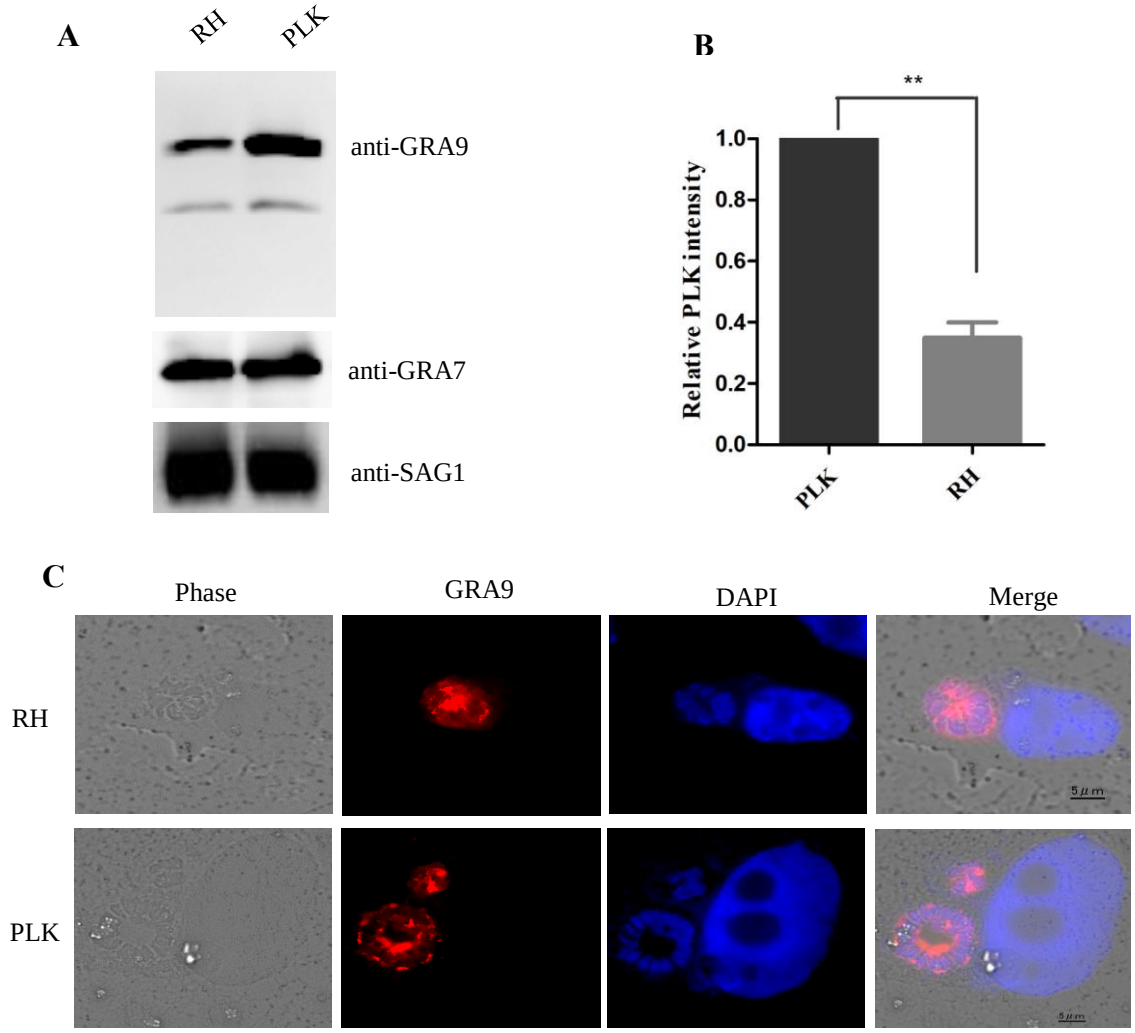


Fig. 5. Expression of native GRA9 protein. (A) Western blot analysis of wild-type RH and PLK strain lysates. GRA9 was detected using mouse anti-GRA9 serum. SAG1 and GRA7 were detected using mouse anti-SAG1 serum and rabbit anti-GRA7 serum, respectively, as loading control. (B) Quantification of GRA9 expression level. The intensity of the PLK band was set to 1 and used for normalization. Means ± SEM of n = 3 independent experiments are shown. **, $P < 0.01$, determined by unpaired Student's *t* test. (C) Indirect immunofluorescence detection of GRA9 in the RH and PLK strains. GRA9 was stained with mouse anti-GRA9 serum. Host cell and parasite nuclei were visualized with DAPI (blue).

Chapter 2

Functional analysis of dense granule protein 9 in *Toxoplasma gondii* using CRISPR/Cas9 technology

2-1. Introduction

Toxoplasma gondii is an obligate intracellular apicomplexan parasite which is capable of virtually infecting any kind of warm-blooded animal, including humans. *T. gondii* is divided into three lineages: types I, II and III in North America and Europe according to its virulence in laboratory mouse strains (Howe and Sibley, 1995). Type I strains are virulent, type II exhibits reduced virulence and type III is avirulent. The lytic cycle drives both *T. gondii*'s propagation and pathogenesis. The parasite enters the host cell depending on the secretion of proteins from its specialized secretory organelles known as the micronemes, rhoptries and dense granules which are important for the formation of the parasitophorous vacuole (PV), in which it replicates and divides (Carruthers and Sibley, 1997; Hakansson et al., 2001). Among these, proteins secreted from the microneme play an important role in the recognition, adhesion and invasion of the host cell by parasites (Meissner et al., 2002). Secreted rhoptry neck (RON) proteins, such as RON2, 4, 5 and 8 participate in formation of a moving junction by which the motile parasite penetrates the host cell (Besteiro et al., 2011). In addition,

secreted rhoptry (ROP) proteins are injected into the host cell cytosol during invasion and eventually localize in the host cell nucleus or PV to subvert and co-opt host cell functions (Hakansson et al., 2001; Boothroyd and Dubremetz, 2008; Hunter and Sibley, 2012; Kemp et al., 2013). At the end of the invasion process and shortly after the formation of the PV, the dense granule proteins (GRAs) are massively secreted into the PV lumen.

GRA proteins are believed to play important roles in the lytic cycle of *T. gondii*. Hence, understanding their function is important for the control of toxoplasmosis. GRA2, 4 and 6 interact in a macromolecular complex to stabilize the network membrane (Labruyere et al., 1999; LaFavers et al., 2017). GRA10 plays a significant role in the growth and propagation of intracellular *T. gondii* (Witola et al., 2014). Recently, a novel dense granule protein, GRA41, has been shown to regulate timing of egress which relates with calcium fluxes (LaFavers et al., 2017). However, the role of several dense granule proteins including GRA9 in the lytic cycle remains unclear.

ROP and GRA proteins are also potentially related to the difference in virulence observed between type I and type II strains. The different virulence between strains could be partially explained by the genetic difference that elicits different cell modulation in the host cell and different immune response in the host (Rosowski et al., 2011; Ma et al., 2014; Behnke et al., 2015a). For example, ROP5 and ROP18 were major virulence factors in genetically divergent strains from North and South America (Behnke et al., 2015a). GRA6 was recently shown to participate in the manipulation of host immune responses by regulating the activation of the host transcription factor nuclear factor of activated NFAT4 in a strain-dependent manner (Ma et al., 2014). Furthermore, as a result of the polymorphism of GRA15 protein, type II strains of *T. gondii* activate more NF-κB to the modulate host immune response than type I strains

(Rosowski et al., 2011). However, the precise role of GRA9 in different *T. gondii* strains is still unclear. Therefore, in this chapter, I sought to investigate the impact of knocking out GRA9 gene on the growth and virulence of *T. gondii* type I RH and type II PLK strains.

2-2. Materials and methods

Ethics statement

The recommendations in the Guide for the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan were strictly followed. The protocol of this chapter was approved by the Committee on the Ethics of Animal Experiments at the Obihiro University of Agriculture and Veterinary Medicine, Japan (permission numbers: 29-134, 201711).

Animals

Japanese white rabbit was obtained from Clea Japan for the preparation of polyclonal antibodies against *T. gondii*. Six-week-old female BALB/c mice were purchased from Clea Japan for parasite virulence assays. All the mice were housed in the animal facility of National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. All animal experiments started one week after habituation.

Cells and parasites

T. gondii type I RH strain with hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) deficiency, type II PLK strain and their derivatives were maintained in monkey kidney adherent epithelial (Vero) cells in Eagle's minimum

essential medium (EMEM) (Sigma, USA) supplemented with 8% fetal bovine serum (Biowest, Japan), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA) at 37 ℃ and 5% CO_2. To purify tachyzoites, the parasites and host cells were washed in cold PBS, and the final pellet was resuspended in cold PBS and passed through a 27-gauge needle syringe three times. Then the parasites were filtered through a 5.0-μm pore filter (Millipore, USA) and were counted.

Production of polyclonal antibody

pGEX-4T-3-SAG1 plasmid from previous study (Kimbita et al., 2001) was used to produce recombinant SAG1-GST protein. Recombinant protein was expressed in *Escherichia coli* BL21 DE3 cells. Purified recombinant protein SAG1-GST (1 mg) emulsified in Freund's complete adjuvant (Sigma, USA) was intraperitoneally injected into female Japanese white rabbit on day 0. Then, this protein mixed with Freund's incomplete adjuvant (Sigma, USA) were injected into the rabbit on days 14 and 28 after the first immunization. Serum was collected 14 days after the last immunization. Specificity of the serum was analyzed by Western blot analysis.

Western blot analysis

Lysates from purified parasites tachyzoites mixed with 5 × SDS gel loading buffer (250 mM Tris-HCl pH 6.8, 10% (w/v) SDS, 0.5% (w/v) bromophenol bule, 50% (w/v) glycerol and 5% (w/v) β-mercaptoethanol) were used for Western blot analysis as previously described (Parussini ET AL., 2012). Mouse anti-GRA9, anti-SAG1, rabbit anti-SAG1 and anti-GRA7 polyclonal antibodies were used. Secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (Amersham Pharmacia Biotech, USA) were used, respectively. The

blot was scanned using VersaDoc™ imaging system (Nippon Bio-Rad Laboratories, Japan) according to the manufacturer's recommendations.

Generation of GRA9-knockout parasite lines

To disrupt GRA9 in RH and PLK strains, a GRA9-targeting guide RNA (gRNA) sequence was designed using E-CRISP (E-Crisp.org). Then, knockout plasmids, pHX-RH-GRA9 and pDF-PLK-GRA9 were generated and used for disruption of GRA9 in RH and PLK strains, respectively. The vector backbones contained Cas9, GFP, gRNA of targeted gene and dihydrofolate reductase (DHFR)/HXGPRT expression cassettes (Zheng et al., 2018). The original gRNA was replaced by GRA9 gRNA in the two vector backbones. The fragment containing GRA9 gRNA was amplified by PCR using primers shown in Table 1. RH and PLK parasites (10^7 tachyzoites) were transfected with 10 μg of the pHX-RH-GRA9 and pDF-PLK-GRA9 plasmids, respectively, as previously described (Soldati and Boothroyd, 1993). Selection of stable transformants based on mycophenolic acid/xanthine (25 μg/ml; 50 μg/ml) or pyrimethamine (1 μM) was performed as described previously (Donald and Roos, 1998; Reynolds et al., 2001). Stable clones were isolated by limiting dilution in 96-well plates, and confirmed by PCR using primers shown in Table 2 and Western blot analyses.

Complementation of PLK GRA9-knockout parasite

To complement the GRA9-deficient PLK strain, I generated a pHX-UPRT plasmid with the gRNA of non-essential uracil phosphoribosyl transferase (UPRT) gene and a pB-synoGRA9 plasmid containing UPRT homology arms (UPRT 5' and UPRT 3'), the 5' flanking region of GRA1 gene (GRA1 5'UTR) and the 3' flanking region of GRA2 (GRA2 3'UTR). UPRT 5' and UPRT 3' homology arms were

amplified by PCR from PLK genomic DNA using primers sets described in Table 1. The GRA1 5'UTR and the GRA2 3'UTR were amplified from the plasmid pDF-PLK-GRA9. GRA9 fragment was amplified from PLK cDNA by overlap PCR. The GRA9 product also contained synonymous codons mutated at target sites for gRNA. Then, GRA1 5'UTR, GRA2 3'UTR and GRA9 products were merged into one fragment using overlap PCR and GRA15'UTR-GRA9-GRA23'UTR was generated. The UPRT 5' and UPRT 3' homology arms were cloned into the *Sac*I-*Bam*HI and *Eco*RV-*Xho*I sites of pBluescript KS (-) vector successively using the ClonExpress™ II One Step Cloning Kit (Vazyme, USA), and pBlu-UPRT was obtained. GRA15'UTR-GRA9-GRA23'UTR was cloned into *Bam*HI-*Eco*RV sites of pBlu-UPRT using the ClonExpress™ II One Step Cloning Kit (Vazyme, USA), and pB-synoGRA9 plasmid was obtained. The GRA9-deficient PLK parasites were transfected with pHX-UPRT and linearized pB-synoGRA9 by *Kpn* I (mass ratio, 1:5) to complement GRA9 by insertion at the UPRT locus. Parasites were screened with 10 μM 5-Fluorouracil (Sigma, USA). Positive clones were confirmed as described above.

Plaque assay

To evaluate the growth of parasites *in vitro,* monolayers of Vero cells seeded in 12-well plates were infected with *T. gondii* wild type (RH and PLK) and mutant (RHΔGRA9, PLKΔGRA9 and complement) strains. The detailed protocol is as follows: 1×10^5 Vero cells were inoculated in each of the well and cultured at 37 ℃ in 5% CO_2 atmosphere for 24 hours. The cells were inoculated with 100 RH tachyzoites/well or 300 PLK tachyzoites/well and cultured until plaque occurred (RH parasites for about 7 days and PLK for about 13 days). The medium was then aspirated from the well and cells infected with parasites were washed 3 times using 1 ml phosphate-buffered saline (PBS). Later, 3% paraformaldehyde was added to the well

and cells were allowed to fix for 20 min at room temperature (RT). Then the 3% paraformaldehyde was removed and cells were washed 3 times using PBS. The cells were later stained with 0.1% crystal violet diluted with distilled water for 30 min at RT. The 0.1% crystal violet was removed and cells were washed 3 times. Finally, plaques were observed using microscope.

Attachment/invasion assay

The attachment/invasion assay was confirmed by indirect fluorescent antibody test (IFAT) (Wang et al., 2017). Vero cells were plated at 1×10^5 cells/well in 12-well plates and incubated for 24 hs. Parasites were inoculated onto Vero cell monolayers in 12-well plates (2×10^5 parasites per well), and allowed to invade the cells for 2 hs under normal growth conditions. Then, coverslips were collected, and washed 6 times with PBS to remove extracellular parasites. After washing, the coverslips were blocked with 3% bovine serum albumin (BSA) for 30 min at RT. To count the number of attached parasites, the coverslips were incubated with mouse anti-SAG1 polyclonal antibody diluted 1:100 in 3% BSA in PBS for 1 h at RT. After washing 5 times with PBS, the coverslips were incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (Sigma, USA) diluted 1:1,000. They were washed 5 times with PBS, permeabilized for 6 min with 0.3% Triton X-100/PBS, blocked with 3% BSA for 30 min and then incubated with rabbit anti-SAG1 polyclonal antibody to count the number of invading parasites. After washing 5 times, the coverslips were incubated for 45 min at RT in Alexa Fluor 488-conjugated goat anti-rabbit IgG (Sigma, USA). Samples were examined using an All-in-one Fluorescence Microscope (BZ-900, Keyence, Japan). Cells that were stained both red and green were scored as attached parasites, whereas those stained only in green were scored as invaded parasites. Eight fields were

randomly counted for each coverslip. Experiments were performed in triplicate and repeated at least three times.

Intracellular replication rate assay

Parasite growth rate was evaluated by counting the number of parasites per vacuole as previously described (Wang et al., 2017). Purified parasites were inoculated into Vero cell monolayers in 12-well plates (2×10^5 parasites per well) and were allowed to invade the cells for 2 hs under normal growth conditions. Vero cell monolayers were then washed 5 times with PBS to remove extracellular parasites. At appointed time post-infection, tachyzoites in vacuoles were marked with mouse anti-SAG1 by IFAT as described above and counted in at least 100 vacuoles. Experiments were conducted in triplicate and repeated three times.

Egress assay

In primary type II PruΔku80 PVs, tachyzoites egress at ~72 hs post-infection (Rommereim et al., 2013). Vero cells were infected at a multiplicity of infection of 1 and cultured under normal growth conditions for 32 hs (Wang et al., 2017). The monolayers were washed three times in PBS to remove extracellular parasites, and then incubated for 5 min with 3 μM A23187 (Sigma, USA), a calcium ionophore. After incubation, parasites were fixed and IFAT was performed with mouse anti-SAG1 and rabbit anti-GRA7 to measure the percentage of intact and egressed PVs. At least 300 vacuoles were counted per slip.

Fatality assay

Fresh tachyzoites were purified and resuspended in EMEM as described above. 10^2 RH or 10^5 PLK tachyzoites were injected into female BALB/c mice by

intraperitoneal (i.p.) injection. Survival rate was monitored for 30 days. Each experiment was repeated at least two times.

Statistical analysis

To analyze the data, GraphPad Prism 5 software (GraphPad Software Inc., USA) was used. Statistical analyses were performed using an unpaired Student's *t*-test, Tukey's multiple comparison test or Chi-square test. Data represent the mean ± standard error of mean. Survival curves were generated using the Kaplan-Meier method and statistical comparisons were made by the log-rank method. A *P*-value < 0.05 was considered statistically significant.

2-3. Results

Disruption of GRA9 gene in RH and PLK strains using CRISPR/Cas9 system

RH and PLK GRA9 knockout strains (RHΔGRA9 and PLKΔGRA9) were generated using the knockout plasmids pHX-RH-GRA9 and pDF-PLK-GRA9 with HXGPRT and DHFR selectable marker, respectively (The structure of the plasmids is shown in Fig. 6A). Mutations in the GRA9 locus in knockout parasites were confirmed by PCR using GRA9 specific primers and knockout plasmid specific primers (Table 2). The GRA9 fragment was not amplified whereas the different fragments within plasmids were amplified in RHΔGRA9 and PLKΔGRA9 parasites (Fig. 6B). Furthermore, approximately 9,200 bp and at least 3,000 bp foreign fragments derived from the transfected plasmids integrated into the GRA9 gRNA locus in RHΔGRA9 and PLKΔGRA9, respectively (Fig. 6C). This may be related to the fact that CRISPR/CAS9 can enhance site-specific nonhomologous integration of foreign DNA. Subsequent

Western blot analysis confirmed the loss of GRA9 expression in RHΔGRA9 and PLKΔGRA9 (Fig. 6D).

Complementary expression of synoGRA9 by PLKΔGRA9

A complementing GRA9 expression plasmid was constructed by replacing the original codons with synonymous codons (pB-synoGRA9), resulting in an allele of the GRA9 gene that could avoid CRISPR/Cas9 knockout (Fig. 7A). The pHX-UPRT plasmid was also generated (Fig. 7B). The integration of synoGRA9 at the UPRT locus was verified by PCR and a 1,371 bp UPRT fragment (PCR1) was amplified in PLK strain whereas it was not amplified in complement due to insertion of synoGRA9 (Fig. 7C). Other two UPRT fragments containing 5' end and 3' end of synoGAR9 respectively (PCR2 and PCR3) were amplified in complement (Fig. 7C). These data demonstrate homologous integration and insertion of synoGRA9 in UPRT site in representative complement. Subsequent Western blot analysis confirmed the expression of GRA9 in complement (Fig. 7D).

Loss of strain-specific GRA9 results in decreased plaquing efficiency

The ability of RHΔGRA9 and PLKΔGRA9 strains to form plaques on Vero cells monolayers was evaluated. Both RHΔGRA9 and PLKΔGRA9 strains were able to produce plaques (Fig. 8A), indicating that GRA9 was not essential for parasite growth. However, PLKΔGRA9 parasites showed a statistically significant decrease in the number of plaques (40.8 $\pm$ 1.2) as compared to PLK wild type (72.8 $\pm$ 1.9, $P <$ 0.0001, Fig. 8B) without noticeable difference in plaque size. Compensation of GRA9 restores the defect in plaquing efficiency. There was no significant difference in the numbers of plaques between RHΔGRA9 and RH wild type (Fig. 8B). To better define the phenotype associated with the loss of GRA9, the effect of GRA9 in invasion of the

extracellular parasites and replication of the intracellular parasites was evaluated. Surprisingly, the invasive ability of both RHΔGRA9 and PLKΔGRA9 strains was inhibited (Fig. 9A) whereas the percentages of vacuoles containing pointed tachyzoite numbers in both knockout parasites were similar to that of the wild type parasites (Fig. 9B). These results suggest that GRA9 is dispensable during replication of parasites in host cells, and that parasite growth ability is impaired after deletion of GRA9 in a strain-dependent manner.

Loss of GRA9 in PLK strain inhibits parasite egress *in vitro*

Egress assay was performed to examine whether GRA9 affects the egress of intracellular parasites. The morphology of the PV at 5 min post-addition of calcium ionophore A21387 is shown in Fig. 10A. Tachyzoites were marked in green by mouse anti-SAG1 polyclonal antibody, whereas PV was marked in red by rabbit anti-GRA7 polyclonal antibody. The IFAT image of PLKΔGRA9 seemed different from the intact PV. The SAG1-labelled PLK GRA9 mutants may be close to the GRA7 intra vacuolar signal and not widely scattered around it, but they do not appear to be properly organized within a vacuole either. The egress rate of all the strains was quantified and no difference was observed in egress percentage between RHΔGRA9 and the parental strain, whereas the egress ability of PLKΔGRA9 was inhibited (74.1% $\pm$ 1.9%) and the PLK wild type parasites significantly egressed faster (89.5% $\pm$ 1.2%) (Fig. 10B). To confirm this phenotype, the ratio of PVs that contained only one parasite was calculated (Fig. 10C). Consistent with the results above (Fig. 9B), no significant difference was seen in the percentage of vacuoles containing different number of tachyzoites at 24 hs post infection. This indicates that PLKΔGRA9 could replicate at a normal rate. However, the percentage of vacuoles containing one tachyzoite in the PLKΔGRA9 was significantly lower than that in the parental strain at 33 hs post infection (Fig. 10C).

This may have been due to the timing of egress in PLKΔGRA9 strain being later than in the parental strain, and that the egressed PLK parasite re-infected surrounding host cells. All these observations taken into consideration, the PLKΔGRA9 strain had a strain-specific delayed egress.

PLKΔGRA9 parasites exhibit attenuated virulence in mice

Based on the decreased plaquing efficiency and delayed egress ability, I evaluated the effect of GRA9 on parasite virulence in mice. The survival rate was not significantly different between BALB/c mice infected with RH and RHΔGRA9 strains (Fig. 11A). In addition, all mice that were challenged with PLK or complement strains succumbed within 7 to 12 days' post-infection (Fig. 11B). On the contrary, mice infected with PLKΔGRA9 showed relatively higher survival rate (93.3%) (Fig. 11B). Of the 15 mice challenged with PLKΔGRA9, only one died at 9 days post-challenged. The death of this mouse should be related to contingency factors because others remained alive until 30 days' post-infection. The sera of surviving mice were positive for anti-TgGRA7 antibodies by ELISA (Fig. 12). These data indicate that GRA9 is important for the virulence of PLK strain *in vivo*.

2-4. Discussion

Although, *T. gondii* GRA9 has been identified as a secreted 41 kDa dense granule protein in previous studies, the role of GRA9 in parasite lytic cycle is still unclear except that the deletion of GRA9 in RH strain reduced the infection rate *in vitro* (Adjogble et al., 2004; Rommereim et al., 2016). Therefore, this chapter reports the phenotypic analysis of *T. gondii* type I and type II knockout mutants that lack expression of the dense granule protein GRA9. These mutants were obtained using

CRISPR/Cas9 technology. Despite NHEJ being the preferred mechanism for insertion of foreign DNA into the *T. gondii* genome, facilitating gene knockouts and tagging endogenous loci in *T. gondii* were developed using homologous recombination by knocking out the critical component KU80 to inactivate the NHEJ (Fox et al., 2009; Huynh and Carruthers, 2009). However, this approach is limited to *T. gondii* KU80 knockout strains (Fox et al., 2009; Fox et al., 2011). CRISPR/Cas9 has been shown as a highly efficient method for disruption of genes of several *T. gondii* strains (Shen et al., 2014).

Like other GRAs, GRA9 contains an amphipathic alpha-helix and two hydrophobic alpha-helices and it is secreted from the anterior pole of the parasites shortly after invasion of the host cell and finally locates at the membranous nanotubular network (MNN) (Adjogble et al., 2004; Mercier and Cesbron-Delauw, 2015). Previous studies of GRA-genes knockout parasites showed that GRA proteins are important for the PV maturation (Bougdour et al., 2014). In addition to the important functions in the maturation of PV, phenotypic analyses of these knockout parasites indicated that both GRA2 and GRA6 were involved in the formation of the membranous tubules which constitute the MNN (Mercier et al., 2002; Travier et al., 2008. Gendrin et al., 2010). GRA9 is also related to the intravacuolar network of tubular membranes (Adjogble et al., 2004). Based on its pattern of secretion and its location at the MNN, GRA9 may play a role in nutrient acquisition or host-pathogen interaction. The plaque assay revealed significant growth defect in the PLKΔGRA9 parasites. However, I did not detect any significant replication defect in GRA9 knockout parasite. These results suggest that the GRA9 was dispensable during replication of parasites in host cells, and parasite growth ability was impaired after deletion of GRA9 in a strain-dependent

manner. Many other GRAs including GRA9 were also shown to have no effect on the replication of the RH strain parasites in the PV (Rommereim et al., 2016).

Calcium-dependent signaling is important for the propagation of parasites of the phylum Apicomplexa, including *T. gondii* (Moreno et al., 2011). The invasion, motility and egress of these parasites are based on calcium-dependent events. One study showed that the knockout of GRA41 in the RH strain led to an earlier egress of the parasites from the PV and indicated that it was involved in regulating calcium homeostasis (LaFavers et al., 2017). In this chapter, deletion of GRA9 in PLK strain led to a delayed egress of the parasite from the PV under induction by the calcium ionophore A23187 while no deficiency in egress ability was observed in RHΔGRA9 strain. This indicates that GRA9 may be involved in parasite egress from PV in type II strains. The delayed egress caused by the loss of GRA9 may be related to the calcium-dependent signaling process as calcium signaling coordinates apicomplexan parasites' egress (Moreno et al., 2011; Lourido et al., 2012).

Although most of the GRAs are heavily expressed at tachyzoite stage, deletion of GRA2, GRA3, GRA6, GRA7 or GRA9 in type I strains did not cause any significant defect in virulence following intraperitoneal infection of mice (Mercier et al., 2007; Rommereim et al., 2016). A dramatic reduction in virulence in the PLKΔGRA9 parasites was observed in this study. This indicates that GRA9 is required for virulence of the parasites. The result may be explained by the slow growth of the mutant strain or GRA9 role in manipulation of host immunity, like GRA6, GRA7 and GRA15, which are reported to play important roles in the manipulation of host immunity by activating the signaling pathway (Rosowski et al., 2011; Ma et al., 2014).

2-5. Summary

Some identified dense granule proteins play major structural functions within the PV and the cyst wall. Moreover, their particular location within the PV allows them to be involved in various interactions between parasites and the host cells. Dense granule protein 9 gene has been identified in *T. gondii*, although its role in lytic cycle and virulence remains unclear. In this chapter, functional analysis of GRA9 was performed using the popular CRISPR/Cas9 technology. This chapter reported for the first time the GRA9 knockout in the type II PLK strain. First, derivatives of *T. gondii* strains RH and PLK with a null mutation in TgGRA9 were generated and their phenotypes were analyzed. However, only PLKΔGRA9 parasites showed defect in plaque formation. Furthermore, deficiency of GRA9 in PLK strain moderately decreased egress ability relative to the PLK wild type parasite. Mouse experiments demonstrated that loss of GRA9 in PLK strain significantly decreased the pathogenicity of *T. gondii*. No any different phenotype was observed in RH and RHΔGRA9 strain. These findings suggest that GRA9 protein may be involved in the PLK strain virulence.

Table. 1 Primers used for generating GRA9-deficient and complemented strains.

Primer name	Sequence (5'→ 3')	Use
RHGRA9gRNA-F1	CGAATTGGAGCTCCA CCGCGGGAGCTCCAAGTAAGCAGAAG	Construction of pHX-RH-GRA9/pHX-UPRT
RH/PLKGRA9gRNA-R1	CACCTCGTCCCATAAGGCGTAACTTGACATCCCCATTTAC	Construction of pHX-RH-GRA9/pDF-PLK-GRA9
RH/PLKGRA9gRNA-F2	ACGCCTTATGGGACGAGGTGGTTTTAGAGCTAGAAATAGC	Construction of pHX-RH-GRA9/pDF-PLK-GRA9
RHGRA9gRNA-R2	TCTAGAGCGGCCGCCACCGCGG GAGCTGATACCGCTCGCC	Construction of pHX-RH-GRA9/pHX-UPRT
PLKGRA9gRNA-F1	TTCCAGTCGACTAGTTCTAGAGAGCTCCAAGTAAGCAGAAG	Construction of pDF-PLK-GRA9
PLKGRA9gRNA-R2	TACAGCCTTCGAAGCTCTAGAGAGCTGATACCGCTCGCC	Construction of pDF-PLK-GRA9
UPRTgRNA-R1	CCAGCTCTCACAATCGAGACAACTTGACATCCCCATTTAC	Construction of pHX-UPRT
UPRTgRNA-F2	GTCTCGATTGTGAGAGCTGGGTTTTAGAGCTAGAAATAGC	Construction of pHX-UPRT
5'UPRThomology-F	CTATAGGGGCGAATTGGAGCTCGGATA TATATGGACGCCA	Construction of pBlu-UPRT
5'UPRThomology-R	TTCCTGCAGCCCGGGGGATCC GATCTTGGAATAGAAGGA	Construction of pBlu-UPRT
3'UPRThomology-F	GGGCTGCAGGAATTC**GATATC**CGAGTCGATGGAAAGCGG	Construction of pBlu-UPRT
3'UPRThomology-R	GGTACCGGGCCCCCCCTCGAGATGTACGTTCGC**AAAACT**	Construction of pBlu-UPRT
GRA1 5'UTR-F	TTCTATTCCAAGATCGGATCCCGAGGCTGCTAGTACTGG	Construction of pB-synoGRA9
GRA1 5'UTR-R	ATGAATTC CTTGCTTGATTTCTTCAAAG	Construction of pB-synoGRA9
PLKsynoGRA9-F1	CTTTGAAGAAATCAAGCAAGGAATTCATGCGGTCACTCAAGTCAAT	Construction of pB-synoGRA9
PLKsynoGRA9-R1	CGCCGCGTCCCATAAGGCGTCTCATCATTTCATGCACTTG	Construction of pB-synoGRA9
PLKsynoGRA9-F2	ACGCCTTATGGGACGCGGCGTGGATGGATTTGGCCCGTCT	Construction of pB-synoGRA9
PLKsynoGRA9-R2	GCATCACTTTCGTCGTAGTCGAATTCTCAGAGTCCTCGGTCTTCCT	Construction of pB-synoGRA9
GRA2 3'UTR-F	AAGAATTCGACTACGACGAAAGTGATGC	Construction of pB-synoGRA9
GRA2 3'UTR-R	CTTTCCATCGACTCG GATATCGTCGACTGGAACATCGGT	Construction of pB-synoGRA9

Table. 2 Primers used for validating ΔTgGRA9 and complement strains.

Primer name	Sequence (5'→ 3')	Use	Product
P1	ATCGGGAGTCTACTTATT	Validation of RHΔGRA9/PLKΔTgGRA9	P1-P2/ P1-P3/ P1-P4/P1-P7/P1-P8
P2	GTGCCAGTTTTCTCGAGC	Validation of RHΔGRA9/PLKΔTgGRA9	P1-P2/ P5-P2
P3	CGCCTTTGAGTGAGCTGATA	Validation of RHΔGRA9	P1-P3
P4	CTGGGAAAGAGTGGTACCAG	Validation of RHΔGRA9	P1-P4
P5	CGAATTGGAGCTCCACCGCGGGAGCTCCAAGTAAGCAGAAG	Validation of RHΔGRA9/PLKΔTgGRA9	P5-P6/ P5-P2
P6	TCTAGAGCGGCCGCCACCGCGGGAGCTGATACCGCTCGCC	Validation of RHΔGRA9/PLKΔTgGRA9	P5-P6
P7	ACTTAATGTCCACGTAGTTC	Validation of PLKΔTgGRA9	P1-P7
P8	CTGGTCGTTCACGTAGAACT	Validation of PLKΔTgGRA9	P1-P8
PLKcoCheck-1F	TCTTCTACGCCGACCGCCTGATT	Validation of PLK complement	PCR1
PLKcoCheck-1R	CAGGCAGCTTCTCGTAGATCAG	Validation of PLK complement	PCR1
PLKcoCheck-2F	TTCAGACTCTCTGTGGTCGGCGAG	Validation of PLK complement	PCR2
PLKcoCheck-2R	ATGGTCAACAAAACAGCATATTCCTCCC	Validation of PLK complement	PCR2
PLKcoCheck-3F	GTAGAGAGGACCAAAAGACGATTGC	Validation of PLK complement	PCR3
PLKcoCheck-3R	CGAACCGATATAAATGCATGGCAT	Validation of PLK complement	PCR3

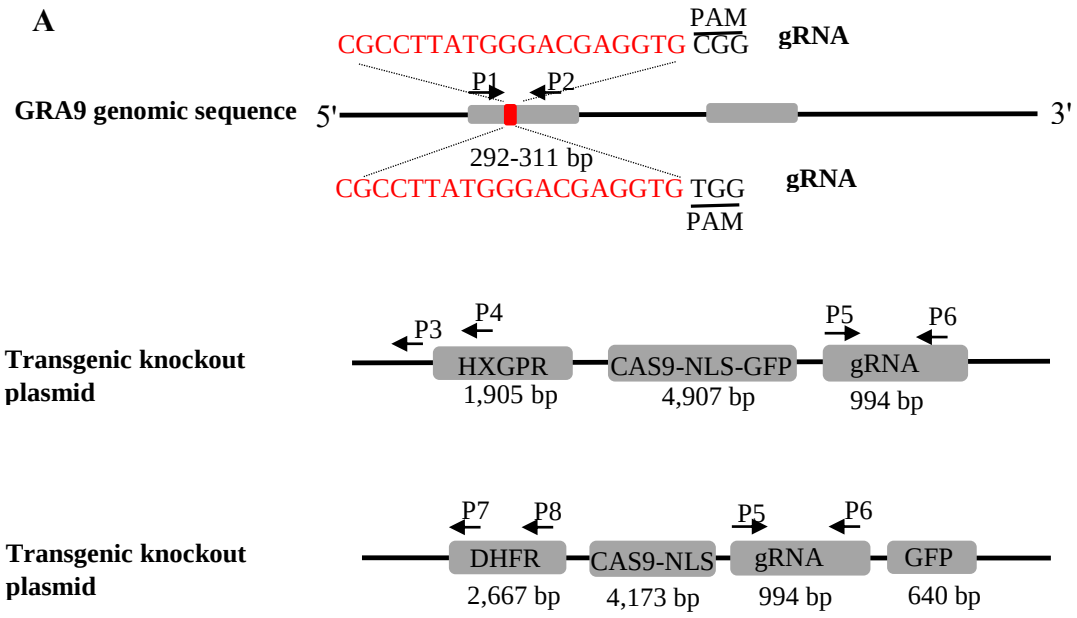


Fig. 6. CRISPR/Cas9-mediated gene disruption of the GRA9 locus. (A) Schematic illustration of the knockout plasmids. The red bar in GRA9 gene represents the region targeted by the gRNA. Both the pHX-RH-GRA9 and pDF-PLK-GRA9 plasmids express CAS, gRNA targeting the GRA9 gene in *T. gondii* and selected marker. The position of the primers used to identify genome editing within the GRA9 locus are represented by arrows. PAM, protospacer-adjacent motif; NLS, nuclear localization signal.

Note: P1-P8, the primers used for identification of disruption of the GRA9 locus in RHΔGRA9 and PLKΔGRA9 parasites.

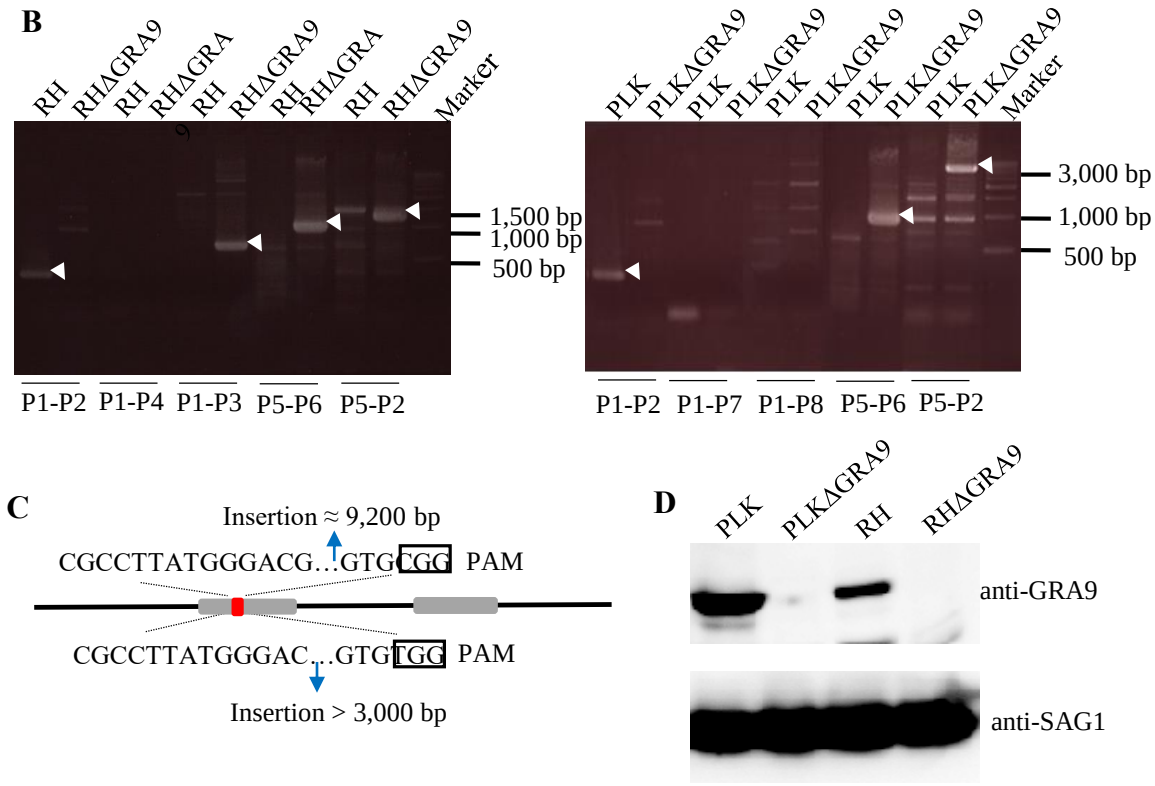


Fig. 6. CRISPR/Cas9-mediated gene disruption of the GRA9 locus. (B) PCR demonstrating nonhomologous integration and gene disruption in representative clones (RHΔGRA9 and PLKΔGRA9) compared with the parental lines RH and PLK. PCR of P1-P2 amplified a 300 bp fragment in parental lines that was lost in RHΔGRA9 and PLKΔGRA9 due to the integration of larger fragments from knockout plasmids. PCR (P1-P3 and P5-P2) provides evidence of site nonhomologous integration based on specific primers for GRA9 and knockout plasmids. (C) Gene editing within the GRA9 locus of RHΔGRA9 and PLKΔGRA9 lines. (D) Western blot analysis of GRA9-knockout parasites. GRA9 could be detected using the mouse anti-GRA9 serum in the parental lines but not in the RHΔGRA9 and PLKΔGRA9 lines.

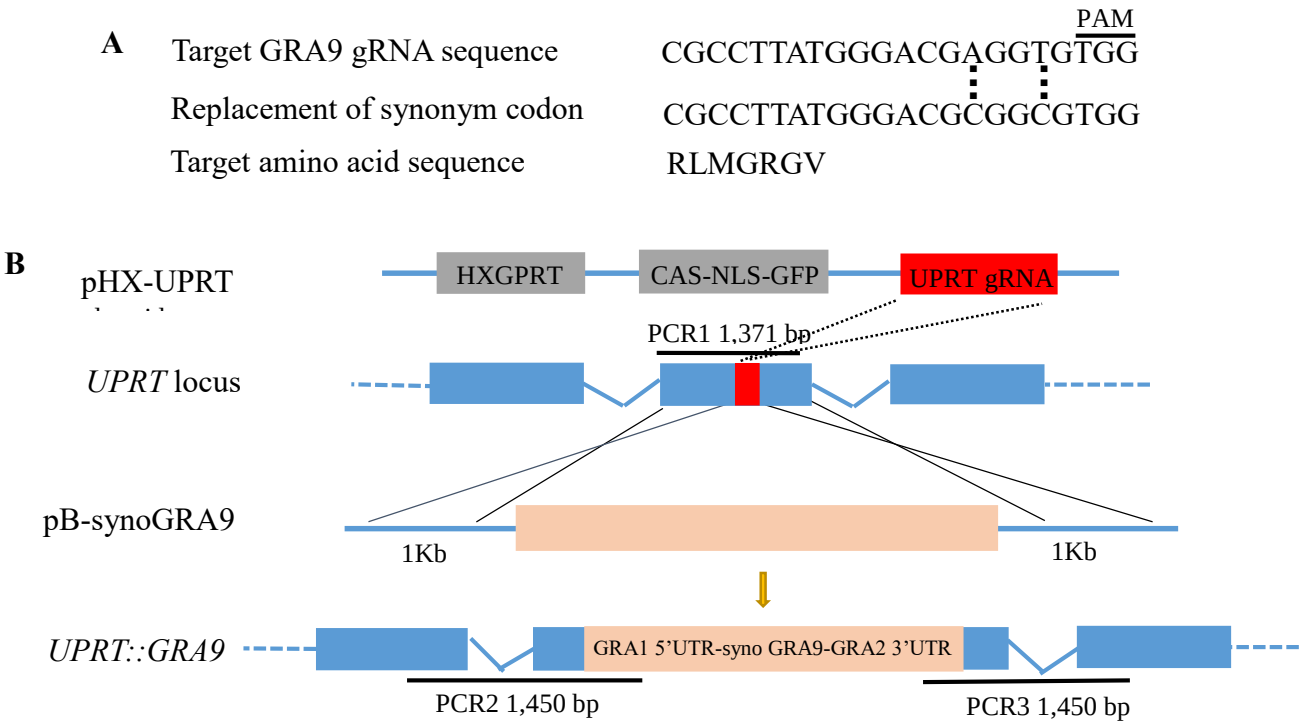


Fig. 7. Complementary expression of GRA9. (A) Synonymous mutation in complementary GRA9 gRNA sequence. Due to the introduction of knockout plasmid pDF-PLK-GRA9 into PLKΔGRA9 parasites to avoid recognition of complementary GRA9 by Cas9, nucleotide A and T in the complementary GRA9 target located near the PAM were mutated to C, resulting in no change in amino acid sequence. (B) Schematic of CRISPR/Cas9 strategy for insertion of synoGRA9 in UPRT locus. The red bar in UPRT gene represents the region targeted by the gRNA.

Noe: PCR1, PCR2 and PCR3: the length of fragments amplified from PLK or complement parasites.

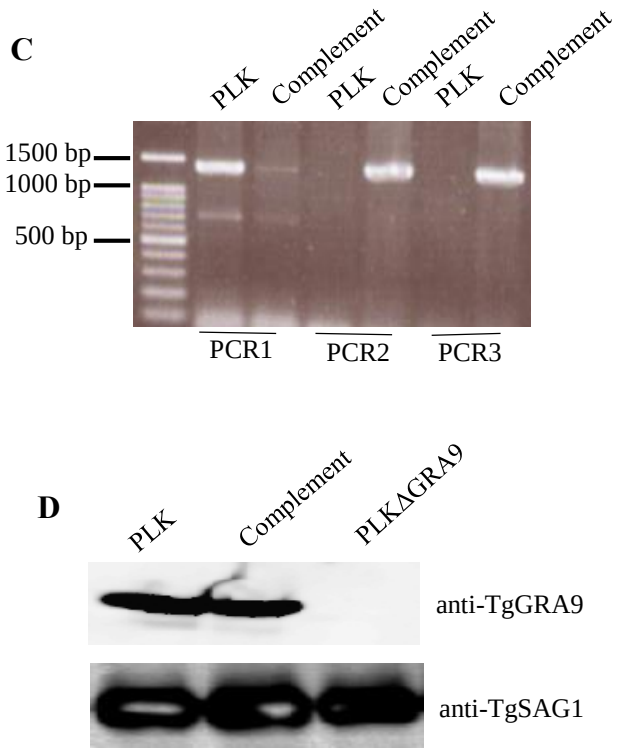


Fig. 7. Complementary expression of GRA9. (C) PCR demonstrating homologous integration and insertion in representative complement compared with the parental line PLK. PCR1 amplified a 1,371 bp fragment in PLK strain that was lost due to insertion of synoGRA9 in complement. Both PCR2 and PCR3 amplified 1,450 bp fragments and provided evidence of homologous integration. (D) Expression of GRA9 in PLKΔGRA9 and complement parasites determined by Western blot. SAG1 was used as a loading control.

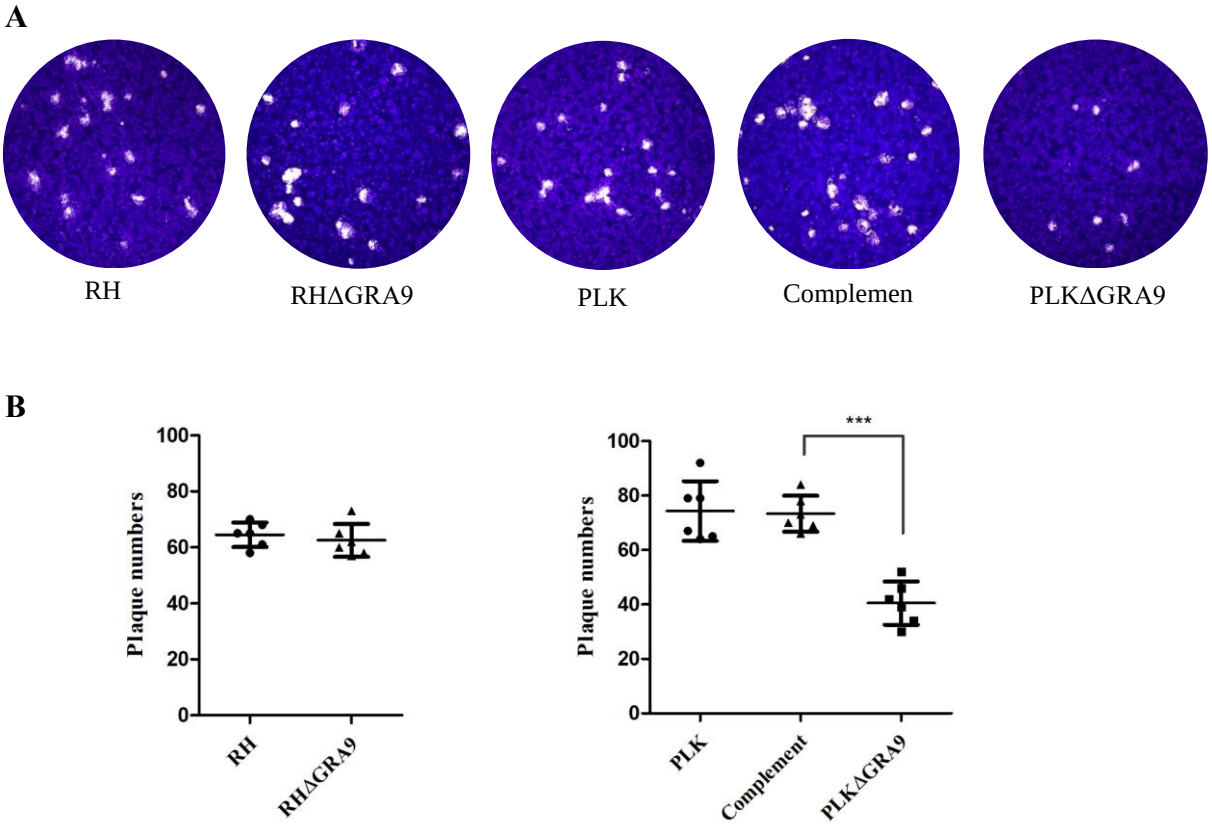


Fig. 8. Plaque assays. (A) Representative images of plaque. Vero cells were infected with 100 tachyzoites of RH strain or 300 tachyzoites of PLK strain. Plaques were visualized by staining with 0.1% crystal violet 7 or 13 days after infection and photographed. (B) Total numbers of plaques. Error bars represent means ± SEM of n = 3 independent experiments. ***, $P < 0.001$, determined by Tukey's multiple comparison test.

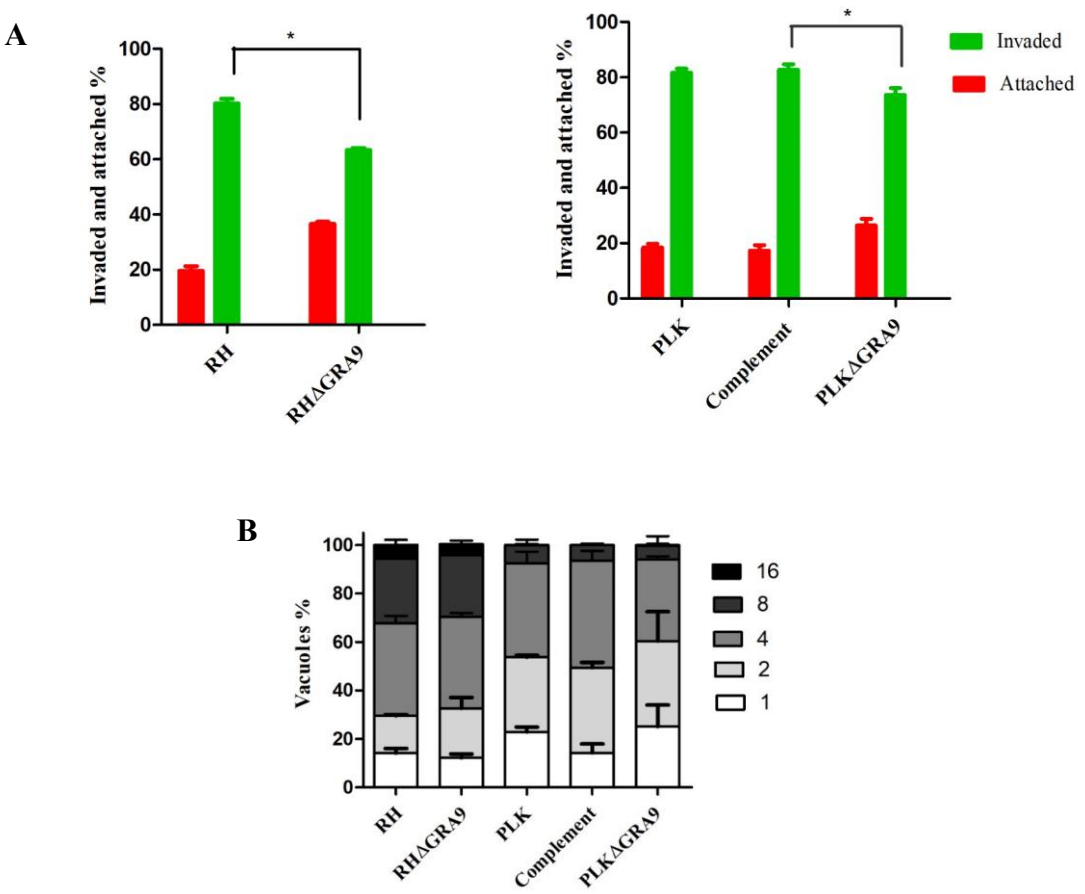


Fig. 9. Growth assay. (A) Invasion assay. Parasites invaded cells for 2 hs. Red bars represent external, attached tachyzoites, and green bars represent internal, penetrated tachyzoites. Data are the mean values ± SEM of n = 3 independent experiments. *, $P < 0.05$, determined by Chi-square test. (B) Replication assays. For intracellular growth, replication was analyzed 24 hs after infection. Error bars represent means ± SEM of n = 3 independent experiments.

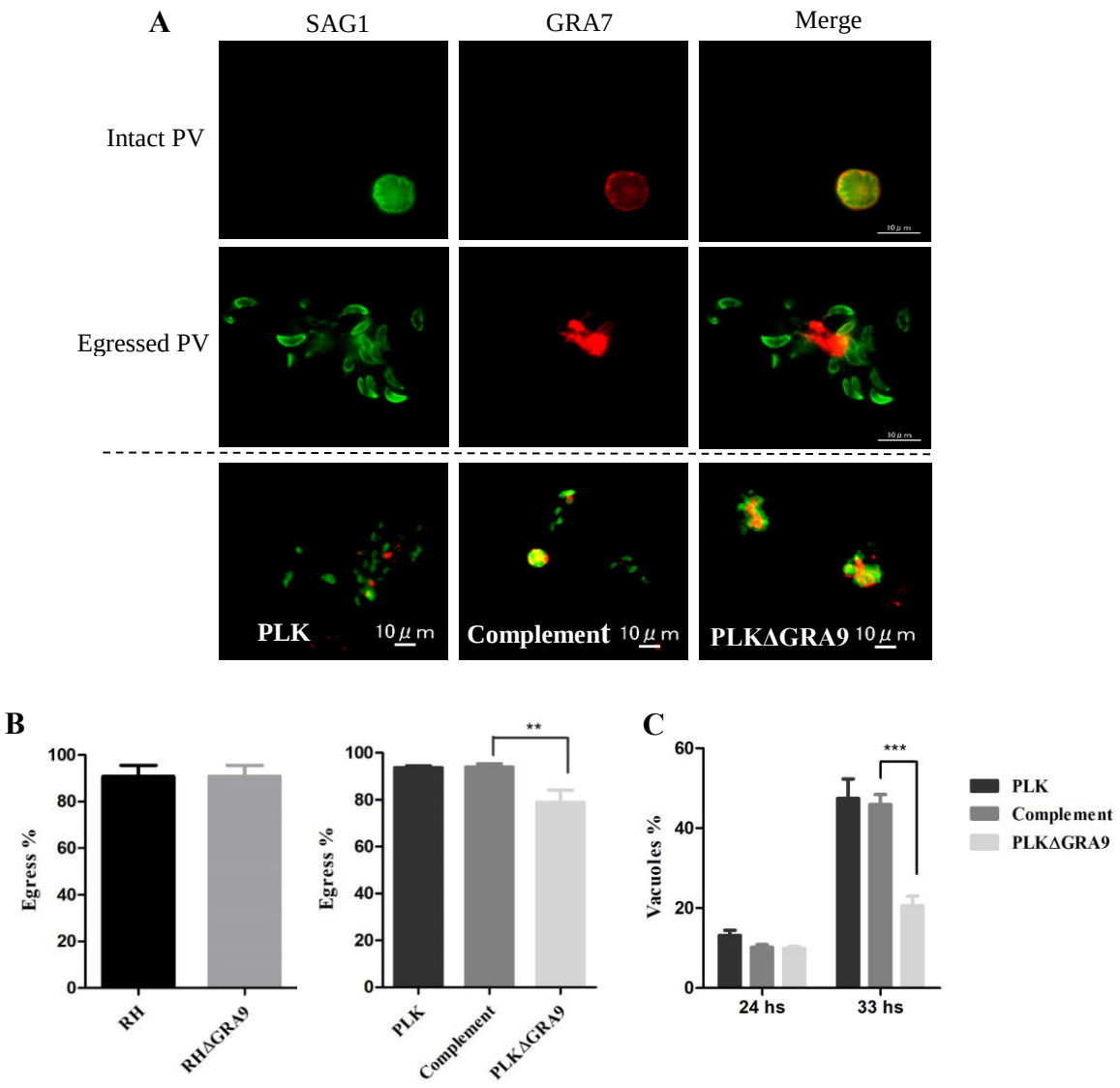


Fig. 10. Egress assay. (A) Representative images of intact and egressed PV, and each strain. The infected Vero cells were incubated with A23187, and then analyzed by IFAT to evaluate the egress rate. SAG1 was stained green; GRA7 was stained red. (B) Percentage of egress was calculated for each strain following treatment with A23187 at 32 hs post infection. Error bars represent means ± SEM of n = 3 independent experiments. **, $P < 0.01$, determined by Tukey's multiple comparison test. (C) Percentage of PVs that contained one parasite was calculated at point time. Data are the mean values ± SEM of n = 3 independent experiments. ***, $P < 0.001$, determined by Chi-square test.

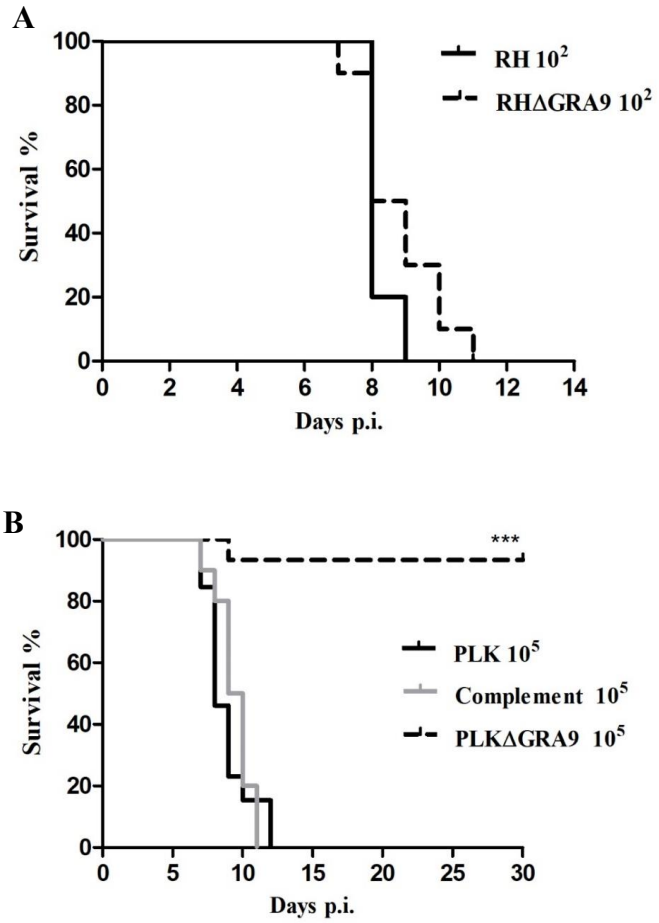


Fig. 11. Survival curves of BALB/c mice infected with parasites. (A) Mice were infected i.p. with 10^2 RH (n = 10) or 10^2 RHΔGRA9 (n = 10). (B) Mice were infected with 10^5 PLK (n = 13), 10^5 complement (n = 10) or 10^5 PLKΔGRA9 (n =15) tachyzoites by i.p.. ***, $P < 0.0001$, determined by the log-rank test.

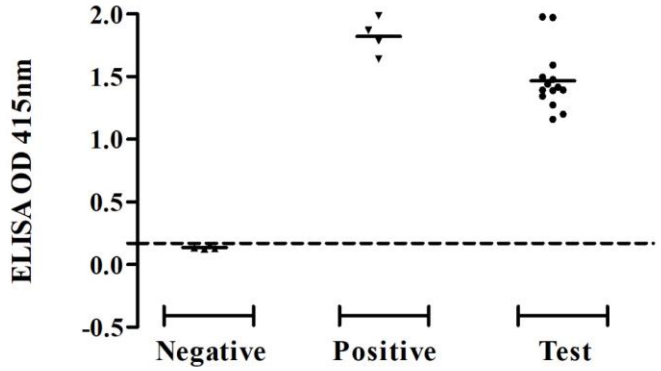


Fig. 12. Results of ELISA assay confirming *T. gondii* infection in mice. Sera were collected from surviving mice (n = 14) two weeks post infection. *T. gondii* antibody in sera was evaluated using ELISA by recombinant GRA7 protein. The sera from normal mice (n = 4) were used as negative control and sera from mice (n = 4) experimentally infected by *T. gondii* were used as positive control.

General discussion

Toxoplasma gondii is a global protozoan parasite that infects up to one-third of the world's human population causing toxoplasmosis (Montoya and Liesenfeld, 2004). Toxoplasmosis can induce heath problem in human beings and cause economic losses in animals, and is considered as the third most common food-borne parasitic infection requiring hospitalization (Vaillant et al., 2005). Identification of the growth and virulence factors of this parasite is important for the development of control strategies. In the current study, in order to better understand the function of GRA9 in *T. gondii*, molecular characterization of GRA9 and phenotypes associated with knockout of GRA9 in RH and PLK strains were analyzed.

Phylogenetic analysis of GRA9 proteins of different *T. gondii* strains and other species indicated GRA9 to be relatively conserved in the different strains of *T. gondii*. Predicted amino acid sequence alignment indicated that there was only one polymorphic amino acid in the sequence of GRA9 of RH and PLK strains, indicating that GRA9 is not a polymorphic protein. In this study, GRA9 expression level was found significantly higher in the PLK strain than in the RH strain. Although, most of GRA proteins are heavily expressed at tachyzoite stages (Mercier et al., 2007), none of the single or double deletion of GRA2-9 type I RH strains showed significant defects in virulence in CD-1 mice following i.p. infection (Rommereim et al., 2016). Similarly, RHΔGRA9 showed same pathogenicity with parental strain in BALB/c mice in this study. These data indicate that GRA2-9 appear to be dispensable for type I strains' virulence. In contrast, type I ΔGRA6 and ΔGRA7 strains were previously reported to exhibit decreased virulence phenotype in mice following infection at a typical sites (subcutaneously or in the footpad) (Alaganan et al., 2014; Ma et al., 2014). Surprisingly,

loss of GRA9 in PLK strain caused attenuated virulence in BALB/c mice following i.p. injection. This indicates that GRA9 is important for the virulence of type II PLK strain. However, these results could not explain the lower virulence of type II PLK strain in comparison to the highly virulent type I RH strain (Sibley and Ajioka, 2008).

In this study, deletion of GRA9 in PLK strain led to a delayed egress of the parasite from the parasitophorous vacuole (PV) under induction by the calcium ionophore A23187 while no deficiency in egress ability was observed in RHΔGRA9 strain. This may be related to the low expression of GRA9 in RH strain. This indicates that GRA9 may be involved in parasite egress from PV in type II strains. Previous study showed that the knockout of GRA41 in the RH strain led to an earlier egress of the parasites from the PV and indicated that it was involved in regulating calcium homeostasis (LaFavers et al., 2017). Therefore, the delayed egress caused by the loss of GRA9 may be related to the calcium-dependent signaling process as calcium signaling coordinates apicomplexan parasites' egress (Moreno et al., 2011; Lourido et al., 2012). GRAs are important for the formation of the PV. The IFAT images of egress assay showed some differences between PLKΔGRA9 and intact PV control. The loss of GRA9 may have disrupted the PVM and parasites egressed but failed to move away from the vacuole. However, it is difficult to clarify this point using IFAT egress assays. Further studies using for instance video microscopy as described previously (Lourido et al., 2012), would be needed to measure egress and parasitophorous membrane permeabilization and elucidate the role of GRA9 during egress.

The phenotype of PLKΔGRA9 strain was not linked to an immediate defect in parasite replication because the replication defect was not observed at 24 hs post infection, yet 13-day-old plaques were significantly reduced in plaque numbers. The plaque formation is the result of many rounds of parasite invasion, replication, motility

and egress (Lourido et al., 2012). Thus, here type II GRA9 mutant showed defect in initial invasion and egress, which may contributes to decreased plaquing efficiency. However, a decrease in plaque numbers but not in plaque area usually reflects a default in survival as extracellular parasites, or maybe in the parasites' capacity to invade. Though, a slight but statistically significant reduction in percentage of invaded parasites was observed in both RHΔGRA9 and PLKΔGRA9, the level of reduction is not sufficient to explain the significant reduction of PLKΔGRA9 plaquing efficiency. Except for the egress, it is possible that GRA9 is involved in other processes of PLK strain, such as motility during transfer from one cell to another (Long et al., 2017). Obviously, the slightly but significantly reduced invasive ability of RHΔGRA9 is not enough to cause significant reduced plaquing efficiency. In the current study, the delayed egress of PLKΔGRA9 did not result in apparent decrease of plaque size. Consistent with our result, GRA41 could regulate timing of parasite egress and knockout of GRA41 led to statistically significant decrease in the number of plaques as compared to the parental strain without any noticeable differences in plaque size (LaFavers et al., 2017). The defective egress may just lead to low density of parasites in plaque (Okada et al., 2013).

General summary

The protozoan parasite *Toxoplasma gondii* is an obligate intracellular pathogen belonging to the phylum Apicomplexa and virtually infect any kind of warm-blooded animal, including humans. Approximately 25-30% of the world's population is infected with *T. gondii*. *T. gondii* is acquired orally by ingestion of raw or undercooked meat containing cysts, or after ingestion of fresh water or vegetables spoiled with oocysts. Toxoplasmosis is an important worldwide zoonosis. However, at present, no safe and effective vaccine exists for prevention against toxoplasmosis. Thus, identification of the growth and virulence factors of this parasite is important for the development of control strategies.

The main purpose of this study was to identify new factors that are important for parasite growth and virulence. An in-depth understanding of a protein's molecular character is useful for evaluating the function of the protein. Furthermore, it is a popular method to evaluate the protein's function by knocking out the gene. Dense granule proteins play major structural functions within the PV and the cyst of *T. gondii*. Moreover, their particular location within the PV allows them to be involved in various interactions between parasites and the host cells. GRA9 protein has been identified in *T. gondii*, although its role in the lytic cycle remains unclear.

In chapter 1, phylogenetic analysis was performed with GRA9 protein from different *T. gondii* strains and other species indicating GRA9 to be relatively conserved in the different strains of *T. gondii*. Sequence analysis showed that the nucleotide sequences of GRA9 in the type I RH and type II PLK strains were both 957 bp, which were consistent with the predicted sequences of type I (TGGT1_251540) and type II (TGME49_251540), respectively. Furthermore, alignment of predicted amino acid

sequences showed that RH GRA9 contains one amino acid substitution compared to that of PLK GRA9. All these information are useful for further elucidating the function of GRA9 protein in *T. gondii*.

In chapter 2, derivatives of *T. gondii* RH and PLK strains with a null mutation in GRA9 were generated using CRISPR/Cas9 system. The phenotypes of GRA9 in wild types, knockout and complemented strains were analyzed *in vitro* and *in vivo* using Vero cells and BALB/c mice, respectively. The phenotype analysis revealed that knockout of GRA9 in PLK parasites inhibited plaque formation and egress from PV. Both the plaque formation and egress ability of PLKΔGRA9 strain were restored by complementation with a synonymous allele of PLK strain GRA9. Mouse experiments demonstrated that loss of GRA9 in PLK strain significantly reduced the pathogenicity of *T. gondii*. However, there was no phenotypic differences between RH and RHΔGRA9 strains except the defect in host cell invasion. Overall, *T. gondii* GRA9 knockout only influenced the growth and virulence of PLK strain. These results indicate that GRA9 may be involved in parasite egress and virulence in mice in a strain-specific manner.

Overall, in the current study, a detailed molecular characterization of GRA9 was provided and its function in type I RH and type II PLK strains *in vitro* and *in vivo* was characterized. These results provide useful information for further understanding the mechanisms of how GRA9 is involved in the lytic cycle and virulence of *T. gondii*.

和文要約

Toxoplasma gondii は、アピコンプレクサ門に属する偏性細胞内寄生性の病原性原虫であり、ヒトを含むほぼすべての温血動物に感染する。感染率は世界人口のおよそ 25~30%に上り、シストが感染した生あるいは加熱不十分な肉の喫摂食、オーシストに汚染された水や野菜の摂取により感染する。これらの点から、トキソプラズマ症は世界的に重要な人獣共通感染症である。しかし、現在まで、トキソプラズマ症を予防する安全かつ効果的なワクチンは開発されていない。したがって、原虫の増殖・病原性因子の同定がその対策の開発には重要である。

本研究の主な目的は原虫の増殖および病原性に重要な新規因子を同定することである。タンパク質の分子特性の詳細な理解は当該タンパク質の機能評価に有用である。また、遺伝子のノックアウトによるタンパク質機能の評価もよく利用される手法である。デンス グラニュールタンパク質 (GRA) は *T. gondii* の寄生胞やシストの構造を維持する主要な因子としての機能を果たしている。さらに、その寄生胞内における特異的な局在は寄生虫・宿主間の様々な相互作用への関与を可能にしている。GRA9 は *T. gondii* において同定されたが、その増殖過程における役割は明らかになっていない。

第 1 章では、*T. gondii* 株間および種間での GRA9 タンパク質の系統解析を実施し、GRA9 が *T. gondii* 株間において比較的保存されていることを示した。配列解析の結果 Type I RH 株および Type II PLK 株における GRA9 の核

酸配列はともに 957 bp であり、Type I (TGGT1_251540) および Type II (TGMR49_251540) について予測された配列と一致することが示された。さらに、予測されたアミノ酸配列のアラインメントにより、RH 株 GRA9 と PLK 株 GRA9 との間に 1 アミノ酸の置換があることが明らかになった。これらは *T. gondii* の GRA9 タンパク質の機能をさらに解明するために有用な知見である。

第 2 章では、GRA9 遺伝子を欠損した *T. gondii* RH 株および PLK 株派生株を CRISPR/Cas9 法により作製した。野生型、欠損株および補完株における GRA9 の表現型は *in vitro* および *in vivo* でそれぞれ Vero 細胞と BALB/c マウスを用いて解析した。表現型解析の結果、PLK 株における GRA9 のノックアウトは原虫によるプラーク形成と寄生胞からの脱出を阻害することが明らかになった。PLKΔGRA9 株のプラーク形成能および脱出能はともに PLK 株 GRA9 の同義アリルを再導入することで回復した。マウスを用いた実験では、PLK 株における GRA9 の欠損は *T. gondii* の病原性を有意に低下させた。しかし、RH 株と RHΔGRA9 株の間では、宿主細胞への侵入率の低下以外には表現型の違いは認められなかった。全般的に見て、GRA9 の欠損は PLK 株においてのみ増殖と病原性に影響した。これらの結果は GRA9 が株特異的に原虫の脱出やマウスへの病原性に関与することを示唆している。

以上のように、本研究では GRA9 の詳細な分子特性を明らかにし、Type I RH 株と Type II PLK 株における *in vitro* と *in vivo* における当該タンパク質の機能を解析した。これらの結果は GRA9 が *T. gondii* の増殖過程や病原性に関わるメカニズムをさらに理解するにあたって有益な知見を提供するものである。

Acknowledgements

I would like to express my deepest thanks and gratitude to my supervisor, Prof. Xuenan Xuan, for giving me the golden opportunity to pursue a Ph.D. in the Program of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine (Obihiro Univ. Agri. Vet. Med.), and to join the Research Unit for Host Defense at the National Research Center for Protozoan Diseases (NRCPD). I am indebted him for intellectual guidance, constructive criticism, invaluable suggestions, patiently revising this dissertation and tireless support throughout my study.

I also would like to thank Prof. Yoshifumi Nishikawa (Obihiro Univ. Agri. Vet. Med.) for his scientific guidance, kind support, and encouragement during my study. My special thanks to Prof. Makoto Igarashi (Obihiro Univ. Agri. Vet. Med.), Prof. Shin-ichiro Kawazu (Obihiro Univ. Agri. Vet. Med.) and Prof. Naoaki Yokoyama (Obihiro Univ. Agri. Vet. Med.) for their valuable advice, suggestions and comments.

I am deeply grateful to Dr. Paul Franck Adjou Moumouni, Dr. Liu Mingming, Dr. Gao Yang, Dr. Lee Seung-Hun, Galon Eloiza May, Tumwebaze Maria Agnes, and Byamukama Benedicto for their kind assistance, careful reading and commenting on countless revisions of my thesis. I am also grateful Dr. Fumiaki Ihara, Dr. Kousuke Umeda, Dr. Ragab M. Fereig and Dr. Arpron Leesombun for their laboratory supports and excellent guidance during my study.

I would like to acknowledge all of the formers and present staffs and students of NRCPD for their kind support.

My deep thanks for China Scholarship Council and the Japanese Government, for the financial support of my scholarship to complete my study and stay in Japan.

I am greatly indebted to associate professor Jia Honglin (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences) for his excellent guidance and kind suggestions in publishing of my academic paper. Special thanks to Dr. Du Jige and Dr. Ding Jiabo for their help during my internship in China.

Finally, I am indebted to my parents, my husband Gao Yang and my daughter Gao Yinuo, for their continuous inspiration during my study in Japan. Without your encouragement, I would not have come this far. Thank you very much.

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