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Functional analysis of enolase in Toxoplasma gondii.

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Functional Analysis of Enolase in *Toxoplasma gondii*

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ABSTRACT

Toxoplasma gondii is an intracellular parasite of humans in the phylum Apicomplexa. In an intermediate host (including human and other mammals), *T. gondii* occurs in an actively dividing form (tachyzoite) and a dormant form (bradyzoite). My research is focused on the function of enolase, which is present in two isoforms known as ENO1 and ENO2. Previous study showed that enolase is expressed in stage-specific manner. ENO2 is expressed only in tachyzoites, and ENO1 is specifically expressed in bradyzoites. Moreover, both isoforms of enolase are be localized in nucleus as well as in cytoplasm of *T. gondii*. This research is aimed to dissect the role of ENO2 in the growth of *T. gondii*. I used homologous dsRNA to induce ENO1 or ENO2 gene silencing. I generated two transgenic parasite lines expressing ENO1 or ENO2 dsRNA, and used them in the loss-of-function study. Although the reduction of ENO2 expression was observed, I did not detect any changes in the growth of parasites, as monitored by two different methods, vacuole counting and plaque assay. I postulated that another protein or other metabolic pathway might compensate for the loss of ENO2 function.

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ABBREVIATIONS

ATP	Adenosine triphosphate
bp	base pair
BAG1	bradyzoite surface antigen 1
BSA	bovine serum albumin
CAT	chloramphenicol acetyl tranferase
cDNA	complementary DNA
DAPI	4', 6 diamidino-2-phenylindole
DEPC	diethlypyrocarbonate
DHFR-TS	dihydrofolate reductase-thymidylate synthase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
dsRNA	Double-stranded RNA
EDTA	ethylenediaminetetra-acetic acid disodium salt
ENO	enolase
FBS	fetal bovine serum
FITC	Fluorescein iso-thiocyanate
GFP	Green fluorescent protein
G6PI	glucose-6-phosphate isomerases
HFF	human foreskin fibroblasts
HXGPRT	Hypoxanthine-guanine phosphoribosyltransferase
INF	Interferon
LDH	lactate dehydrogenase
LacZ	B-galactosidase
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MPA	mycophenolic acid
mRNA	Messenger RNA
miRNA	Micro-RNA
nt	nucleotide
rNTP	ribonucleoside triphosphate
Oligo	Oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polynerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RNase	Ribonulease

ROP1	rhoptry protein 1
RT	reverse transcription
RT-PCR	reverse transcription PCR
RISC	RNA induced silencing complex
siRNA	small interfering RNA
SAG1	surface antigen 1
SDS	sodium dodecyl sulphate
SSC	sodium chloride-sodium citrate
Taq	*Thermus Aquaticus*
TEMED	N,N,N',N'-Tetramethylethylenediamine
TUB	Tubulin
6TX	6 thioxanthine
UPRT	uracil phosphoribosyltransferase
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 *Toxoplasma gondii*

Toxoplasma gondii is an intracellular pathogenic protozoa parasite. It belongs to the phylum Apicomplexa and is the only known member of the genus *Toxoplasma*. It was first discovered in a rabbit, by Splendore in Brazil in 1908, and also by Nicolle and Manceaux in a northern African rodent *Ctenodactylus gundi* (Black and Boothroyd, 2000). As most of the Apicomplexa parasites, *T. gondii* is an important pathogenic agent of humans and animals. Its hosts include the vast majority of warm-blooded animals. In 2000, it was reported that, 15.8% of the population between 12 to 49 years old in United States were infected with *T. gondii* (Jones *et al.*, 2003). The infection causes the disease called toxoplasmosis, which is usually asymptomatic in healthy individuals. However, for immuno-compromised patients, such as AIDS patients and those who have recently received organ transplantation, infection by *T. gondii* can cause severe complications such as hepatitis, pneumonia, blindness, and neurological disorders and may lead to behavioral alteration. Furthermore, an acute infection during pregnancy can cause *in utero* infection as well, resulting in a spontaneous abortion, a stillborn child, or a child that is born with some degree of mental or physical retardation (Dubey, 1996).

While residing in the hosts, *T. gondii* converts between tachyzoite, the rapid proliferating stage, and bradyzoite, the slowly replicating stage. Normally, uncompromised host immune response can quickly remove tachyzoites during the acute

infection. In most cases of human toxoplasmosis, tachyzoites can differentiate into bradyzoites, which stay within the tissue cysts. Parasites are thus able to escape the host immune response for the remainder of the host's lifespan, leading to the chronic infection. When the patients become immuno-compromised, bradyzoites are released from the cysts and differentiate into tachyzoites, resulting in a recurrent acute infection (Gross *et al.*, 1996). Current treatment of toxoplasmosis using a cocktail of pulfonamide (dihydropteroate synthetase inhibitor) and pyrimethamine (dihydrofolate reductase inhibitor) is only effective for the acute infection and may cause harsh side effects (Black and Boothroyd, 2000). Although a combination of atovaquone and clindamycin is effective for treatment of the chronic toxoplasmosis in mouse and some other animals, their effects have not been re-produced in human (Alves and Vitor, 2005; Djurkovic-Djakovic *et al.*, 2002). To develop new drug targets and strategies to eradicate its infection, we need to learn more about the parasite, especially its metabolism and interaction with host organisms.

1.1.1 Life cycle of *T. gondii*

Toxoplasma gondii multiplies by sexual and asexual cycles. The sexual life cycle occurs exclusively in the intestine of its definite host, felines. The cycle begins when a cat ingests food containing *T. gondii*-tissue cysts. The parasites are released from the cyst in the small intestine and infect epithelial cells, initiating gametogenesis and differentiation into micro- and macrogametes. Then, two gametes fuse and produce an oocyst, which is

later secreted in feces. Millions of oocysts can be generated within 2 weeks after oocyst formation. Within 2-3 days after being released from the intestine, oocysts undergo sporogony and generate the infective haploid stage known as sporozoites. The mature sporozoites are very stable in the environment and can survive for months in cold and dry climates (Dubey, 1994). When the sporozoites are ingested by the felines, they continue to propagate by the sexual life cycle. Ingested by other animals, referred to intermediate hosts, the parasites will multiply using their asexual life cycle.

The asexual life cycle occurs in any infected warm-blooded animals. The transmission can usually begin following the ingestion of sporulated oocysts or bradyzoites in tissues cysts in raw or contaminated meat products. Then the parasites transform into tachyzoites and reproduce asexually within infected cells. After several rounds of synchronous division of the parasites, the infected cells that cannot support the parasite load will be destroyed. Newly formed tachyzoites are then released to infect other cells. During this phase of infection, the tachyzoites can spread to every organ of the host. Approximately 2-3 weeks following infection and in response to the host immunity, the tachyzoites convert to slow-replicating bradyzoites within the tissue cysts (Dubey *et al.*, 1998; Soete *et al.*, 1993).

Figure 1.1

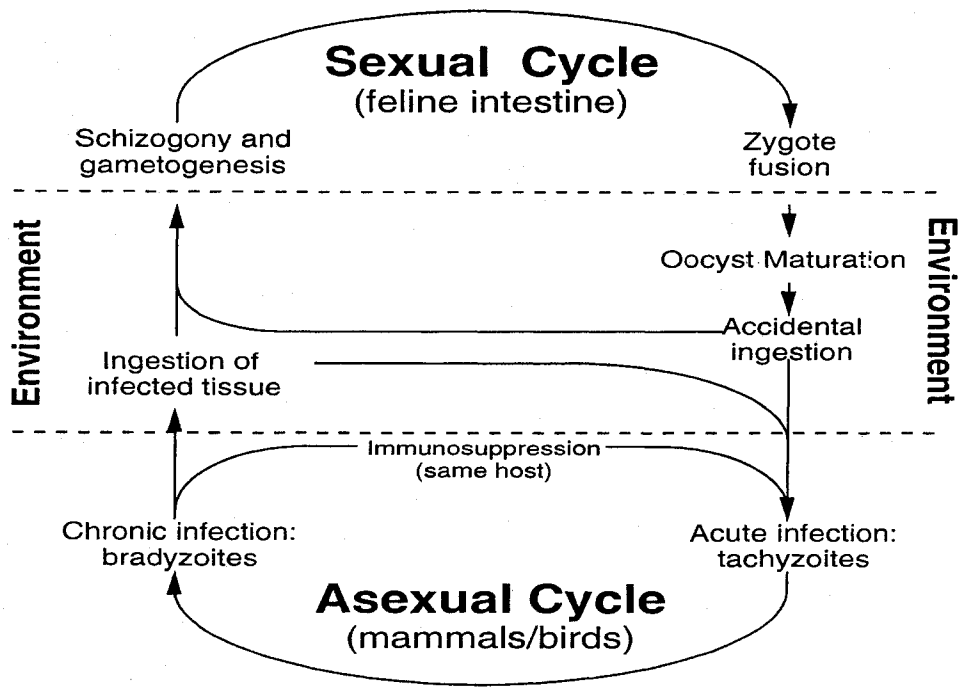
Life cycle of *Toxoplasma gondii*

Legend

The life cycle of *T. gondii* consists of two phases: sexual and asexual. The sexual phase occurs only in the intestine of felines (definite host) while the asexual phase takes place in birds and animals (Yang and Parmley, 1997). The figure is obtained from Black and Boothroyd, 2000.

Figure 1.1

Life cycle of *Toxoplasma gondii*



1.1.2 Differences between tachyzoites and bradyzoites

The conversion between tachyzoites and bradyzoites is accompanied by morphological and molecular biological changes, including the expression of stage-specific antigen and the alterations of basal metabolism (Ferguson and Hutchison, 1987; Denton *et al.*, 1996).

The fast-growing tachyzoite is usually surrounded by a sac-like membrane, called a parasitophorous vacuole, which originates from both the host and parasite cell membranes (Gross *et al.*, 1996). The tachyzoites within a single vacuole usually divide synchronously, leading to the formation of a rosette structure. The bradyzoites stay in the tissue cysts that can be as large as 100μm in length and are mainly localized in the brain and muscle tissues. The tissue cysts are surrounded by an elastic thin wall which contains β-(1, 4) linkaged N-acetyl-glucosamine residues called chitin without glycogen and other polysaccharides (Gross *et al.*, 1996). The cyst wall is able to bind with lectins such as those found in the seed of *Dolichos biflorus* and wheat-germ (Boothroyd *et al.*, 1997; Tomavo, 2001; Cleary *et al.*, 2002). Thus these lectins are used as the detection reagents for the tissue cysts and bradyzoites (Ferguson *et al.*, 2002). Within the cyst wall, the bradyzoites are less susceptible proteolytic enzymes and gastric acid in the stomach (Freyre, 1995). Within the cytosol of the bradyzoites, several amylopectin granules are located and supposedly act as energy source. Having non-functional mitochondria, bradyzoites lack a functional TCA cycle and respiratory chain. As a result, bradyzoites are not susceptible to mitochondrion electron transport inhibitors such as atovaquone. On

the other hand, tachyzoites have functional mitochondria and both aerobic and anaerobic respiration (Denton *et al.*, 1996; Dando *et al.*, 2001). Thus glycolysis is essential for both tachyzoites and bradyzoites. Therefore, the enzymes catalyzing the reactions in the glycolysis pathway, such as enolase, glucose-6-phosphate isomerases and glyceraldehyde-3-phosphate dehydrogenase, have been considered as promising drug targets in *T. gondii* and many other apicomplexa parasites (Verlinde *et al.*, 2001; Roos, 2005).

Differential expressions of heat shock proteins (HSP) were reported in the tachyzoite and bradyzoite stages. For example, two differentially spliced forms of HSP60 are present in both life cycle stages. Using polyclonal antibodies raised against HSP60, it was found that HSP60 is localized in the mitochondria in tachyzoite stage, while in two vesicular bodies during the stage of bradyzoites (Toursel *et al.*, 2000). Bradyzoite specific antigen 1 (BAG1) or HSP30, which is homologous to small plant HSP, is only expressed in bradyzoites, but not in tachyzoites (Bohne and Roos, 1997; Parmley *et al.*, 1995). In addition, many surface antigens, including CST1, SAG2A and SAG2B, are stage-specific, suggesting they might be important for the interaction between the parasites and their host cells (Zhang *et al.*, 2001; Cleary *et al.*, 2002; Lekutis *et al.*, 2000). Moreover, a number of metabolic enzymes, including those of glycolysis and gluconeogenesis such as lactate dehydrogenase (Dando *et al.*, 2001), glucose-6-phosphate isomerases (G6PI) and enolase (ENO), were reported to be differentially expressed in tachyzoite and bradyzoite, suggesting that their association with changes in metabolism rates and environment

adaptation of the parasites (Yang and Parmley, 1997; Tomavo, 2001; Dzierszinski et al., 2001; Dzierszinski *et al.*, 1999).

1.1.3 Interconversion between tachyzoite and bradyzoite

The conversion between tachyzoite and bradyzoite is essential for *T. gondii* and plays a key role in its pathogenesis. A better understanding of the interconversion mechanism could greatly facilitate a design for new chemotherapeutic agents capable of eliminating tissue cysts (Boothroyd *et al.*, 1997). *In vivo*, the stage conversion is related to the host inmmune responses. It has been suggested that the switch between tachyzoites and bradyzoites is related to the expression of heat shock proteins and parasites' stress responses.

Most experimental strains of *T. gondii* can switch between tachyzoites and bradyzoites in cell cultures. About 10-20% of cysts are formed by spontaneous conversion. External stresses mimicking the host immune responses can induce tissue cyst formation and increase the formation of cyst *in vitro* (Soete *et al.*, 1993). The cyst-induction includes growing the parasites in the alkaline environment (pH 8.2-8.4) or acid conditions (pH 6.6-6.8), increasing culture temperature (42 °C instead of 37 °C). These methods rely on the stress response of the parasites (Soete *et al.*, 1993; Weiss *et al.*, 1995; Weiss and Kim, 2000). Inhibitors of mitochondrial function, such as oligomycin, antimycin A, atovaquone and rotenone, as well as inducers of oxidative stress including nitric oxide and Nitroprusside, an exogenous source of nitric oxide, can also induce the

encystment of *in vitro* cultures (Soete *et al.*, 1993; Bohne *et al.*, 1994). It was also shown that the elevation of cAMP or cGMP could play a role in the bradyzoite induction (Kirkman *et al.*, 2001). Immunological factors, including interferon (IFN)-γ and tumour necrosis factor (TNF)-α, play a role in controlling tachyzoite growth and could indirectly control stage conversion. Although evidence suggest the indirect effect of NO-induced stress, the underlining mechanism has yet been determined (Alexander and Hunter, 1998).

Additionally, it was reported that conditions favouring the formation of bradyzoites were associated with the up-regulation of heat shock proteins (HSPs), suggesting that HSPs are essential during stress-induced stage conversion (Weiss *et al.*, 1998). Knockout of the *BAG1* (*HSP30*) gene can decrease, but not completely prevent *in vivo* cyst formation of the parasites. This evidence further validates the involvement of HSPs in the process of stage conversion (Bohne *et al.*, 1998).

1.1.4 *T. gondii* culture conditions and parasite strains

Toxoplasma gondii can be *in vitro* cultured in different cell types. Human foreskin fibroblasts (HFF) are the most widely used because they quickly propagate and survive in the presence of many growth-inhibiting drugs, such as those used during the selection of transgenic parasites. Additionally, they allow the parasites to replicate for several cycles before lysis (Freyre, 1995; Roos *et al.*, 1994). Various cell cultures, namely Vero cells,

Hela cells and T cells, are commonly used in the culture of *T. gondii* (Cleary *et al.*, 2002; Boothroyd *et al.*, 1997).

There are several experimental strains of *T. gondii*. Each strain exhibits different replication rates, virulence and ability to form *in vitro* cysts and are thus suitable for different experimental design (Freyre, 1995). An experimental strain called RH is highly virulent and fast-duplicating and the most commonly used in biochemical experiments, particularly in the characterization of parasite virulence and production drug-resistant mutant lines (Roos *et al.*, 1994). However, the RH strain exhibits less ability to form tissue-cysts, thus produces few tissue cysts in mouse when cultured *in vitro*. Moreover the RH strain can not undergo the sexual replication in the definite hosts (Freyre, 1995). Other strains, such as ME49 produce more tissue cysts and are thus ideal for study on bradyzoite development and differentiation. A subclone of ME49 strain, referred to as the PLK strain, shows slower replicating rates and is widely used for the study of life cycle and stage conversion of *T. gondii*, due to its ability to undergo complete sexual life cycle and to convert between tachyzoites and brayzoites (Soete *et al.*, 1993; Boothroyd *et al.*, 1997).

1.1.5 *T. gondii* as an experimental model for apicomplexan parasites

Many members of Apicomplexa are pathogenic. For example, Plasmodium falciparum is the causing agent of malaria. It is difficult to culture and maintain in the laboratory, thus impeding the experimental studies (Sibley, 2003). T. gondii, on the other

hand, can be easily maintained in vitro using standard cell culture techniques. In addition, this parasite is amenable for molecular transformation and is thus ideal for genetic manipulation and analysis. Genetic manipulation by molecular transformation, including both transient transformation and stable transformation, has been widely employed as a tool to verify gene function and molecular events at various stages of *T. gondii* (Kim and Weiss, 2004). The modulations of *T. gondii* gene expressions by molecular tools such as insertional mutagenesis, tetracycline-repressor-based inducible systems, are extensively utilized to analyze gene functions (Donald and Roos, 1995; Nakaar *et al.*, 2000; Meissner *et al.*, 2001). *T. gondii* has an 80 Mb haploid genome consisting of 14 chromosomes during its asexual life cycle, which facilitates the generation of loss-of-function mutants (Black and Boothroyd, 1998; Khan *et al.*, 2005). Additionally, most of the genome sequence of *T. gondii* has been revealed (ToxoDB, http://www.toxodb.org, (Kissinger *et al.*, 2003). Several genes of *T. gondii* are representive of related genes found in other related Apicomplexans. Thus this parasite is widely used as an experimental model (Roos *et al.*, 1994; Black and Boothroyd, 2000; Kim and Weiss, 2004).

1.1.6 Genetic manipulation of *T. gondii*

1.1.6.1 Transient and stable transformation

The development of molecular transformation of *T. gondii* has made genetic manipulation a highly employed tool to study gene functions and molecular events of the parasites. Electroporation, introducing exogenous DNA or RNA into cells by the pulse of

current, is the most efficient technique to introduce nucleic acids into *T. gondii*. In transient transformation, several reporter genes such as β-galactosidase (LacZ) and chloramphenicol acetyltransferase (CAT) have been successfully employed. Their expressions are efficient when driven by *T. gondii* promoters such as those of the major surface antigen (Lekutis *et al.*, 2000), the rhoptry protein (Soldati *et al.*, 1995) and the β-tubulin (TUB1) (Roos *et al.*, 1994; Soldati *et al.*, 1995)

Stable transformation, resulting in the generation of parasite lines that permanently express transgenes by directed (homologous) or random integration (non-homologues recombinants) into the parasite genome, has widely been used (Donald and Roos, 1994). Moreover, Black and Boothroyd (1998) developed an episomal vector, which is able to replicate autonomously in *T. gondii* without integrating into the genome. They found that, when they randomly used DNA fragments isolated from the *Tox*oplasma genomic DNA to construct into transforming plasmids, some of these sequences permit the episomal maintenance of the plasmid in the parasites. These episomal vectors avoid the possibility of introducing mutations into the genome by non-homologous recombination. Episomal vectors also offer the advantage of allowing easy analysis of the activity attributed to the transformed DNA. This is accomplished by isolating the episome to re-transform the parental strain or by selecting against the episome using a negative selectable marker. The development of this molecule tool may help to understand the events critical to *T. gondii* such as invasion, intracellular replication, and differentiation (Black and Boothroyd, 1998).

1.1.6.2 Selectable markers for stable transformation

Selectable markers are required for gene transformation that cannot be selected directly. Two non-essential enzymes involved in the nucleotide pathways of *T. gondii*, uracil phosphoribosyl transferase (Al-Anouti *et al.*, 2003) and hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT), as well as other enzymes like dihydrofolate reductase-thymidylate synthase (DHFR-TS) and chloramphenicol acetyltransferase (CAT) are utilized efficiently as the selectable markers for *T. gondii* transformation (Donald and Roos, 1994).

HXGPRT is important for the purine salvage pathway of *T. gondii*. It catalyzes the conversion from hypoxanthine, xanthine, and guanine into inosine monophosphate (IMP), xanthosine monophosphate (XMP) and guanine monophosphate (GMP) respectively. XMP can be further utilized for the synthesis of GMP, and can also be generated from IMP by IMP dehydrogenase in the absence of xanthine or HXGPRT (Fig. 1.2). HXGPRT can also catalyze the conversion from 6-thioxanthine (6-TX) to 6-thioxanthosine-5'-phosphate, which is toxic to the parasites. However, 6-TX cannot be recognized by mammalian host cells that lack the XPRT activity. Thus HXGPRT can be utilized as a negative selectable marker using 6-TX as the selection (Chaudhary *et al.*, 2004; Donald *et al.*, 1996). The generation of *HXGPRT* knockout *T. gondii* strains (RHΔ *HXGPRT*, PLKΔ*HXGPRT*) makes it possible to use HXGPRT as a positive selectable marker for molecular transformation. The positive selection requires the existence of

mycophenic acid (MPA) and xanthine. MPA inhibits the activity of IMP dehydrogenase and thus prevents the formation of XMP and subsequent GMP, which is essential for *T. gondii* survival using this pathway. Parasites with HXGPRT expression can utilize HXGPRT to generate XMP from xanthine and maintain their lives, while those without HXGPRT expression would be killed (Donald *et al.*, 1996). The *HXGPRT* knockout strains can be used in transformation with plasmids that express HXGPRT and the medium supplemented with MPA and xanthine is effective to select transformed parasites from those without.

Figure 1.2

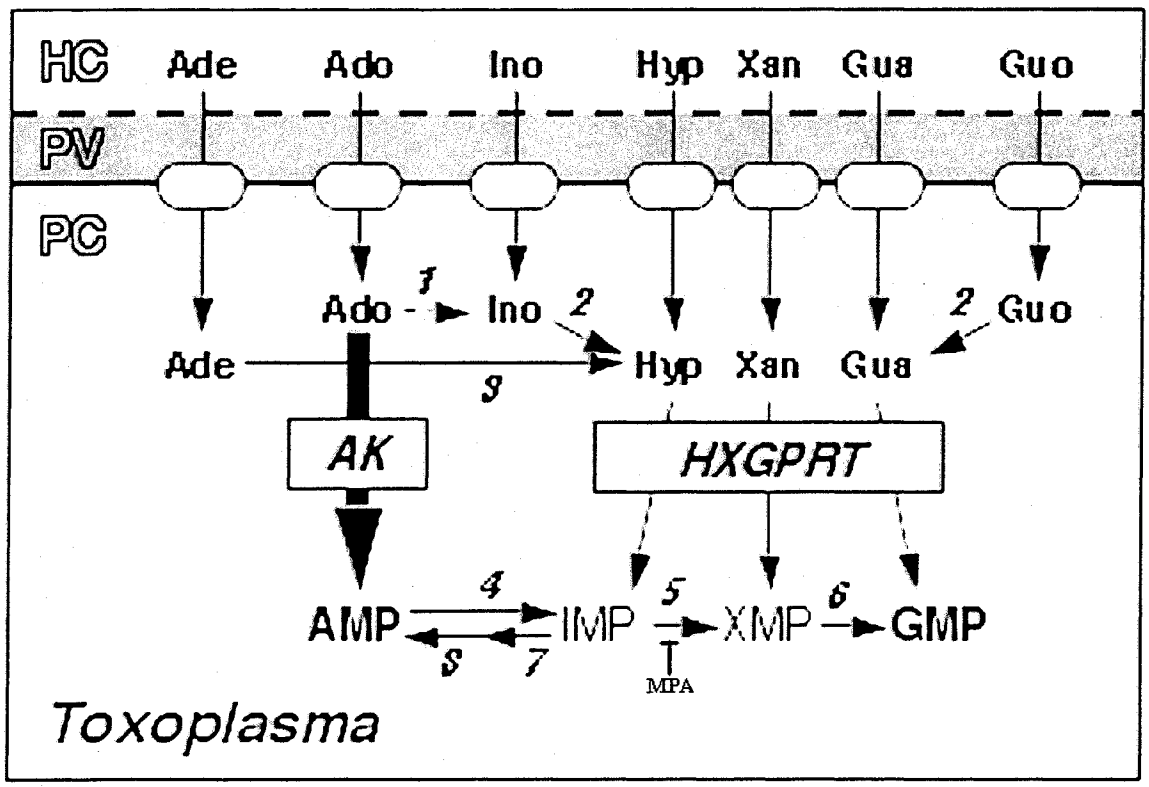
Purine Salvage Pathway of *Toxoplasma gondii*

Legend

Schematic representation of purine salvage pathway of *T. gondii*. Enzymes are indicated by *arrows* labeled AK (adenosine kinase), HXGPRT, or with *numbers* in the *Toxoplasma* panel (unless otherwise indicated): 1, adenosine deaminase; 2, purine nucleoside phosphorylase; 3, adenine deaminase; 4, AMP deaminase; 5, IMP dehydrogenase (inhibition target of MPA); 6, GMP synthetase; 7, adenylosuccinate synthetase; 8, adenylosuccinate lyase. *HC*, host cell cytoplasm; *PV*, parasitophorous vacuole; *PC*, protist cytoplasm (Chaudhary *et al.*, 2004).

Figure 1.2

Purine Salvage Pathway of *Toxoplasma gondii*



1.1.6.3 RNA tools in *T.gondii*

RNA tools, including ribozyme and antisese RNA, have been used for the down-regulation of gene expression in many organisms including *T. gondii* (Al-Anouti and Ananvoranich, 2002; Sheng *et al.*, 2004; Nakaar *et al.*, 2000; Nakaar *et al.*, 1999). They are efficient for the study on essential genes functions, because these RNA tools have the advantage that they do not disrupt gene expression at the DNA level as the DNA transformation (Lamond and Sproat, 1993). Moreover, these RNA tools can be used in suppressing gene expression and in phenotypic studies.

Antisense RNA functions by base-pairing with target mRNA to interrupt the downstream process (i.e. translation) or by destroying the target mRNA by RNase H (Lamond and Sproat, 1993; Hostomsky *et al.*, 1994). It was employed to modify the expression of triphosphoate hydrolase (NTPase) as well as HXGPRT in *T. gondii* (Nakaar *et al.*, 1999). Ribozyme, first discovered by Cech and Altman in the 1980s, are RNA molecules that can catalyze RNA cleavage in a site-specific manner (Tanner, 1999). In *T. gondii*, engineered delta ribozymes were successfully utilized to reduce the expression of UPRT and HXGPRT (Sheng *et al.*, 2004).

RNA interference (RNAi), an endogenous machinery of gene regulation, is functional in many eukaryotic organisms (Hannon, 2002; Fire *et al.*, 1998). In *T. gondii*, when dsRNA homologous to *UPRT* is introduced into the parasites, the expression level of *UPRT* is lowered (Al-Anouti *et al.*, 2003). The efficacies in gene silencing of dsRNA

and delta ribozyme are similar and are much higher than that of antisense RNA (Al-Anouti and Ananvoranich, 2002).

1.2 Enolase

Enolase (2 phospho-D-glycerate hydrolase, EC 4.2.1.11) is a glycolytic metal-activated enzyme. It belongs to the enolase superfamily comprising among other carboxyphosphonoenolpyruvate synthase. The enzyme catalyzes the Mg^{2+}-dependent removal of one water molecule from 2-phosphoglycerate (2PGA) to yield phosphoenolpyruvate (PEP), the penultimate step in the conversion of glucose to pyruvate. This reaction step is the only dehydration reaction in the glycolysis pathway. Moreover, enolase also catalyzes the reverse reaction during the process of gluconeogenesis. This glycolytic enzyme usually exists as a dimer of 45–48 kDa subunits, in which one magnesium ion is required for each subunit (Lebioda and Stec, 1991). Moreover, this enzyme is found to be highly conserved among different species from archaebacteria to mammals.

1.2.1 Enolase isoforms in *T. gondii*

In *T. gondii,* two isoforms of enolases are known as ENO1 and ENO2. Genes encoding these two enolase isoforms, showing 65.8% in open reading frame (ORF) nucleotide sequence identity, are localized on Chromosome VIII of *T. gondii*, residing closely to each other. ENO1 and ENO2 exhibit 73.8% amino acid similarity. ENO1 is

expressed only in bradyzoites and ENO2 is specifically expressed in tachyzoites. Compared to enolases from other organisms, the *T. gondii* enolase isoforms carry a pentapeptide EWGY(W)S(C) (103-107 aa) insertion and a dipeptide E(D)K (263 and 264 aa) and are thus considered highly related to those of plants (Dzierszinski *et al.*, 1999). During differentiation between tachyzoites and bradyzoites, enolase is important for glycolysis and anaerobic respiration. It has thus been hypothesized that ENO1 and ENO2 might be a promising target for developing drugs for chronic toxoplasmosis.

Comparing characterizations of these two enolase isoforms using recombinant proteins, it was found that ENO1 and ENO2 exhibit similar kinetic parameters: Michaelis constant (Kirkman *et al.*, 2001) of 76.8μM vs. 77.7μM. However, ENO2 had threefold higher specific activity (V_{max} = 89.2 mmol x min^{-1} x mg^{-1}) than ENO1 ($V_{max.}$ = 34.1 mmol x min^{-1} x mg^{-1}). Both isoforms have a similar optimum pH at pH 7.2. But ENO1 is slightly more stable than ENO2. The denaturation temperature of ENO1 was estimated at 64.0 °C, while that of ENO2 at 57.0 °C (Dzierszinski *et al.*, 2001). The different expression patterns as well as different enzymatic characterizations of ENO1 and ENO2 suggest that these two isoforms might be related to the different metabolism rates of tachyzoites and bradyzoites. ENO2, which is more active and less stable, is expressed only in the virulent tachyzoite stage, supposedly important for rapid development. ENO1, less active but more stable than ENO2, is more suitable for the encysted dormant bradyzoite with less energetic requirements. ENO1 and ENO2 are distinct in their antigenic properties. The polyclonal antibodies raised against ENO1 do not cross-react

with ENO2, vice versa, despite the high degree of amino acid homology (Dzierszinski *et al.*, 2001). It hypothesized that the difference in the expression pattern of enolase isoforms, as well as other stage-specific metabolism enzymes such as LDH and GP6I, is necessary for environmental adaptation and metabolic requirements of the two different parasitic stages. However, little is known about how the expressions of these stage specific enzymes are regulated during the stage conversion.

1.2.2 Structural features of enolase

Similar to enolases in other organisms, the two enolase isoforms of *T. gondii* contain conserved amino acid residues important for their catalytic activity. As indicated in Figure 1.3, these amino acids are Glu174, Glu217 and Lys355, which are involved in the dehydratation step; Asp252, Glu303 and Asp330, whose carboxylate groups coordinate the conformational metal ion ligand (Mg^{2+}) required for substrate binding; Arg384 interacting with the phosphate group of 2-PGA; and His383 and Lys406 interacting with the carboxylic group of 2-PGA (Dzierszinski *et al.*, 2001; Lebioda *et al.*, 1989; Lebioda and Stec, 1991). The different characterization of ENO1 and ENO2 might be due to the difference in the residues surrounding or neighboring those residues directly involved in the substrate binding or catalysis.

The deletion of the two plant-like motifs in ENO1 decreases its activity (Dzierszinski *et al.*, 2001). These two plant-like peptide insertions are positioned in highly conserved regions. The dipeptide insertion localized on the amino acids 263 and

264 aa is in one of the connection loops intruding into the active site of ENO2 and ENO1. The pentapeptide insertion found at position 103-107 aa increases the length of a connecting loop in the C-terminal domain and makes it close to the active site of the enzyme. These structural features provide possible explanation to the significance of these two insertions in the activity of enzyme. These plant-like motifs are also found in enolase of *Plasmodium falciparum*, another member of Apicomplexa family (Read *et al.*, 1994). This feature may suggest that Apicomplexa and plants are derived from a common ancestor, or Apicomplexa acquired their enolase by an endosymbiotic gene transfer from the cyanobacterial ancestors of the green algal plastid in their cytosol.

Figure 1.3

Sequence Alignment of ENO1 and ENO2

Legend

The amino acid sequnce of *T. gondii* ENO1 and ENO2 is aligned using LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html). Two plant-like motifs (103-107 aa EWGY(W)S(C) , 263 and 264 aa E(D)K) are shown in green colour. And conserved amino acids (Glu174, Glu217, Asp252, Glu303, Asp330, Lys355, His383, Arg384 and Lys406) important for catalytic activity are shown in red colour

Figure 1.3 Sequence alignment of ENO1 and ENO2

```
                10        20        30        40        50        60
ENO1    MVVIKDIVAREILDSRGNPTIEVDVSTEGGVFRAAVPSGASTGIYEALELRDKDPKRYLG
        ::.:::::.::.:::::::::::. :.:: ::::::::::::::::::::::::  ...:
ENO2    MVAIKDITARQILDSRGNPTVEVDLLTDGGCFRAAVPSGASTGIYEALELRDKDQTKFMG
                10        20        30        40        50        60

                70        80        90       100       110       120
ENO1    KGVLNAVEIVRQEIKPALLGKDPCDQKGIDMLMVEQLDGTKNEWGYSKSKLGANAILGVS
        :::..::: ... :::::.:::::::::::: ::::.:::::::::. ::::::::::.::
ENO2    KGVMKAVENIHKIIKPALIGKDPCDQKGIDKLMVEELDGTKNEWGWCKSKLGANAILAVS
                70        80        90       100       110       120

               130       140       150       160       170       180
ENO1    IACCRAGAASKGLPLYKYIATLAGKTIDKMVMPVPFFNVINGGEHAGNGLALQEFLIAPV
        .:::::::::.::.::::::::::::. ::::::::::::::::: :::: .:.:::.::::
ENO2    MACCRAGAAAKGMPLYKYIATLAGNPTDKMVMPVPFFNVINGGSHAGNKVAMQEFMIAPV
               130       140       150       160       170       180

               190       200       210       220       230       240
ENO1    GAPNIREAIRYGSETYHHLKNVIKNKYGLDATNVGDEGGFAPNVATAEEALNLLVEAIKA
        :: .:.::::. :.:.:.::: :::.:::::::::::::::::::::: : :::.::.::::.
ENO2    GASTIQEAIQIGAEVYQHLKVVIKKKYGLDATNVGDEGGFAPNISGATEALDLLMEAIKV
               190       200       210       220       230       240

               250       260       270       280       290       300
ENO1    AGYEGKIKIAFDAAASEFYKQDEKKYDLDYKCKTKNASKHLTGEKLKEVYEGWLKKYPII
        .:.:::.::: :.::::::. ::.: :::::.:  ... :.. :::.:...:.   .::::..
ENO2    SGHEGKVKIAADVAASEFFLQDDKVYDLDFKTPNNDKSQRKTGEELRNLYKDLCQKYPFV
               250       260       270       280       290       300

               310       320       330       340       350       360
ENO1    SVEDPFDQDDFASFSAFTKDVGEKTQVIGDDILVTNILRIEKALKDKACNCLLLKVNQIG
        :.:::::::::: :.. .:.:::::::.:::::::: ::::::.:::: :::::::::
ENO2    SIEDPFDQDDFHSYAQLTNEVGEKVQIVGDDLLVTNPTRIEKAVQEKACNGLLLKVNQIG
               310       320       330       340       350       360

               370       380       390       400       410       420
```

```
ENO1   SVTEAIEACLLAQKSGWGVQVSHRSGETEDSFIADLVVGLRCGQIKSGSPCRSERLCKYN
       .:.:.:::: :::::. :::.:::::::::::::::::::::: :::::.:::::::::::::
ENO2   TVSESIEACQLAQKNKWGVMVSHRSGETEDSFIADLVVGLRTGQIKTGAPCRSERLCKYN
              370        380       390       400        410       420

              430       440
ENO1   QLMRIEESLGADCVYAGESFRHPK
       ::::::::::.:: ::: .:::::.
ENO2   QLMRIEESLGSDCQYAGAGFRHPN
              430       440
```

1.2.3 Multiple functions of enolase

As a glycolytic enzyme, enolases localize in the cytoplasm, where they can be free or easy to associate with the cytoskeleton and other glycolytic enzymes. In *T. gondii*, ENO1 and ENO2 are localized in the cytoplasm and nuclei, although there is no classic nuclear localization signal found in their amino acid sequences (Ferguson *et al.*, 2002). This discovery suggests that, *T. gondii* enolase might have some additional functions, such as nuclear activities (i.e. division) and regulation of gene expression.

In other organisms, enolase has also been reported to perform multiple functions. For example, α-enolase, one of the three isoforms of enolase found in animals, is identified as the eye lens crystallin in reptiles and birds (Piatigorsky and Wistow, 1989). In HeLa cells, α-enolase acts as a component of the centrosome, and one of its alternative transcript products known as *myc*-promoter binding protein 1 (Feo *et al.*, 2000) can bind to *c-myc* promoter and negatively regulate transcription of this protooncogene (Johnstone et al., 1992; Ghosh et al., 1999). In human peripheral blood cells, α-enolase functions as a plasminogen receptor (Redlitz *et al.*, 1995), and is one of the hypoxia-inducible proteins (Semenza *et al.*, 1996). In fusion yeast *Saccharomyces cerevisiae*, enolase is the heat shock protein HSP48 and involved in thermal tolerance and growth control in this organism. Thus yeast enolase is recruited as a cofactor of tRNA targeting toward mitochondria (Iida and Yahara, 1985).

Apart from their metabolitic functions, it is not yet known whether *T. gondii* enolase isoforms have additional functions. One of the focuses of this study is to reveal the physiological functions of ENO1 and ENO2 in the development of *T. gondii*.

1.3 Gene silencing in *T. gondii*

In order to study the roles of ENO2 in *T. gondii*, I chose to suppress or silence the expression of ENO2 in the parasites. Upon the silencing of ENO2, resultant loss-of-function phenotypes may provide useful information on the functions of this enzyme. In this section, I will review some techniques used in loss-of-functions phenotype analysis in *T. gondii*.

1.3.1 Gene targeting in *T. gondii*

Gene targeting, including gene knockout and knockin, has been widely used in many organisms such as yeast, *Arabidopsis, Drosophila* and mouse for analysis of gene functions. Gene targeting is based on homologous recombination, which involves the exchange of DNA between sequences of perfect or near perfect homology over several hundreds of base pairs. The process of homologous recombination plays essential roles in the mitotic and meiotic cell cycles of most eukaryotic organisms. However, in most of the eukaryotes, the opportunity of homologous recombination is extremely low when compared with non-homologous recombination, rendering the efficiency of gene targeting to be low as well (Muller, 1999).

Toxoplasma gondii, which has haploid genome during its asexual life cycle, is very amendable for gene silencing and replacement. In gene targeting, constructs carrying the desired mutated allele of the interested gene with selectable markers are used in the transformation of parasites, and the homologous DNA fragment targets would guide and direct the homologous recombination to occur at corresponding chromosome locus. Thus, researchers can disrupt or delete the target gene or part of it (so-called knockout, KO), or replace the original gene with either a mutated one or another gene (so-called knockin, KI) (Koller and Smithies, 1992; Hanin and Paszkowski, 2003; Rong, 2002). Up to now, several genes of *T. gondii*, including *HXGPRT*, *UPRT*, dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) and *BAG1* were reported to be knocked-out or replaced (Donald and Roos, 1998; Bohne *et al.*, 1998; Bohne *et al.*, 1994; Donald and Roos, 1994).

One of the often-utilized methods to increase the efficiency of gene targeting is increasing the length of homologous regions required for homologous recombination. At the locus of *T. gondii* DHFR-TS, when genomic fragments of 8 kb homology were used as a circular plasmid in the transformation, ~50% of the transformed parasites harbored transgenes integrated by homologous transformation. When a 16 kb genomic fragment was utilized, >80% homologous recombination was observed (Donald and Roos, 1994).

Furthermore, advancements of positive-negative selection system and efficient molecular screening methods have made gene targeting more effective in many organisms (Mansour *et al.*, 1988). Additionally, the development of conditional gene

targeting including tissue- or cell-type specific gene targeting and temporal-inducible gene targeting has rendered gene targeting a more flexible and promising tools for the study of various genes (Sauer, 1998; Utomo *et al.*, 1999).

It is possible to generate null mutants by gene knockout in *T. gondii,* which enables the study of gene function that can lead to the identification of potential drug targets. Although the frequency of homologous recombination in *T. gondii* is relatively high, it is not convenient to silence enolase isoforms by gene targeting becausetheir genomic loci are almost overlapped. To create a knockout or a knockin of either *ENO1* or *ENO2* would interfere with the other. Moreover, it is highly likely that the null mutant would be lethal. To circumvent these potential difficulties, RNA interference is used. The introduction of dsRNA would attenuate the expression of enolases and allow us to dissect their functions.

1.3.2 RNA interference

RNAi is one of the most remarkable biological discoveries in the last 20 years. It is an evolutionary conserved mechanism in which double-stranded RNA (dsRNA) or small interference RNA (siRNA) initiates the specific silencing of homologous genes. It is a mechanism that is widely found in eukaryotes, including protozoa, insects and mammals (Fire *et al.*, 1998). It may function as a defensive mechanism to block the expression of aberrant or harmful genes originating from viruses and transposons (Hannon, 2002). RNAi also plays a fundamental role in genome rearrangement, chromosome remodeling and stem cell maintenance (Sugiyama *et al.*, 2005; Hatfield *et al.*, 2005; Mochizuki and

Gorovsky, 2005). Most importantly, RNAi is one of the most powerful and convenient tools to knock down specific gene expression and has a potential for gene therapy (Novina and Sharp, 2004; Harper *et al.*, 2005).

1.3.2.1 The discovery of RNA interference

The first observed phenomenon of RNAi took place in 1990 in plant research when scientists introduced a transgene designed to overexpress the enzyme chalcone synthase in petunias aimed to deepen floral color. Unexpectedly these researchers found white patchy flowers instead of the expected increased flower pigmentation (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). They thought that it was the transgene that silenced the expression of the plant purple-flower genes. The phenomenon was named as co-suppression, because the exogenous transgene suppressed the expression of itself as well as its endogenous homolog. The phenomenon remained obscure until similar gene silencing effects (known as quelling) was observed in *Caenorhabditis elegans* upon injection of dsRNA (Fire *et al.*, 1998). It was found that it was the dsRNA that lead to the degradation of target mRNA, known as post-transcriptional gene silencing (PTGS). Later it was reported that RNAi was also functional in other organisms including fusion yeast *Schizosaccharomyces pombe*, *Drosophila melanogaste*, *Arabidopsis thaliana*, *Neurospora crassa* as well as mammals (Hannon, 2002). Furthermore, it was also reported that RNAi activity can persist through cell division rounds and growth, and that RNAi is inheritable and transmissible (Fire *et al.*, 1998).

1.3.2.2 The mechanism of RNA interference

It is suggested that the suppressive effects of RNAi can operate on several different levels. The co-suppression effects in plants is triggered by DNA methylation of the genes which are homologous to dsRNA at cytosine residues, and thus these modified genes are prevented from being transcribed (Wassenegger, 2005). However, the cleavage of mRNA homologous to dsRNA in post-transcriptional level is usually thought to be the most essential pathway for the silencing effects of dsRNA.

Genetic and biochemical studies have contributed tremendously to the understanding of the mechanism of RNAi. Using extracts from *Drosophila* embryos and tissue-cultured cells transfected with dsRNA, a nuclease capable of degrading exogenous homologous mRNA, was partially purified. This nuclease was co-purified with small RNAs of 21-25 nt long consisting of sense and antisense sequences derived from within the used regions (Tuschl *et al.*, 1999; Elbashir *et al.*, 2001; Yang *et al.*, 2000). Incubation of a cell-free system from *Drosophila* embryos with both the dsRNA and its homologous mRNA exhibited RNAi activity as found *in vivo*. And preincubation of the cell-free lysate with the dsRNA potentiated the degrading activity of the lysate on the target mRNA *in vitro* (Hammond *et al.*, 2000; Zamore *et al.*, 2000). These series of experiments led to a model of RNAi pathways consisting of initiation and effecter steps.

In the initiation step, an RNase III like enzyme known as Dicer recognizes and digests dsRNA into small interference RNA (siRNA) in an ATP-dependent manner. Dicer,

a highly conserved protein found in worms, flies, plants, fungi and mammals, basically consists of a dsRNA binding domain and one or two RNase III nuclease domains (Elbashir *et al.*, 2001). These 21-25 nt siRNA, RNA duplex carrying 5'-phosphate and 3'-hydroxyl termini with 2-nt overhanging 3′ end, can later incorporates into a ~360 kDa ribonucleoprotein nuclease-complex called the RNA induced silencing complex (RISC) (Hannon, 2002). The exact components of RISC are not clear now, with the exception of the Argonaute family protein that is supposed to be the essential element in the complex. The Argonaute proteins are divided into two sub-families, known as Argonaute1-like (after the *Arabidopsis* Argonaute1) and Piwi-like (after the *Drosophila* Piwi), depending on the existence of N-terminal PAZ domain in addition to the highly conserved C-terminal Piwi domain found in all Argonaute proteins (Liu *et al.*, 2004). Argonaute proteins were reported to be able to bind with Dicer, suggesting that their interaction is important for the loading of siRNA into RISC. In the effecter step, the siRNA is first unwound in an ATP-dependent process. The formed single-stranded small RNA guides the RISC to homologous mRNA and subsequently cleaves it at 10-11 nt away from the 3' terminus of the guide RNA. The 5′phosphate group on the siRNA is essential for assembly with the RISC and subsequent target cleavage (Elbashir *et al.*, 2001; Hannon, 2002). Moreover, heterochromatin formation is also one possible pathway to inhibit target gene expression (Wassenegger, 2005).

Furthermore, Dicer can digest hairpin RNA precursors into micro RNA (miRNA), 20-25 nt single-stranded RNA that is able to mediate translation repression. miRNA can

incorporate into a protein complex known as miRNA-containing ribonucloprotein (miRNP) complex, which also contains a Argonaute family protein. In some organisms such as human, miRNP can guide miRNA to bind with homologous mRNA and inhibit its translation without affecting mRNA levels (Nelson *et al.*, 2004).

Other components of RNAi machinery, including R2D2, a Dicer interacting protein found to be important to load siRNA into RISC, were also reported. Among them, RNA dependent RNA polymerase (RdRP), which was reported to be able to amplify RNAi signals, is suggested to be essential for RNAi in some organisms, such as *Neurospora crassa*, and *Schizosaccharomyces pombe* (Cogoni and Macino, 1999; Hall *et al.*, 2002). The siRNA can bind with the target transcript as primer, and RdRP can extend the primers and generate the complementary RNA strand, resulting in a dsRNA which may serve as a new substrate for Dicer (Martienssen, 2003). Moreover, in *S. pombe*, it was also shown that RdRP was essential for RNAi-mediated heterochromatin assembly (Sugiyama *et al.*, 2005; Hall *et al.*, 2002). However, RdRP may be not required for RNAi in some other organisms. In *Caenorhabditis elegans*, it was reported that the mutant for the gene *ego-1* encoding its RdRP homologue showed similar RNAi activity when compared to the wild type (Sijen *et al.*, 2001). *Drosophila* and mammals, containing no RdRP homologues in its genome, still exhibit gene-silencing efficiency at the post-transcriptional level (Cottrell and Doering, 2003). It appears that RdRP is not a universal component of the RNAi pathway and that its function can be organism specific.

However, it is also possible that these organisms have distinct or divergent RdRP expression.

Figure 1.4

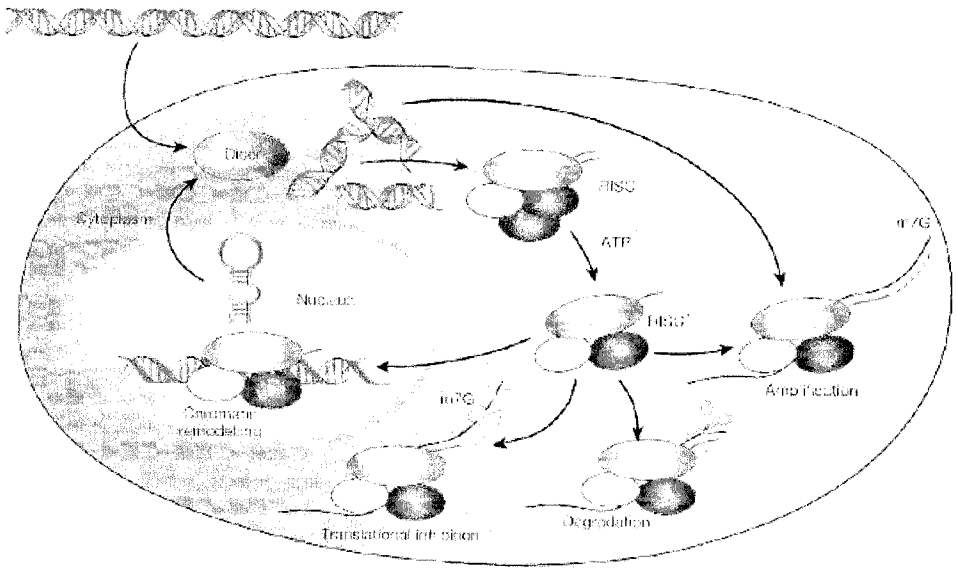
The mechanism of RNA interference

Legend

The first commintting step of the RNA interference pathway is catalyzed by Dicer which digests endogenous and exogenous dsRNA and generates siRNAs. The siRNA is loaded into RISC and directs the complex to silence gene by mRNA degradation, translational inhibition, or chromatin remodeling. In some organisms, RdRP-dependent synthesis of new dsRNA may be used to amplify RNAi signals. The figure is adopted from Hannon GJ, 2002

Figure 1.4

The mechanism of RNA interference



1.3.2.3 The application of RNA interference

RNAi is a powerful tool to suppress specific gene expression for reverse genetic studies and to elucidate the functions of genes in different organisms. The emergence of high throughput RNAi microarray chips has provided an important tool to analyze the functions of new genes coming from high throughput sequencing centers (Silva *et al.*, 2004). Moreover, RNAi technology is frequently employed in identify novel drug targets and is a potential therapy for some diseases. Many pharmaceutical companies and research centers have launched RNAi-therapy programs. Actually, any diseases could be potential targets for RNAi-therapy. For example, in mice, siRNA homologous tumor necrosis factor α (TNFα) was reported to be able to inhibit joint inflammation caused by collagen induced arthritis (CIA) (Schiffelers *et al.*, 2005). In addition, by using siRNA expression vector to inhibit the expression of HIV cellular receptor CD4 and coreceptors CXCR4 and CCR5, HIV infection on T cells can be significantly reduced (Anderson and Akkina, 2005). Specific siRNA is also reported to be able to inhibit growth of cancer cells (Takei *et al.*, 2004; Leng and Mixson, 2005).

For the application of RNAi in research, many parameters need to be considered including specificities and length of the dsRNA utilized, types of cells employed, as well as the method of dsRNA delivery. In tissue culture, methods such as electroporation, microinjection, and lipid mediated gene delivery are often used to deliver dsRNA or siRNA to cells (Parrish and Fire, 2001; Elbashir *et al.*, 2001). For *Caenorhabditis elegans,* feeding on dsRNA-expressing bacteria or even soaking in the solution of dsRNA can

successfully deliver dsRNA into cells (Timmons and Fire, 1998; Tabara *et al.*, 1998). Moreover, stable transformations, which can produce stable RNAi effects, with vectors expressing dsRNA and siRNA, have been put into practice. The target sequence can be inserted into a plasmid as inverted repeats so that the dsRNA is expressed as a hairpin dsRNA *in vivo* (Sui *et al.*, 2002; Paddison *et al.*, 2002). A target sequence can also be placed between two promoters arranged in a head-to-head fashion (Tschudi *et al.*, 2003; Al-Anouti *et al.*, 2004; LaCount *et al.*, 2000). In addition, inducible expression systems such as tetracycline-inducible vectors, and stage- and tissue-specific expression systems, were reported to be utilized in RNA silencing experiment and may have great prospect in the future study (Cottrell and Doering, 2003; Anderson and Akkina, 2005).

However, there is some concerns about using dsRNA in mammalian systems due to a protective antiviral response system which leads to a non-specific inhibition of host gene expression (Huppi *et al.*, 2005). But this difficulty can be avoided by using embryonic cells instead of non-embryonic cells, or by using siRNA instead of long dsRNA (Yang *et al.*, 2002; Elbashir *et al.*, 2001; Huppi *et al.*, 2005).

1.3.2.4 RNAi in protozoa parasites

When RNAi was first put into practice for the down-regulation of gene expression, it was used in the protozoa *Trypanosome brucei* (Ngo *et al.*, 1998). Genome searching has shown that *T. brucei* contains members of Argonaute family proteins but no Dicer homologues (Shi *et al.*, 2004; Ullu *et al.*, 2004). For other members of the

Trypanosomatidae family, *Trypanosoma congonlese* was found to have RNAi functions, while *T. cruzi* and *Leishmania major*, were revealed to be RNAi negative and their genome databases show no homologues of Dicer and Argonaute (Ullu *et al.*, 2004). In addition, RNAi was reported to be functional in *T. gondii*, as well as in *Plasmodium falciparum*, another member of Apicomplexa family (Malhotra *et al.*, 2002; McRobert and McConkey, 2002; Al-Anouti *et al.*, 2003). The presence of classical RNAi genes, including potential homologues of Argonaute, Dicer and RdRP, were reported from database mining of the *T. gondii* predicted coding regions. The presence of Argonaute family protein was reported in *T. gondii*, further supporting the functioning of RNAi in the parasites (Riyahi *et al.*,).

Objectives

Stage-specific expression and nuclear localization of enolase isoforms in *Toxoplasma gondii* suggest additional important functions of this glycolotic enzyme in the parasites and provide potential drug targets for toxoplasmosis treatment. But limited knowledge has been acquired about enolase up to now. The objective of this study was to investigate the possible role of ENO2 in the development of *T. gondii*, including their involvement in parasite growth and stage differentiation. We would also like to investigate the effectiveness and specificity of dsRNA induced gene silencing in *T. gondii*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Parasite Strains

Toxoplasma gondii strains RHΔHX and RH were obtained from the AIDS Research and Reference Reagent Program, NIH. RHΔHX strain is generated from RH strain and contains a deleted HXGPRT gene which allows for the slection of transfected parasites (Donald and Roos, 1998).

2.2 Materials and Chemicals

Chemicals and reagents that were used in this study are listed below.

Amersham-Pharmacia Biotech (Baie d'Urfe, Quebec).

Calf intestinal alkaline phosphatase (CIAP), T7 RNA polymerase, 2'-deoxyribonucleoside 5'-triphosphates (dNTPs), ribonucleoside triphosphates (rNTPs), nitrocellulose membranes, and ECL western blot detection reagent.

Baxter Diagnostics Corp. (Toronto, ON)

Nalgene ™ disposable 25mm syringe filters (0.2μm pore size) and glass Pasteur pipettes

Bio-Rad Laboratories (Mississauga, ON)

Bromophenol Blue, Coomassie Brilliant Blue R-250, Xylene Cyanol, and Bio-Rad protein assay dye reagents

Gelman Sciences (Ann Arbor, MI)

The Vacucap ^{TM} disposable bottle-top filter for sterilization of cell media

GibcoBRL (Burlington, ON)

Cell culture media Dulbecco's modified medium (DMEM), minimal essential medium (MEM), dialyzed fetal bovine serum (dFBS), Dulbecco's phosphate buffer supplemented with calcium (DPBS), Trypan Blue (4%) Trypsin-EDTA (0.25% Tripsin, 1mM EDTA) and penicillin-streptomycin (10,000 u/ml)

Hyclone (Logan, UT)

10% cosmic calf serum

Invitrogen Corporation (Burlington, ON)

Proteinase K, Trizol, and RNase Out ribonuclease inhibitor, TOP10 *E.coli* competent cells and BL21 (DE3) competent cells

Molecular Probes (Eugene, OR)

Goat anti-rabbit IgG conjugated to rhodamine

New England BioLabs Inc.

Restriction enzymes *Kpn*I, *Nsi*I, *Nde*I and *Xba*I

Omega Bio-tek (Doraville, GA)

EaZy Nucleic Acid Isolation Plasmid Midiprep Kit

Perkin Elmer (Norwalk, CT)

[^{3}H] uracil and [^{3}H] hypoxanthine monohydrogen chloride

Promega (Madison, WI)

Agarose, DNA polymerase (Klenow) fragments, Moloney murine leukemia virus (M-MLV) reverse transcriptase, RQ1 RNase-free DNase, Multicore buffer, Calf intestinal alkaline phosphatase (CIAP), T4 DNA ligase and restriction enzymes *Sac*I, *Hind*III, *Hinc*II, *Bam*HI, *EcoR*I, *Pvu*II and *Xho*I

Qiagen (Mississauga, ON)

The Qiaex ® II Gel Extraction Kit

Roche Diagnostics (Laval, Qubec)

Taq DNA polymerase

Sarstedt Incorporation (Newton, NC, USA)

Conical 15 ml and 50 ml graduated polypropylene centrifuge tubes, disposable pipette tips, 24 and 96 well plates, Petri dishes, cell scrapers, and T75 and T25 tissue culture flasks

Sigma-Aldrich (Oakville, ON)

Acetic acid, ampicillin, chloroform, DNA ladders, 4′,6 diamidino-2-phenylindole (DAPI), *Dolichos biflorus* conjugated to fluorescein isothiocyanate (Cormack *et al.*, 1996), dimethylsulfoxide (DMSO), dithiothreitol (DTT), diethylpolycarbonate (DEPC), ethanol, ethylenediaminetetra-acetic acid disodium salt (EDTA), ethidium bromide, formamide, formaldehyde, glycine, glycerol, hydrochloric acid, methanol, morpholinopropanesulfonic acid (MOPS), mycophenolic acid (MPA), pepsin, polyoxyethylene sorbitan monolaurate (Tween-20), 2-propanol, phenylmethyl sulfonyl fluoride (PMSF), pyruvate, scintillation fluid, sodium citrate, sodium chloride, sodium

acetate, N,N,N',N' tetramethyl ethylenediamine (TEMED), tris-hydroymethylaminomethane (Tris), Triton X-100, Trichloroacetic acid, xanthine

TaKaRa Bio Inc.

Mung Bean Nuclease, T4 DNA ligase, restriction enzymes *Sac*II, *Hinc*II, and *BamH*I

USB (Cleveland, OH)

Acrylamide, bis-acrylamide, ammonium persulfate (APS), equilibrated phenol, RNase A and sodium dodecyl sulphate (SDS)

Wisent (Montreal)

Dialyzed fetal bovine serum (FBS)

2.3 Apparatus and instrumentation

Agarose gel electrophoresis of DNA and RNA was carried out using the Miniature Horizontal Gel System MLB-06 from Tyler Research Instruments. AlphaImager™ 2200 Light Imaging System with AlphaEase software was used to view gels and take images. Sodium dodecyl sulfute Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the vertical gel electrophoresis system, including all the glasses, Teflon combs and spacers, from Bethesa Research Laboratories (BRL).

Centrifugations were carried out in J2-HS Centrifuge (Beckman), the desktop Eppendorf Model 5415C microcentrifuge from Desaga (Sarstedt Gruppe, Germany), or the Br4i centrifuge (Jouan, SA). DNA, RNA and protein concentrations were quantified

using Shimadzu UV-Visible Recording Spectrophotometer UV-160 (Agilent Technologies, ON). The quartz cuvettes used were from Sigma.

PCR reactions were performed using the 20-well Techgene Thermal Cycler (Techne, Cambridge, UK). Ligation reactions and reverse transcription (RT) reactions were incubated in the 48-well Perkin Elmer Cetus DNA thermal Cycler (Perkin Elmer, Norwalk, CT).

Parasite and cell culturing was performed under the Class II type A/B3 Biosafety cabinet (Jouan, SA). All cultures were maintained in Thermo Forma CO_2 incubator (Thermo Forma). Electroporations were conducted using the BTX model 600 Electro Cell Manipulator (Genetronics). Bacterial cultures were grown in the New Brunswick Scientific G-25R shaking incubator

2.4 Cell cultures

Cell and parasite culture was always conducted under sterile conditions in the Class II type A/B3 Biosafety cabinet (Jouan, SA), and all cultures were maintained in a CO_2 incubator (Thermo Forma).

2.4.1 Human foreskin fibroblasts (HFF) culture

Normal Human foreskin fibroblasts (HFF, obtained from Dr. D. Roos, University of Pennsylvania) were used as the host for *T. gondii*. HFF cell monolayers were cultured in D10 complete medium (composed of Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 10% cosmic calf serum, 5 μg/μl streptomycin, 5 units/ml penicillin) and grown in 5% CO_2 atmosphere at 37 °C.

Confluent HFF cell monolayers were subcultured using trypsin solution (0.25% trypsin, 0.03% EDTA) followed by 1 minute incubation at 37 °C. Un-adhered cells were resuspended in fresh D10 complete media and split into 4-5x area of the old culture.

HHF were stored using a solution containing 12.5% dimethyl sulfoxideand 10% dialyzed fetal bovine serum (FBS) in D10 medium. The cells were then aliqouted into cryogenic vials and stored at –80 °C and later in liquid nitrogen for future use.

2.4.2 *T. gondii* culture

Toxoplasma gondii was propagated in HFF grown in ED1 complete medium (Modified Eagle Medium (MEM) containing 1% dialyzed FBS, 5 μg/μl streptomycin and 5 units/ml penicillin) at 37 °C in 5% CO_2 atmosphere. When the parasites completely lysed the monolayers, the plate was scraped and newly released *T. gondii* was used to infect another confluent HFF monolayer.

To store the parasites for future use, the parasites were re-suspended in solution containing 12.5% DMSO and 10% dialyzed FBS in ED1 medium. The cells were then placed in freezing vials and stored at –80 °C and later in liquid nitrogen for future use.

2.5 Construction of dsRNA expression plasmid

The DNA fragments encoding 1-542 bp nucleotides starting from start codon of *ENO1* and *ENO2* open reading frames (ORFs) were amplified from pENO1 and pENO2 expression plasmids acquired form Dr. S. Tomovo (Université des Sciences et Technologies de Lille, France) (Dzierszinski *et al.*, 2001), and oligonucleotide primers called 5'-ENOx and 3'-ENOx. This set of primers match perfectly with the *ENO1* gene sequence. They also show high homology with the *ENO2* gene sequence and are found to be able to amplify 1-542 bp *ENO2* ORF DNA fragment by PCR.

The plasmid producing dsRNA was designed to put the target sequence between two promoters arranged as an inverted repeat that allowed transcription of both strands of the DNA sequence located between them. Such construction was reported to be functional in many organisms such as *Trypanosoma congolense*, *Trypanosoma brucei* as well as *T. gondii* (Bannai H *et al.*, 2003, LaCount *et al.*, 2000, Al-Anouti *et al.*, 2003). 542 bp DNA fragments of *ENO1* and *ENO2* were placed into the plasmid vector $p(TUB8)_2$, between two TUB8 promoters respectively. And to allow selection for the positively transformed parasites with medium containing mycophenolic acid (MPA) and xanthine, an expression cassette of hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) was also cloned into the constructed plasmids, producing plasmids named as $p(TUB8)_2$*ENO1-HX*, and $p(TUB8)_2$*ENO2-HX* respectively. An ampicillin resistant gene also existed in the plasmids for bacterial selection under antibiotic pressure. To serve as a negative control, a 533 bp DNA fragments of green

fluorescence protein (Cormack *et al.*, 1996) gene amplified from plasmid pTub8mycHis*GFP-HX* by PCR using T7-5*GFP*mut2 and T7-3*GFP*mut2 primers was also cloned into the vector to generate the plasmid p(TUB8)$_2$*GFP-HX*. The Scheme of the plasmids constructed is shown in Fig. 3.2. Appendix III.

The coding sequences of *ENO1* and *ENO2* and rhoptry protein 1 (Soldati *et al.*, 1995) as well as Argonaut (*AGO*) were obtained from the *T. gondii* sequence database (http://www.toxodb.org) (Kissinger *et al.*, 2003) and were used to design oligonucleotide primers (T7on5'*ROP*, T7on3'*ROP*, 5'*ENOx*, 3'*ENOx*, T7on5'*ENOx* and T7on3'*ENOx*, all sequences are listed in Appendix II) for PCR reaction.

2.5.1 RNA extraction from *T. gondii*

Freshly released *T. gondii* were passed through a 27G1/2 syringe needle, filtered through a 3 μm polycarbonate filter to remove host cell debris and harvested by centrifugation at 3,000x g for 10 minutes. The parasites were lysed using TRIZOL® Reagent and RNA was extracted as per the manufacturer's instruction. The obtained RNA samples were dissolved in 20 μl of DEPC H_2O.

To remove genomic DNA contamination, the RNA samples were treated with 4 units RQ1 RNase-free DNase (Promega) for 30 minutes at 37 °C. The DNase was inactivated by heating at 75 °C for 5 minutes, followed by phenol/chloroform treatment. The RNA samples were recovered from the aqueous phase by precipitating using 2.2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and were

centrifuged at 12000x g for 15 minutes. After washing with 70% ethanol, the RNA pellet was dissolved in 20 µl of DEPC H_2O.

2.5.1.1 Agarose gel electrophoresis

The total RNAs extracted from *T. gondii* were visualized on 1% agarose gel, and the presence of 18s and 28s ribosomal RNA was used as the internal control. To visualize the DNA or RNA in the gel, ethidium bromide was added to the agarose solution to a final concentration of 10 ng/ml. The gel was allowed to solidify in the electrophoresis tray and was placed in the electrophoresis tank filled with 1x TAE buffer. DNA/RNA samples were mixed with gel loading buffer (6x: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in water) and were loaded into the well along. The gel was run in 1x TAE buffer for 0.5 to 1 hour at ~100 v. The gel was then visualized and photographed in Alphaimager 2200 Light Imaging System with AlphaEase software.

2.5.2 Reverse transcription and polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was generated using a reverse transcription reaction and oligodT-primer (Appendix II). The cDNA products were used as templates for PCR.

The concentration of RNA was determined by measuring the absorbance at 260 nm ([RNA] = A_{260nm} x dilution factor x (40 µg/ml)). In order to determine the purity of DNA from proteins, the absorbance was also measured at 280 nm and compared to that at 260

nm.

2.5.2.1 Reverse transcription (RT)

The reverse transcription reactions were carried out using 2 μg of total RNA after DNase treatment as initial templates. The RNA samples were subjected to PCR to confirm no DNA contamination. 5 pmoles of oligodT primer was add to the RNA samples, followed by incubation at 65 °C for 5 minutes to remove secondary structures of RNA, and cooling on ice for 1 minute. After centrifuging briefly, the mixture was finally completed in a total volume of 10 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 1 mM each dNTP, 20 u (0.5 μL) RNase-out RNase inhibitor and 100 u (0.5 μl) Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega). The reactions were incubated at 42 °C for 90 minutes, followed by heating at 70 °C for 15 minutes to inactivate the reverse transcriptase.

2.5.2.2 Polymerase chain reaction (PCR)

Polymerase chain reactions were performed using the 20-well Techgene thermal cycler (Techne, Cambridge, UK). All the oligonucleotide primers used in the study were synthesized by Integrated DNA Technologies (Coralville, IA). The sequences of all the oligonucleotide primers used in the study are listed in Appendix II. 2 μl of RT reaction mixture was used for the subsequent PCR amplification. The PCR was performed in a total volume of 25 μL containing 75 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$,

100 μM dNTPs, 50 pmoles of each oligonucleotide primer (5'ENOx and 3'ENOx), and 0.5 μL of *Taq* DNA polymerase. The reaction condition for *ENO1*and *ENO2* gene fragments amplification was 94 °C 5 min + 35 x (94 °C 30s, 42 °C 30s, 37 °C 75s) + 72 °C 5 min. The PCR products were visualized on 1% agarose gel.

2.5.3 Preparation of vector

The DNA plasmid p(TUB8)$_2$-CAT was digested with *Nsi*I and treated by Mung Bean Nuclease to produce blunt ends for the cloning of the DNA fragments encoding *ENO1* or *ENO2*. The sequences and digestion analyses of these constructs are shown in Appendix III.

2.5.3.1 Plasmid miniprep

An isolated bacterial colony was used to inoculate 2 ml of LB broth supplemented with ampicillin (100 μg/ml). The culture was incubated at 37 °C overnight at 250 rpm. Following incubation, 1.5 ml of the culture was transferred to a 1.5-ml microtube and was centrifuged at 12,000x g for 1 minute to collect the cells. After removing the medium and drying briefly, the pelleted cells were resuspended in 100 μl of a ice-cold solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 10mM EDTA. Then the suspended cells were lysed by the addition of 200 μl of solution containing 0.2 N NaOH, 1% SDS prior to incubation on ice. Following a 2 to 3 minutes incubation, 150 μl of ice-cold 3 M NaOAc, pH 5.2 was added to precipitate chromosomal DNA and

SDS-protein complex. After incubation on ice for 5 minutes, the mixture was centrifuged at 12,000x g for 5 minutes. The clear supernatant (~450 µl) was then transferred to another 1.5-ml tube and 450 µl of TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added. This mixture was vortexed for 30 seconds and then centrifuged at 12,000x g for 1 minute at room temperature. The top aqueous phase was then transferred to a fresh tube and 900 µl of 95% ethanol was added to precipitate the plasmid. This mixture was then vortexed for approximately 30 seconds and left standing at room temperature for 2 minutes, followed by centrifugation at 12,00x g for 10 minutes at 4°C. The resulting DNA pellet was washed with 450 µl of 70% ethanol and the mixture was centrifuged at 7,500x g for approximately 3 minutes. The final pellet was resuspended in 30µl of 1x TE buffer containing 20 µg/ml RNase, and incubated at 37 °C for 15 to 20 minutes to remove RNA.

2.5.3.2 Restriction enzyme digestion

Restriction enzyme digestion was used in the analysis and preparation of plasmids as well as DNA fragments for cloning. The reaction mixture was made by adding 1 µl 10x enzyme reaction buffer provided with the enzyme and 0.5 µl of desired enzyme to an adequate amount of DNA solution (<1 µg), followed by adding of ddH_2O to make the reaction10 µl. The mixture was incubated at 37 °C for 4 to 16 hours, followed by visualization using agarose gel. The treated DNA can be purified by phenol: chloroform treatment and precipitated with ethanol.

2.5.3.3 Mung Bean Nuclease treatment

Mung Bean Nuclease reactions were carried out to create blunt ends for the ligation of incompatible sticky ends. The general reaction involved the use of 25μl of DNA (<1 μg), 3 μl of 10x buffer (0.03 M sodium acetate (pH 5.0), 0.05 M NaCl, 1 mM $ZnCl_2$), 0.5 μl (45 u/μl) of Mung Bean Nuclease (TaKaRa), and 1.5 μl of H_2O. After the addition of Mung Bean Nuclease, the samples were incubated at 37 °C for 15 minutes to let the enzyme work. The treated DNA sample was either purified by phenol: chloroform treatment followed by ethanol precipitation, or subjected to gel purification directly.

2.5.3.4 Calf Intestinal Alkaline phosphatase (CIAP) treatment

In the process of inserting *HXGPRT* cassette into constructed plasmids, CIAP was used to remove the phosphate group of the vectors to prevent the self-ligation in the ligation reaction. Briefly, the reaction was made of 20 μl of DNA (<1 μg), 3 μl of 10x CIAP buffer, 0.5 μl (1 u/μl) of CIAP (Promega), and 6.5 μl of H_2O. The reaction mixture was incubated at 37 °C for 30 minutes, followed by adding another 0.5 μl CIAP and another 30 minutes 37 °C incubation. The treated DNA sample was either purified by phenol: chloroform treatment followed by ethanol precipitation, or subjected to gel purification directly.

2.5.4 Gel purification of DNA fragments

To obtain purified DNA fragments for ligation, the DNA samples were resolved on 1% agarose gel by electrophoresis, the *ENO1* and *ENO2* DNA fragments as well as plasmid vector were excised and purified using QIAEX II Gel Extraction Kit as described by the manufacturer (Qiagen catalog No. 20021). Finally, extracted DNA sample was dissolved in 30 µl of ddH_2O, visualized in 1% agarose gel and frozen at –20 °C for future use.

2.5.5 Ligation

After purification of the DNA fragments of *ENO1* and *ENO2*, they were ligated with gel-purified $p(TUB8)_2$ plasmid vector. 4 µl of insert was mixed with 1 µl of vector, 1 µl 10 x Ligase Buffer (300 mM Tris-HCl (pH 7.8) 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP), 0.5 µl of T4 DNA Ligase (3 u/µl) (Promega), and 3.5 µl of ddH_2O. The reaction was incubated overnight at 16 °C. Half of this reaction mixture was then used for transformation into subcloning-grade competent cells.

2.5.6 Transformation of *E. coli* with plasmids

To maintain sterile conditions throughout bacterial culturing procedures, all broth solutions and glassware were autoclaved prior to use.

2.5.6.1 Preparation of competent bacteria

XL1-Blue MRF' *Escherichia coli* (Stratagene) was grown on an LB agar plate containing tetracycline (100 μg/ml). A single colony was inoculated from the plate into 3 ml LB broth with tetracycline and grown overnight in the shaker incubator at 37 °C at 250 rpm. The second day, 1 ml of the culture was used to inoculate a 100 ml LB broth without antibiotics. The bacteria were shaken at 37 °C for 3 hours to let OD_{595nm} reaches 04-0.6. Then, the bacteria were chilled on ice for 30 minutes and then pelleted at 4000x g for 15 minutes at 4 °C in 50 ml Falcon tubes. 45 ml of the supernatant was discarded and the remaining 10 ml was used to resuspend the bacteria pellet. 30 ml cold sterile 100 mM $CaCl_2$ solution was then added to wash the cell pellet. The suspended pellet was centrifuged again at 4000x g for 15 minutes. The supernatant was removed and the pellet was further washed with 40 ml 100 mM $CaCl_2$ solution for two times followed by centrifugation. The pelleted bacteria were finally resuspended in a 2ml solution containing 100 mM $CaCl_2$ and 25% glycerol, aliquoted into 600 μl microfuge tubes and finally stored at -80°C for at least overnight before use (Sambrook *et al*, 1989).

2.5.6.2 Transformation of competent bacteria with plasmids

Half of the ligation reaction product was added to 25 μl of freshly thawed competent *E. coli*. After 20 minutes of incubation on ice, the cell suspension was heat shocked at 42 °C for 45 seconds and further incubated on ice for another 2 minutes. After the addition of 400 μl LB broth, the bacteria were incubated at 37 °C for 30 to 45 minutes

with shaking at 250 rpm. Approximately 100µl of this culture was spread on LB agar plate containing 100 µg/ml ampicillin. Plates were then incubated overnight at 37 °C (Sambrook *et al*, 1989).

2.5.7 Screening for the correctly constructed plasmids

Isolated colonies of ampicillin resistant bacteria were screened for positive cloning by PCR. Literally, single colonies were picked and grown in 100 µl LB broth supplemented with 100 µg/ml ampicillin at 37 °C overnight. 50 µl of the culture was centrifuged at 12,000x g for 1 minute. The supernatant was removed and the pelleted cells were resuspended in 20 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). The suspension was boiled for 10 minutes and spun down at 12,000x g for 1 minute. Up to 2.5 µl of the supernatant was used as the template for 25 µl PCR using primers flanking the insert DNA fragments. PCR products were then visualized in 1% agarose gel.

The potentially positive clones were further isolated by miniprep and subjected to restriction enzyme digestion analysis to search for the correctly constructed plasmids.

2.5.8 Insertion of *HXGPRT* cassette into plasmid

The *HXGPRT* cassette was also cloned into the plasmids to serve as a positive selectable marker used to select positively transformed parasites from the untransformed ones. Briefly, the *HXGPRT* cassette DNA fragment was digested from the plasmid

pTub8mycHis*GFP-HX* by restriction enzyme *Sac*II. The target plasmid was also subjected to *Sac*II digestion followed by CIAP treatment. The digested *HXGPRT* cassette insert and vector were purified by QIAEX II Gel Extraction Kit and used in ligation reaction as described above. The ligation products were also used to transform XL1-Blue MRF' *E. coli* competent cells, and were subjected to screening to get the expected constructs. The sequences and digestion analyses of these constructs are shown in Appendix III.

2.6 Transient dsRNA electroporation

To study whether dsRNA induced RNAi was available to knockdown the expression of enolase in *T. gondii*, synthesized dsRNAs homologous to two isoforms of enolase were transformed into the parasites by electroporation. And the consequences of the dsRNA electroporation were studied by RT-PCR and western blot.

2.6.1 dsRNA synthesis by *in vitro* transcription

DNA templates of the transcription were synthesized by PCR using ENOx primers set which had 17 nucleotides T7 promoters at 5'-end of both oligonucleotides (T7on5'ENOx and T7on3'ENOx, sequences are listed in Appendix II). And ~550 bp *ENO1* and *ENO2* DNA fragments were used as template for the PCR reaction. The PCR product was purified using phenol/chloroform purification followed by ethanol precipitation, and dissolved in DEPC treated water. 0.2 μg of the PCR product was used

in the *in vitro* transcription reaction as the template. The transcription reaction was carried out in a total volume of 100 µl containing 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 10 mM NaCl, 10 mM DTT, 1/100 dilution pyrophosphate, 50 u RNaseOUT RNAase inhibitor, 2.5 mM rNTP and 200 u T7 RNA polymerase. Transcription was allowed to proceed at 37°C for 2.5 hours. At the end of the incubation period, 2 units of RNase free RQ1 DNase were add to the reaction, following by further incubated for 30 minutes to degrade DNA template. The product was finally purified by phenol: chloroform extraction and the integrity of the RNA sample was be confirmed by resolving the sample on 1% agarose gel stained with ethidium bromide (Sambrook, 1989). The concentration of synthesized RNA was determined by spectrometry as described above. Additionally, dsRNA homologous to *GFP* DNA was also produced with the same approach.

2.6.2 Electroporation of dsRNA into *T. gondii*

After *T. gondii* tachyzoites of strain RH had completely lysed a confluent HFF monolayers plate, the parsites were scraped from culture plate, syringed through a 27G1/2 needle twice, filtered through a 3 µm membrane to remove host cellular debris. The parasites were then harvested by centrifugation at 2000x g for 10 minutes at 4 °C followed by washing twice with Dulbecco's phosphate-buffered saline buffer supplemented with 100 µg/ml Ca^{2+} (DPBS). The parasites were finally centrifuged at 2000x g for 10 minutes at 4 °C to collect the pellets, then resuspended in 800 µl

electroporation buffer which contained 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.6), 25 mM HEPES (pH 7.6), 2 mM EDTA and 5 mM $MgCl_2$, and was freshly supplemented with 2 mM ATP and 5 mM glutathione (GST). 10 μg dsRNA was added to the resuspended *T. gondii*, and the mixture was transferred to the 4 mm electroporation cuvettes (Ultident, St. Laurent, Quebec) and subjected to electroporation using BTX model 600 Electro Cell Manipulator (Genetronics) with current of 25 Ω, 25 μF and 1.8 kEV. The electroporated parasites were then left standing in the electroporation vial at room temperature for 15-30 minutes to restore their viabilities and were then used to infect a new confluent HFF monolayers in ED1 medium in 60mm culture plate (Donald and Roos, 1994).

2.6.3 RT-PCR to study mRNA expression level

The total RNA of *T. gondii* after dsRNA electroporation was extracted as described above. The RNA samples were subjected to DNase treatment and subsequent PCR to confirm no DNA contamination. 2 μg of the total RNA was used in the RT reaction using oligodT, which is complementary to the polyA tails of mRNA, as the primer. 2 μl of the RT product is used in the PCR as the templates. The reaction condition for *ROP1* amplification was 94 °C 5 min + 35 x (94 °C 30s, 55 °C 30s, 37 °C 45s) + 72°C 5 min. The reaction condition for *ENO2* amplification was 94 °C 5 min + 35 x (94 °C 30s, 42 °C 30s, 37 °C 75s) + 72 °C 5 min. The PCR products were subjected to agarose gel electrophoresis to visualize the results. To make the amount of *ROP1* products to be equal,

5.0μL of ds*ENO1* RNA RT-PCR product, 3.5μL of ds*ENO2* RNA RT-PCR product and 6.0μL of ds*GFP* RNA RT product from both *ENO2* and *ROP1* were loaded on the gel.

2.6.4 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

2.6.4.1 Protein quantification by Bradford assay

Freshly released *T. gondii* were passed through a 27G1/2 syringe needle, filtered through a 3 μm polycarbonate filter to remove host cell debris and harvested by centrifugation at 3,000x g for 10 minutes. The pelleted parasites were lysed with the protein lysis buffer containing 50 mM HEPES (pH 7.4), 0.025% Triton X-100, 20% glycerol, 10 mM PMSF, and centrifuged at 12,000x g for 10 minutes at 4 °C. The concentrations of cell lysates were determined by Bradford assay using the Agilent UV-visible spectrophotometer as described on the manual. 10 μl of the protein sample was diluted with 1.59ml of ddH_2O and mixed with 0.4 ml of Bradford reagent (Bio-Rad). After incubation at room temperature for 10 minutes, spectrophotometeric measurements were carried out at 595 nm using the diluted Bradford Reagent as a blank. A standard curve made with standard protein solutions of BSA was used to determine the unknown concentration of protein in the sample (Sambrook *et al*, 1989).

2.6.4.2 SDS-PAGE

Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) was

conducted with a discontinuous buffer system (Laemmli, 1970). The stacking and resolving gels were cast using the vertical gel electrophoresis system from BRL (Bethesda Research Laboratories). The resolving gel solution (2.58 ml ddH_2O, 1.8 ml 30% Acrylamide solution, 1.5 ml 1.0 M Tris-HCl pH 8.8, 60 µl 10%SDS, 60 µl 10% ammonium persulfate, 5 µl TEMED to make total volume to be ~6ml) was poured first and left to polymerize before being overlaid by the stacking gel solution (1.46 ml ddH_2O, 0.25 ml 30% Acrylamide solution, 0.25 ml 1.0M Tris-HCl pH 6.8, 20 µl 10% SDS, 20 µl 10% ammonium persulfate, 3 µl TEMED to make total volume to be ~2 ml).

The protein samples were mixed with gel loading buffer containing 6.25 mM Tris-HCl (pH 6.8), 2% SDS, 10% glucose, 0.05% Bromophenol blue and 720 mM 2-mercaptoethanol, boiled for 5 minutes and loaded onto SDS-PAGE gel. The gel was run in the Tris-glycine running buffer containing 196 mM glycine, 25 mM Tris-HCl (pH 8.4), 0.1% SDS, at 125 volts until the bromophenol blue dye reached the end of the gel (~90min).

2.6.4.3 Protein transfer and immuno-detection

The separated proteins on SDS-PAGE gel were subsequently transferred to nitrocellulose membrane by electro-blotting in transfer buffer containing 192 mM glycine, 25 mM Tris-HCl (pH 7.4), 20% methanol at 80 volts for 1 to 2 hours. The efficiency of transfer was determined by ponceau staining.

The blot was blocked in 5% skim milk in TBS (137 mM NaCl, 20 mM Tris-HCl,

pH 7.6) for 1 hour at room temp and then incubated in 2% skim milk containing the rabbit anti-ENO2 antibody overnight at 4°C. After washing with TBST (0.2% Tween in TBS) for 3x 10 minutes, the blot was incubated in 2% skim milk containing the goat secondary antibody against rabbit IgG linked with horseraddish peroxidase for 1 to 2 hours at room temperature. After another wash with TBST for 3x 10 minutes, the blot is visualized for ENO2 signals with Detection Reagent (Amersham Bioscience, Baie d'Urfe, Quebec). Afterward, the blot was stripped with stripping solution containing 250 mM glycine-HCl (pH 2.0), 1% SDS, treated with LDH1 primary antiboady, and finally anti-rabbit secondary antibody to visualize LDH1 signals.

2.7 Generation of transgenic parasite lines

The constructed plasmids were used to generate transgenic parasite lines. The plasmids were first extracted using E.Z.N.A Plasmid Midiprep Kit (Omega Bio-tek, GA), and then were used to transform *T. gondii* strain RHΔHX. The transformed parasites were cultured under selective pressure to produce stable transgenic parasite lines.

2.7.1 Plasmid extraction by midiprep

The constructed plasmid was extracted from *E. coli* using E.Z.N.A Plasmid Midiprep Kit from Omega Bio-tek according to manufacturer's instruction. Literally, an isolated colony was used to inoculate 100 ml of LB broth supplemented with 100 μg/ml ampicillin. The culture was incubated overnight at 37 °C in shaking incubator at 250 rpm.

Then, 50 ml of the overnight-cultured medium was placed in a 50 ml Falcon tube and the cells were harvested by centrifuging at 4,000x g for 10 minutes. The cells were then resuspended in 2.25 ml of Solution I with RNase A. Then, the bacteria were lysed by adding 2.25 ml of Solution II, followed by gentle inversion for 7 to 10 times for through mixing prior to incubation at room temperature for 5 minutes. The resulting mixture was then neutralized with 3.2 ml of Solution III and mixed by inversion for several times until a flocculent white precipitate formed. The mixture was centrifuged at 12000x g for 10 minutes at room temperature to pellet the cellular debris and genomic DNA. Then, the clear supernatant was transferred to a clean Hibind DNA Midi column assembled in a 15ml collect tube. The binding column was then centrifuged at 5,000x g for 5 minutes to completely pass the lysate through the column (need two times to transfer all the DNA solution to the column). Then 3 ml of the Binding HB buffer was added to the top of the column followed by centrifugation at 5,000x g for 5 minutes. After washing the column with 3.5ml DNA Wash Buffer diluted with ethonal and centrifuging at 5,000x g for 5 minutes, the resulting binding column was washed with 3 ml of 100% ethonal and centrifuged at 5,000x g for 3 minutes. Then, the column is subjected to centrifugation at 5,000x g for 10 minutes again to remove residue ethanol. Finally, 0.75 ml sterile deionized water was added to the column. The column was transferred to a new clean tube and subjected to centrifugation at 5,000x g for 5 minutes to elute out the DNA bind with the column. The elution process was repeated to increase the yield of plasmid DNA. The DNA sample was visualized on 1% agarose gel containing ethidium bromide. The

concentration of the plasmid solution was determined by measuring the absorbance of the DNA sample at 260 nm ([DNA] = A_{260nm} x dilution factor x (50 μg/ml)). In order to determine the purity of DNA from proteins, the absorbance was also measured at 280 nm and compared to that at 260nm.

2.7.2 *T. gondii* transformation using electroporation and selection

To produce the stable ENO1 and ENO2 knowckdown strains, 10μg of the plasmid p(TUB8)$_2$*ENO1-HX*, p(TUB8)$_2$*ENO2-HX* as well as the control plasmid p(TUB8)$_2$*GFP-HX* were used to transform the parasites strain RHΔ*HX* by electroporation respectively. The operation of electropration was performed as described above. After electroporation, the parasites were used to infect confluent HFF monolayers and incubated in ED1 complete medium for 24h at 37 °C in 5% CO_2 atmosphere. Then selection was applied by culturing the parasites in ED1 medium complemented with 25 μg/ml MPA and 50 μg/ml xanthine. Untransformed parasites were killed and the transformed ones survived due to the expression of exogenous *HXGPRT*. The selection pressure was kept to maintain the expression of the transgenes.

2.8 Characterization of transgenic parasite lines

To characterize the generated parasite lines, their genomic DNAs were extracted and used as the template for PCR to check the existence of the transgenes. Then, the production of dsRNA in these parasite lines was studied using RT-PCR. The expression

level of enolase was also monitored in the level of RNA and protein, by RT-PCR and western blot respectively.

2.8.1 Genomic DNA extraction and PCR

When *T. gondii* tachyzoites had completely lysed a confluent HFF monolayers, they were scraped from culture plates, syringed through a 27G1/2 needle twice, filtered through a 3 μm membrane to remove host cell debris. The cells are sedimented by centrifugation at 3,000x g for 10 minutes at room temperature. The collected pellet was washed twice with DPBS and was used for genomic DNA or total RNA extraction. To extract genomic DNA of *T. gondii*, the parasites pellet was resuspended in 250 μl genomic DNA lysis buffer containing100 mM EDTA, 10 mM Tris-HCl (pH 8.0) and 1% SDS and 2 mg/ml Proteinase K. The mixture was incubated at 55-60 °C for 4 to 6 hours to completely lyse the parasites. The suspension was then extracted three times with an equal volume of phenol:chloroform and twice with an equal volume of chloroform. Then, the genomic DNA was obtained from the aqueous phase by precipitating with two volumes 95% ethanol and 1/10 volume 3M sodium acetate (pH 5.2). After 15 minutes centrifugation at 5000x g at 4 °C, the DNA pellet was washed by 250 μl 70% ethonal and dissolved in 30 μl ddH_2O. The genomic DNA sample was resolved on 1% agarose gel, to visualize the existence of high molecular weight DNA band.

0.2 μg genomic DNA was used as the template for PCR. The 25 μl mixture of PCR is prepared as described above. The reaction condition for *ROP1* and *GFP* amplification

was 94 °C 5 min + 35 x (94 °C 30s, 55 °C 30s, 37 °C 45s) + 72°C 5 min. The reaction condition for *ENO1* and *ENO2* amplification was 94 °C 5min + 35 x (94 °C 30s, 42 °C 30s, 37 °C 75s) + 72°C 5min. The PCR products were visualized on 1% agarose gel.

2.8.2 Detection of dsRNA expression by RT-PCR

The total RNAs of different strains of *T. gondii* were extracted as described above. The RNA samples were subjected to DNase treatment and subsequent PCR to confirm no DNA contamination. 2 μg of the total RNA sample was used in the RT reaction using the upper primers of *ENO* primer set (5'ENOx) to produce the cDNA complementary to the ENO antisense RNA, and primer 5'*GFP* was utilized to produce cDNA complementary to *GFP* antisense RNA. The RT reaction was conducted as described above and 2 μl of the RT products was used as the template for PCR to detect the production of dsRNA.

To study the expression level of enolase in the levels of mRNA and protein of the different parasite lines, RT-PCR and western blot were utilized and the operations are performed as described in section 2.6.3 and 2.6.4 for transient enolase knockdown experiments.

2.9 Immunofluorescence Assay

Newly released parasites were inoculated onto confluent HFF monolayers grown on glass slides. For different stages of the parasites, tachyzoites were then allowed to

grow for 2-3 days, while intracellular bradyzoites were cultured for 4 days before analysis. After removing culture media and washed with DPBS for 3 times, the cells were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes and then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. Cells were then blocked with 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature (Yahiaoui *et al.*, 1999). Slides were incubated for 1 hour with the primary antibody in a humidity chamber. After three washes with PBS for 3 x 10 minutes, cells were incubated for another 1 hour with seconday antibody conjugated to rhodamine or Fluorescein isothiocyanate (Cormack *et al.*, 1996) in a dark humidity chamber. Afterward, the slides were washed with PBS for another three times. The nuclei of the cells were stained by incubation in the presence of 4′,6 diamidino-2-phenylindole (DAPI, Sigma) for 10 minutes, and three washings with PBS (Yahiaoui *et al.*, 1999). For cyst staining, the cells were stained with lectin from *Dolichos biflorus* conjugated to FITC (diluted 1:300, Sigma) for 1 hour. The slides were dried in the air and overlaid with fluoromount, followed by incubation at room temperature overnight in the dark. The cells were examined with a Leica DMIRB microscope. All images were taken with a cooled Q-Imaging CCD camera using the Improvision Openlab software.

2.10 Measurement of parasite growth

The growth ability of different parasite lines was analyzed by their amplification ability. Freshly released tachyzoites were used to infect confluent HFF monolayer cells in

ED1 complete medium grown on glass slides. After growing for 24h and 48h, the slides were washed with DPBS three times and were fixed with 3% paraformaldehyde in PBS for 10 minutes. After permeabilization with with 0.2% Triton X-100 in PBS for 15 minutes, the fixed cells and parasites were incubated in DAPI solution for ten minutes to stain their nuclei. After drying in the air, the slides were overlaid with fluoromount, and incubated at room temperature overnight in the dark. The cells were examined with a Leica DMIRB microscope. The numbers of vacuoles containing different numbers of parasites were counted and their ratios in total vacuoles were scored, and plotted.

The growth rates of the transgenic parasite lines as well as the parental strain were also measured by plaque assay. Briefly, newly released parasites of different strains were counted after staining with 0.4% Trypan Blue, and 5,000 live parasites were used to infect confluent HFF monolayers in 35mm culture plates. After 24h incubation at 37 °C in 5% CO_2 atmosphere, the plates were sealed and incubated undisturbed for at least 6 days, shielded from excess vibration. When plaques of adequate size for visualization were formed (usually ~9 days after infection), the cells were rinsed with PBS, fixed in methanol, stained in crystal violet (to make 5 X stock, 25 g of crystal violet was dissolved in 250 ml ethanol and added to 1,000 ml 1% ammonium oxalate) and air-dried. Parasites plaques appeared as irregular clear areas against mottled violet background produced by stained confluent HFF cells. The numbers of plaques were counted under optical microscope.

2.11 *In vitro* differentiation of *T. gondii*

To differentiate the parasites from tachyzoites into bradyzoites, the alkaline method was used. Briefly, freshly released tachyzoites were used to inoculate confluent HFF cells in ED1 complete medium and were allowed to invade the host cells for 4 hours. The medium was subsequently replaced with RPMI1640 medium pH 8.2, containing 25 mM HEPES, 5% fetal bovine serum, 5 μg/μl streptomycin, and 5 units/ml penicillin, and incubated in a humid air atmosphere at 37 °C with 5% CO_2. The medium was replaced every one or two days to maintain the pH. And parasites were harvested immediately after they had been released from HFF monolayers.

After 4–6 days of bradyzoite differentiation using the alkaline method, the cells were scraped and digested with 170 mM NaCl-pepsin (0.1 mg/ml) to free the bradyzoites from the cells. After incubation in 60 mM HCl for 1 minute at 37 °C, the cells suspension was neutralized with 94 mM Na_2CO_3. Collected parasites were passed through a 27G1/2 needle twice and filtered through a 3 μm filter (Nucleopore) to remove host cell debris. Filter-purified parasites were then collected by centrifugation at 3,000x g for 10 minutes and washed twice with DPBS. The collected bradyzoites can be lysed for protein study, or for DNA and RNA extraction. They can also be used to infect confluent host cells in ED1 complete medium to transfer them into tachyzoites.

CHAPTER 3

RESULTS

3.1 Transient knockdown of enolase expression

To investigate the function of enolase isoforms in the development and stage conversion of *T. gondii*, we attenuated the expression of these glycolytic enzymes using RNAi approach. Initially, we conducted transient down-regulation assays of *ENO1* and *ENO2* to evaluate the efficiency of dsRNA induced silencing. *In vitro* synthesized dsRNA homologous to *ENO1* and *ENO2* was electroporated into the parasites. The mock electroporation was also performed using only electroporation buffer. Following the electroporation (3-5 days), newly released parasites were collected to monitor the expression of *ENO1* and *ENO2* using RT-PCR and western blot analysis. And *ROP1* expression was used to establish the baselines for a comparison between different electroporated parasite sets (Fig. 3.1A, *ROP1*). We detected no change in mRNA level of *T. gondii ROP1* after electroporation with dsRNA homologous to *ENO1*, *ENO2* or *GFP*. The *ENO2* dsRNA electroporated parasites showed a decreased level of *ENO2* transcripts ~75 % (Fig. 3.1A, *ENO2*) and contained un-modulated levels of *ROP1* and *Argonaute* (Fig. 3.1A, *AGO*), when these RT-PCR signals were compared with those of the mock electroporation. These results indicate that the dsRNA induced gene silencing is effective to specifically lower the expression of *ENO2*.

To determine whether the transient gene knockdown affected the levels of gene products, western blot analysis was performed using polyclonal antibodies raised against ENO2 and LDH1. We detected that ENO2 was decreased following the *ENO2* dsRNA electroporation (Fig. 3.1B), but was not significantly affected by electroporation with other dsRNAs. The signals revealed by LDH1-antibody were used as loading controls for the correction of protein concentrations used in the study.

Figure 3.1

Transient down regulation of ENO2 expression

Legend

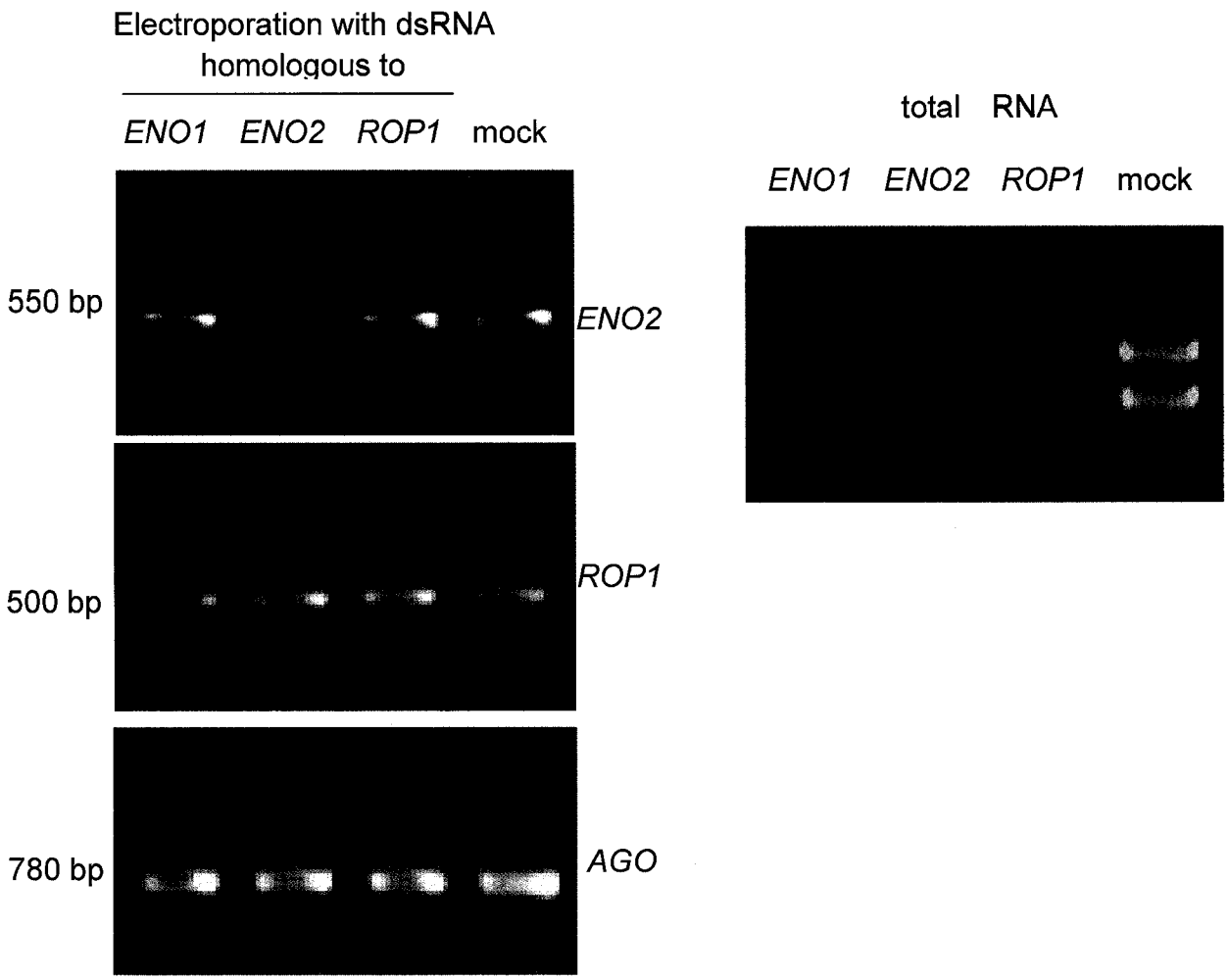
A. Following dsRNA electroporations, total RNAs were isolated from *T. gondii* strain RH and subjected to RT-PCR analysis using the oligonucleotide primers specific to *ENO2*, *ROP1 and AGO*. The RNA samples used in the RT reaction are also shown in the figure (right panel, not quantitative). The amount of each sample was quantified using spot densitometry of AlphaEase software.

B. Western blots were incubated with polyclonal antibodies against ENO2 (upper panel), and LDH1 (lower panel). The amount of each sample was quantified using spot densitometry of AlphaEase software.

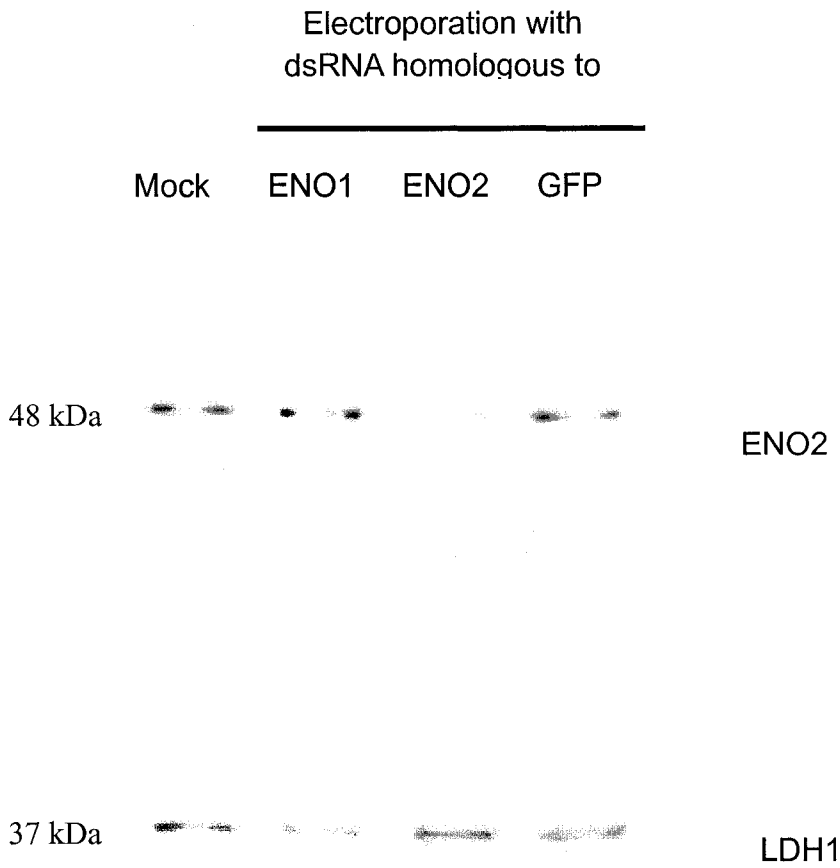
Figure 3.1

Transient down regulation of ENO2 expression

A. RT-PCR



B. Western blot



3.2 Generation of stable ENO1 and ENO2 knockdown parasite lines

The results of transient down-regulation confirmed the effectiveness and specificity of dsRNA induced silencing of enolase expression. However the introduction of dsRNA into *T. gondii* can only produce transitory knockdown effects and is unsuitable for the study of their physiological functions. Therefore we created transgenic parasite lines using RHΔHX as parental parasites to obtain stably expression of *ENO1* or *ENO2* dsRNA to induce gene silecning.

The plasmids expressing dsRNA homologous to *ENO1*, *ENO2* or *GFP* was constructed by placing the target DNA fragment between two head-to-head promoters. Such construction was previously used in the dsRNA expression in *Trypanosoma congolense*, *Trypanosoma brucei* as well as *T. gondii* (Bannai H *et al.*, 2003; LaCount *et al.*, 2000; Al-Anouti *et al.*, 2003). The plasmid construction was described in "Materials and Methods" (Fig. 3.2).

The three constructed plasmids p(TUB8)$_2$*ENO1-HX*, p(TUB8)$_2$*ENO2-HX* and p(TUB8)$_2$*GFP-HX* were individually introduced into *T. gondii* strain RHΔHX using electroporation. Transformed parasites were then cultured using medium supplemented with MPA and xanthine. The selection was maintained to ensure integration of the transforming plasmid into parasite genome. The stable transgenic parasite lines were named as dsENO1, dsENO2 and dsGFP respectively, according to the transforming plasmids used. We also tried to generate stable transgenic parasite lines using another parental strain, named PLKΔHX, which grow slower than RHΔHX and can differentiate

to bradyzoites more efficiently. Using such a strain, we aimed at dissecting the knockdown effect influenced by different genetic makeup. Unfortunately we can only obtain stable parasite line when p(TUB8)$_2$*ENO2-HX* was used, but not with other constructs. This parasite line is named dsENO2P.

Figure 3.2

Schematic representation of construction strategies

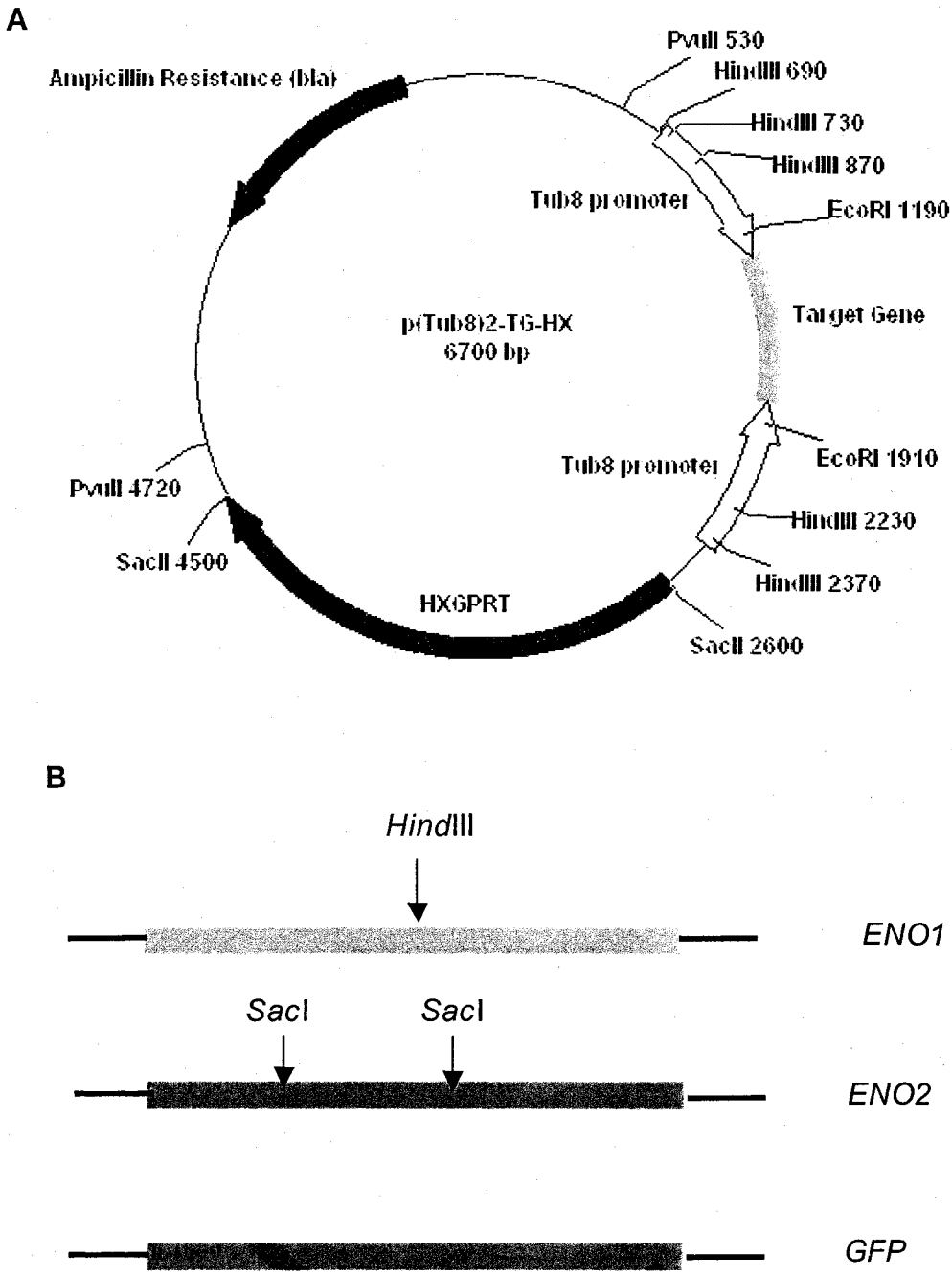
Legend

A. Plasmid used in the generation of parasite lines with dsRNA expression. Two tubulin promoters (Tub8) are arranged head-to-head and the target gene sequence (*ENO1*, *ENO2* or *GFP*) is placed between these two promoters.

B. DNA fragments of target genes cloned into the constructed plasmids. Restriction sites for *Hind*III and *Sac*I are shown in *ENO1* and *ENO2* fragments

Figure 3.2

Scheme of constructed plasmids used to generate parasite lines



3.3 Characterization of the transgenic parasite lines

To confirm the presence of transgenes in the genome of the parasites, total DNAs of the transgenic parasite lines were extracted and subjected to PCR analysis for comparison to the parental parasite strains. The expression of dsRNA in the transgenic parasite lines was monitored by RT-PCR.

3.3.1 Confirmation of transgene integration

To confirm that the ENO1 or ENO2 transgenes were integrated into the parasite genome, we made use of the difference between the cDNA and genomic sequences. In the gene encoding *ENO1*, there is a 493 bp intron separating the first 40 bp exon from the rest of the coding sequence, while *ENO2* gene has a 650 bp intron. We synthesized the oligonucleotide primers flanking these regions (5'ENOx and 3'ENOx). Using these primers, we can easily distinguish the transgenic genes by their smaller size following PCR amplification using isolated genomic DNA as templates and agarose gel electrophoresis.

The results of genomic PCR are shown in Figure 3.3. PCR using 5'*ENO*x and 3'*ENO*x primers, a band of ~1 kb was amplified from all samples, representing the genomic locus of *ENO1* gene. Additional bands of approximately 540 bp, corresponding to the transgenic gene of *ENO1* or *ENO2,* were shown in the PCR products from genomic DNA sample of dsENO1 and dsENO2 strains (Fig. 3.3A lane 3, 4). Since the PCR products of *ENO1* and *ENO2* transgenic DNA were similar in size, these PCR products

were further analyzed by restriction endonuclease digestion to ensure the target genes were properly amplified. For example, *Hind*III site is present in *ENO2* but not *ENO1* gene. Thus HindIII can digest the PCR products derived from *ENO2*, but not those from *ENO1*. Similarly, *Sac*I will digest *ENO1*, but not *ENO2* (Fig. 3.2B, C). The digestion profile confirmed the identification of the two ~540 bp bands generated from the transgenic strains, indicating correct integration of transgene plasmid into the genome of the parasites. Moreover, the genomic DNA samples were also subjected to the PCR reaction using T7-5*GFP*mut2 and T7-3*GFP*mut2 primer set and the band representing the *GFP* transgenic DNA was only generated from PCR using dsGFP gDNA as the template (Fig. 3.3B), demonstrating the exclusive presence of *GFP* transgene in the dsGFP strain. The integration of the transgene in the strain of dsENO2P was also confirmed by genomic DNA PCR.

Figure 3.3

Confirmation of transgene integration

Legend

A. Total DNA samples extracted from generated transgenic parasite lines and parental strains were subjected to PCR to detect presence of *ENO1* and *ENO2* transgenes in parasite genome.

Lane 1: PCR products of gDNA from parental strain RHΔHX

Lane 2: PCR products of gDNA from transgenic strain dsGFP

Lane 3: PCR products of gDNA from transgenic strain dsENO1

Lane 4: PCR products of gDNA from transgenic strain dsENO2

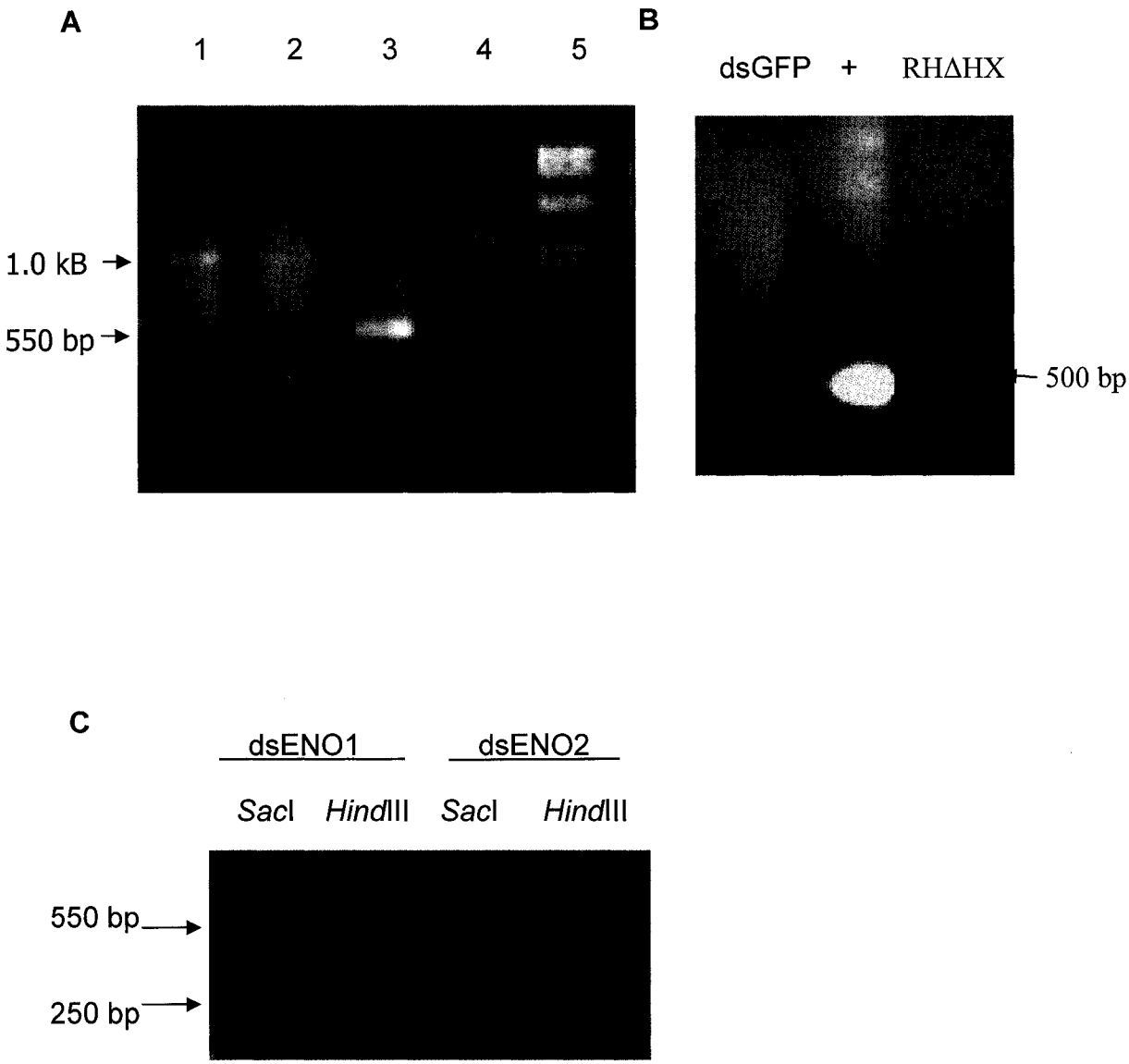
Lane 5: DNA marker

B. Total DNAs extracted from generated transgenic parasite line dsGFP and parental strains RHΔHX were subjected to PCR to detect integration of *GFP* transgene in parasite genome. Constructed plasmid p(TUB8)$_2$*GFP-HX* was also used in PCR to serve as a positive control (+).

C. ~540 bp PCR products from dsENO1 and dsENO2 genomic DNA were subjected to *Sac*I and *Hind*III digestion to confirm their identities.

Figure 3.3

Confirmation of transgene integration



3.3.2 Expression of dsRNA in the transgenic parasites

Investigation of dsRNA expression in the parasite lines is crucial. The constructed plasmids used to generate these parasite lines were designed to express dsRNA homologous to *ENO1*, *ENO2* and *GFP*. Total RNA from the transgenic and parental strains were extracted and used in RT-PCR analysis to detect the expression of dsRNA using 5'-*ENOx* and 3'-*ENOx* primer set as well as T7-5*GFP*mut2 and T7-3*GFP*mut2 primer set.

No significant band was produced in the RT-PCR using *ENOx* primer sets from RNA samples isolated from parental strain RHΔHX and the control strain dsGFP, indicating that antisense RNA as well as dsRNA homologous to *ENO1* or *ENO2* genes was not expressed in these strains. When the total RNA samples from dsENO1 and dsENO2 strains were used in the RT-PCR using *ENOx* primer sets, a band of ~540 bp was detected in both reactions (Fig. 3.4A, top panel). These two PCR products were also subjected to restriction endonuclease digestion to verify their identities. The RT-PCR band generated from dsENO1 RNA can be digested by *Sac*I but not *Hind*III, indicating its homology to *ENO1* gene, while the RT-PCR band obtained from dsENO2 RNA can be cut by *Hind*III but not *Sac*I, showing its *ENO2* homology (Fig. 3.4B). These results showed that dsRNA homologous to *ENO1* is only expressed in dsENO1 strain, and dsRNA homologous to *ENO2* is exclusively expressed in dsENO2 strain. The total RNA samples from these four strains were also subjected to RT-PCR reaction using *GFP* primer set, and positive results were only detected from dsGFP samples (Fig. 3.4A,

middle panel), indicating that *GFP* dsRNA was only produced in this transgenic *T. gondii* strain. Moreover, the expression of dsRNA homologous to *ENO2* in the transgenic strain dsENO2Pwas also detected using the same method.

Figure 3.4

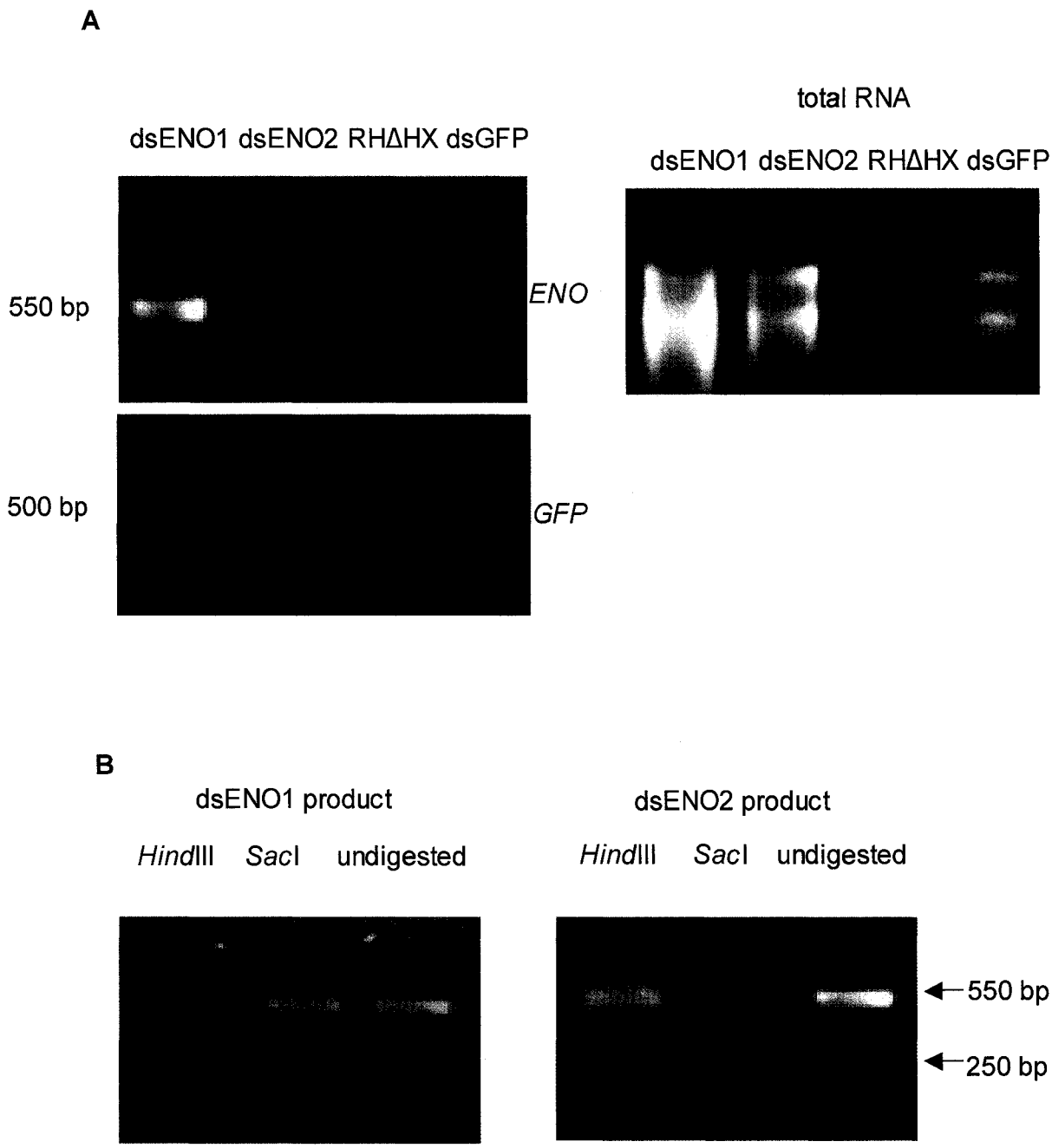
Confirmation of dsRNA expression

Legend

A. Total RNA samples extracted from generated transgenic parasite lines (dsENO1, dsENO2, dsGFP) and parental strains (RHΔHX) were subjected to RT-PCR using *ENOx* (upper panel) and *GFP* (middle panel) specific primers to detect dsRNA production. The RNA samples used in the RT reaction are also shown in the figure (right panel, not quantitative).

B. ~540 bp RT-PCR products from dsENO1 and dsENO2 total RNA were subjected to *Sac*I and *Hind*III digestion to confirm their identities.

Figure 3.4
Expression of dsRNA



3.4 The steady state level of enolase transcripts in transgenic parasite strains

Our transforming plasmids were designed to express dsRNA that would trigger gene silencing of the target genes. Following the confirmation of successful expression of dsRNA, we consequently evaluate whether the expressions of the target genes are knocked down. RT-PCR was used to assess the expression level of these target genes. Total RNAs were isolated from newly released tachyzoites of the transgenic and parental strains and used as templates in RT reaction in which the cDNA synthesis was initated using oligodT as primers. Although oligodT priming might not give high yields as gene specific primers do, it avoid variations among different RT reactions when the cDNA products were used in gene-specific PCR amplification. The results of the RT-PCR are shown in Figure 3.5A, the identities of the bands produced form ENOx RT-PCR is confirmed by restriction endonuclease digestion, and the ratio of *ENO2/ROP1* signals was also calculated for each sample. The expression level of *ENO2* was not changed in the parental strain RHΔHX, the control strain dsGFP as well as the transgenic strain dsENO1 (*ENO2/ROP1*~1.0). In the strain dsENO2, the ratio of *ENO2/ROP1* was ~0.4, showing that the expression of *ENO2* was lowered in the level of mRNA and indicating that the knockdown effects induced by dsRNA was gene specific. For the generated strain dsENO2P, compared with the parental strain PLKΔHX, similar results were also observed (Fig. 3.5B). The expression level of *ENO2* mRNA in dsENO2P strain was lowered, but its *ROP1* mRNA showed comparable amount with PLKΔHX.

To measure the expression level of gene products in the transgenic strains and the

parental strain, western blot analysis was conducted utilizing polyclonal antibodies against enolase isoforms ENO1 and ENO2. Equal amounts (5 μg) of protein extracts from freshly lysed trachyzoites were resolved in SDS-PAGE gel and transferred to nitrocellulose membrane. Figure 3.5C shows the results of the western blot. The lysate from dsENO2 strain contained less amount of ENO2 protein. And no significant difference of ENO2 signal was detected in the control strain dsGFP, and the transgenic strain dsENO1 from the parental strain RHΔHX (Fig. 3.5C, ENO2). Then, the membrane was stripped and LDH1 signals were detect to confirm equal loading amount (Fig.3.5C, LDH1). To further investigate whether ENO1, which is expressed in bradyzoite stage, could compensate the loss of ENO2 protein, an identical blot was prepared and incubated with ENO1-specific antibody. Very faint signals of ENO1 were detected, suggesting that this enolase isoform was not expressed to compensate attenuated ENO2 expression in tachyzoite stage (Fig. 3.5C). The faint bands could result from cross-interaction of ENO2 protein and ENO1- antibody.

Throughout the course of this study, we found that the expression level of ENO2 of transgenic strain dsENO2 was decreasing gradually following passages and selection by MPA and xanthine (Fig. 3.5D). The ratio between ENO2 and LDH1 was ~1.6 after we electroporated the constructed plasmid p(TUB8)$_2$*ENO2-HX* into the parasites and passed three times in HFF monolayers. The ratio decreased to ~1.3 after six passages, and become stable at the level of ~0.6 after nine passages. We hypothesized that if this observation was due to that a more homogenous population was formed following each

passage. Thus after RHΔHX parasites were electroporated with p(TUB8)$_2$*ENO2-HX* plasmid and completely lysed the host cells, we used them to inoculate a 24-well plate covered with HFF monolayers for cloning. About ten clonal parasites were acquired, five of which were used to infect new host cells and passed for three times before they were analyzed by immunoblot. However, western blot results showed that, there was no significant difference between the expression level of ENO2 in those transgenic parasites and that of RHΔHX.

Figure 3.5

Gene expression in transgenic strains

Legend

A. Total RNA extracted from generated transgenic parasite lines (dsENO1, dsENO2, dsGFP) and parental strains (RHΔHX) were subjected to RT-PCR to detect *ROP1* and *ENO2* expression in mRNA level. The amount of each sample was quantified using spot densitometry of AlphaEase software.

B. RNA extracted from generated transgenic parasite lines dsENO2P, and parental strains PLKΔHX were subjected to RT-PCR to detect ROP1 and *ENO2* expression in mRNA level.

C. Western blot analysis was performed using lysate extracted from tachyzoites of the transgenic parasite lines (dsENO1, dsENO2, dsGFP) as well as lysate from the parental strains (RHΔHX). The blot was treated with specific polyclonal antibodies against ENO2 (upper panel) and ENO1 (middle panel). Anti-LDH1 antibody was also used to confirm that comparable amount of sample were loaded (lower panel).

Figure 3.5

Gene expression in transgenic strains

Legend (continue)

D. Western blot analysis showed the decreasing ENO2 expression level in dsENO2 strains after transformation. LDH1 signals were also detected to normalize loading amount.

Lane 1: RHΔHX

Lane 2: dsGFP

Lane 3: dsENO2 after 11 passages

Lane 4: dsENO2 after 9 passages

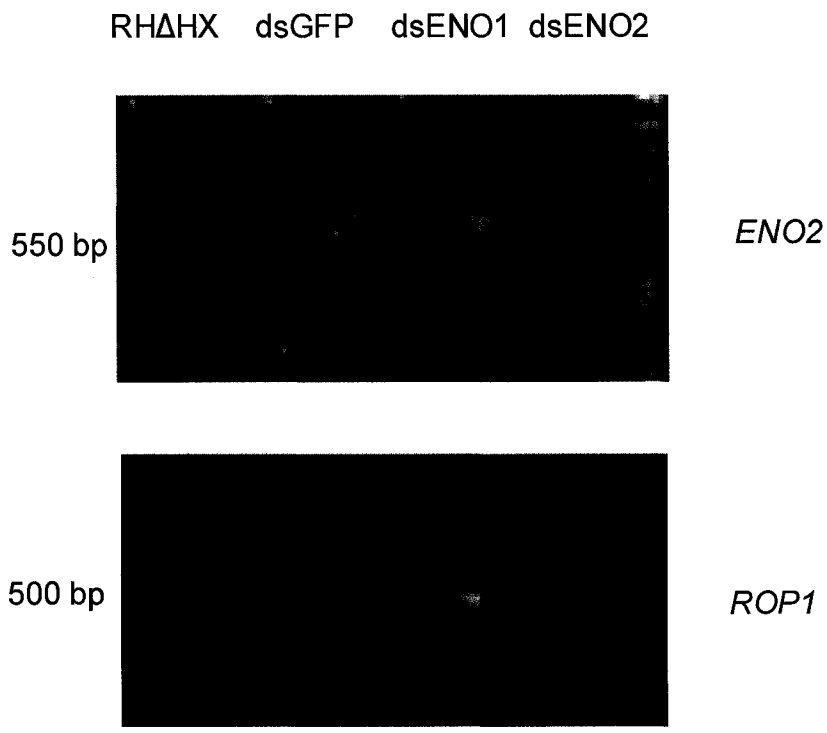
Lane 5: dsENO2 after 6 passages

Lane 6: dsENO2 after 3 passages

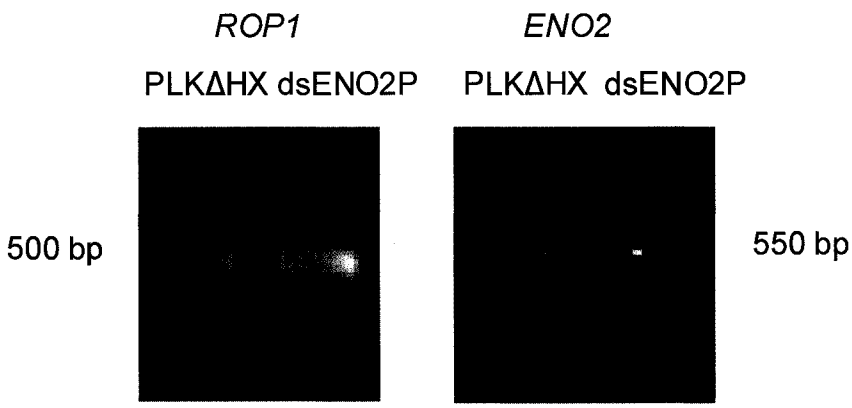
Lane 7: dsENO1

Figure. 3.5
Gene expression in transgenic strains

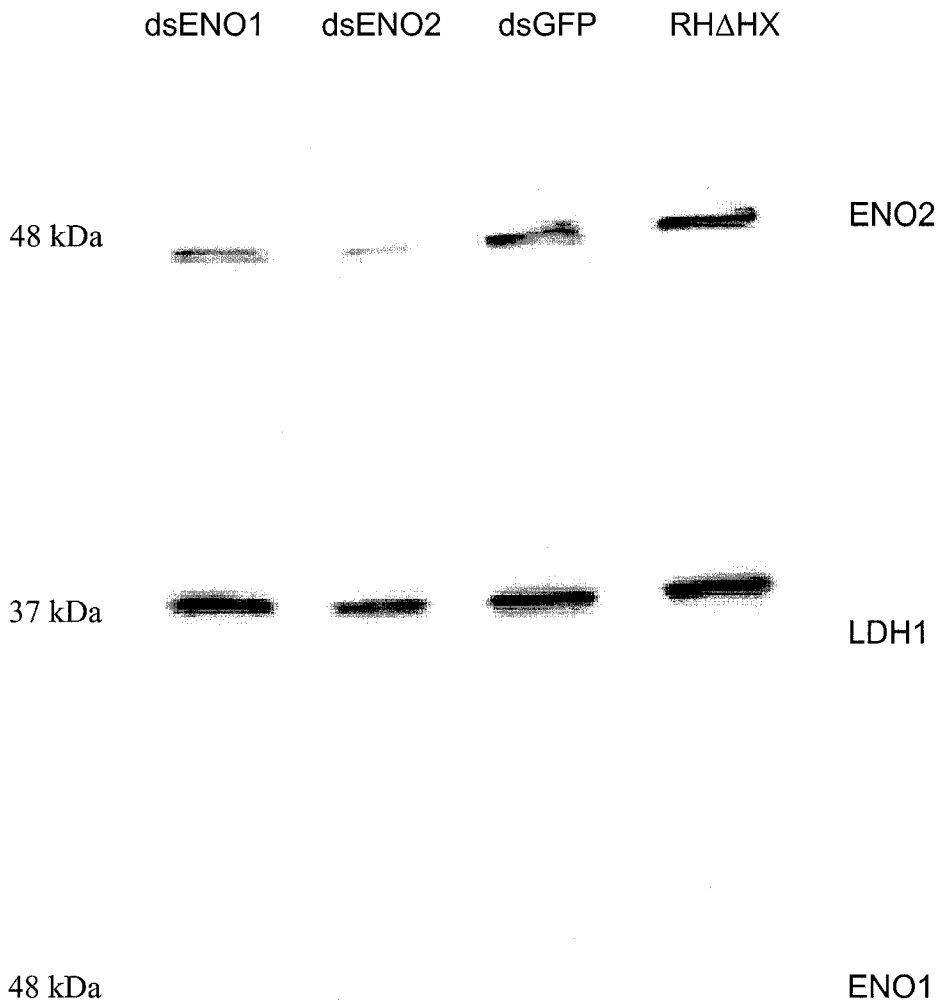
A. RH RT-PCR



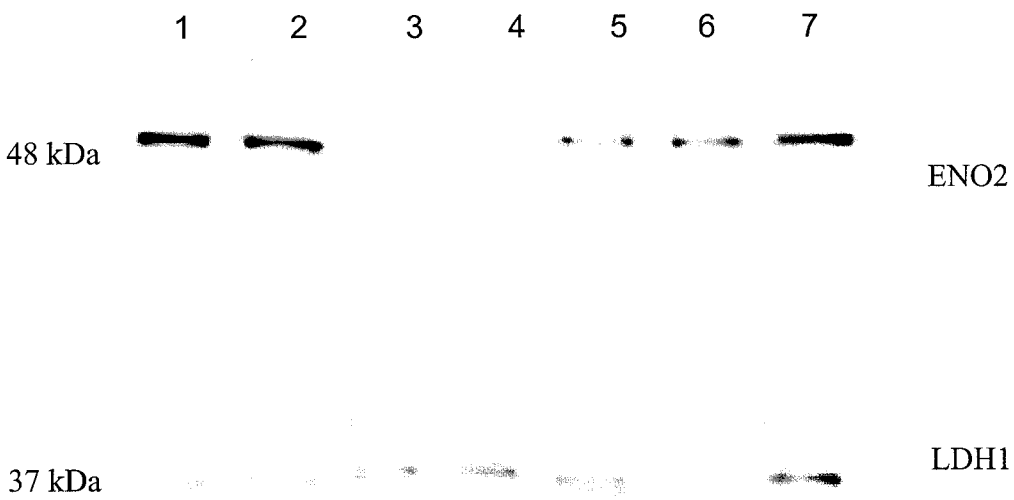
B. PLK RT-PCR



C. Western blot



D. Passage differences



3.5 The localization of enolase

Immunofluorescence assay was performed to monitor the subcellular localization of enolase in the parental type strain RHΔHX, as well as in the generated transgenic strains. In addition to ENO2 (rhodamine-labeled secondary antibody) detection, we also monitored the localization of surface antigen 1 (SAG1 or P30), which is on the membrane of tachyzoites (Fluorescein isothiocyanate (FICT)-labeled secondary antibody, green). Merging with nuclei stained (4', 6 diamidino-2-phenylindole (DAPI), blue) image, we detected that the ENO2 signal is localized in the cytosol and nuclei, similar to previous report on the localization of enolase (Fig. 3.6). In order to estimate the expression level of ENO2 protein, the intensity of the fluorescence signal of the parental and transgenic tachyzoites were quantified and normalized with reference to the background fluorescence signal. The ratio of the ENO2 signals from the tachyzoite parasite lines over those from the parental was calculated and plotted for each parasite line. We detected that the ENO2 signal did not change significantly in the strain dsENO1 or dsGFP when compared to the parental RHΔHX strain. However this signal was attenuated in the strain dsENO2, indicating the efficiency and specificity of the dsRNA induced enolase knockdown effects. Furthermore, in these transgenic parasite strains, ENO2 signal was observed in both nucleus and cytosol of the parasites, suggesting that dsRNA induced RNAi does not affect the localization of ENO2 protein.

Figure 3.6

Localization of ENO2

Legend

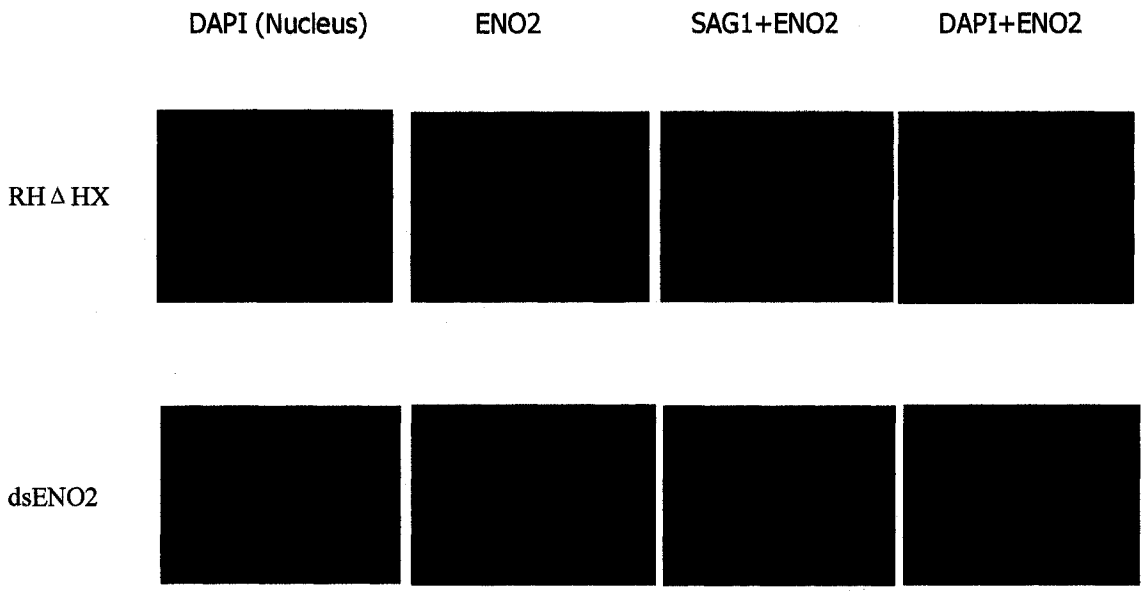
A. Parasites of Parental strain RHΔHX and transgenic strain dsENO2 are subjected to immunofluorescence assay. DAPI was used to label the nucleus of parasites (blue). FITC-labeled goat anti-mouse IgG was used to show the SAG1 localized on the membrane of *T. gondii* (green). Rodamine-labeled goat anti-rabbit IgG was used to reveal the localization of ENO2 (Red).

B. Normalized fluorescence values of ENO2 signals for the parasite lines under tachyzoite culture conditions.

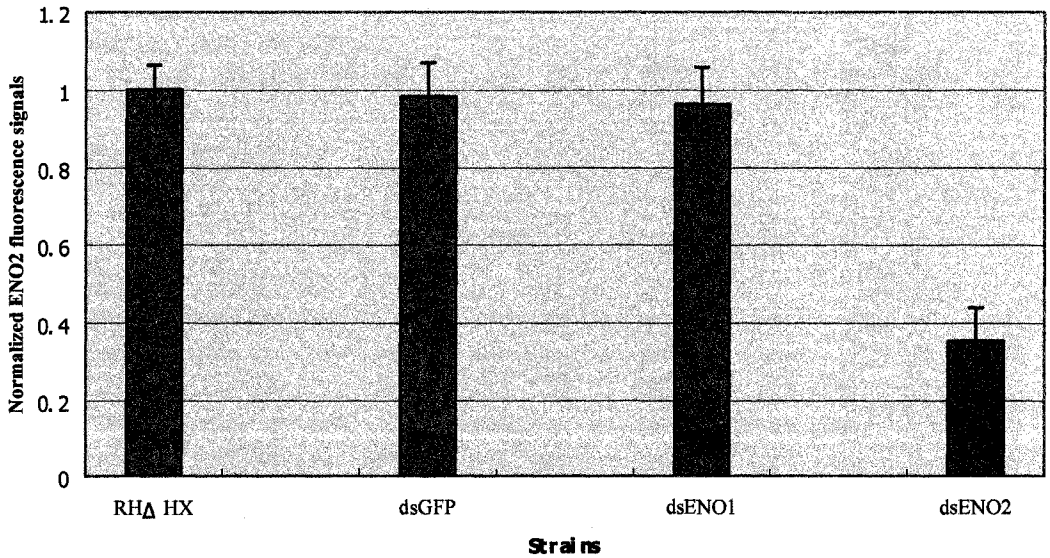
Figure 3.6

Localization of ENO2

A.



B.



3.6 Phenotypic analysis of transgenic parasite strains

We would expect that the knockdown of enolase might affect the growth of *T. gondii*. The multiplication rates of the transgenic strains as well as the parental strain were thus monitored. Since *T. gondii* proliferates by a synchronic unique binary division, the number of parasites per vacuole could reflect the division rate. Following trypan blue staining to evaluate the parasite viability, we used 10,000 viable parasites of each strain, including dsENO1, dsENO2 and RHΔHX, to infect confluent HFF monolayers. The parasites were allowed to invade the host cells for 1 hour, and then non-attached or dead parasites were removed by rinsing with fresh media. At 24 hours and 48 hours post-infection, we counted the numbers of parasites within independent vacuoles (between 150 – 200 vacuoles) The fractions of vacuoles containing 1-, 2-, 4-, 8-, 16 or 2^n parasites were caculated in order for comparing the growth distribution of tested parasite strains as shown in Figure 3.7. There was no significant difference between the transgenic strains dsENO1, dsENO2 and the parental strain RHΔHX, indicating that the suppression of expression of ENO1 and ENO2, two isoforms of enolase, did not affect the growth of *T. gondii,* at least in the stage of tachyzoite.

Plaque formation assay was also employed to monitor the overall proliferation ability of the generated transgenic strains dsENO1, dsENO2, dsGFP as well as the parental strain RHΔHX. Approximately 5,000 viable parasites were used in the infection of HFF monolayers. After changing the medium at 1 hour post-infection, the cells were incubated in tachyzoite culture condition for 6 days. The plates were stained and the

number and sizes of plaques were monitored. No significant differences in the numbers and sizes of formed plaques were detected in the transgenic strains dsENO1 (68 formed plaques), dsENO2 (73 formed plaques) when compared with the parental strain RHΔHX (77 formed plaques). This result further verified that inhibition of enolase expression does not reduce the growth and proliferation ability of *T. gondii*, at least in the RH strain.

Figure 3.7

Parasite growth and replication

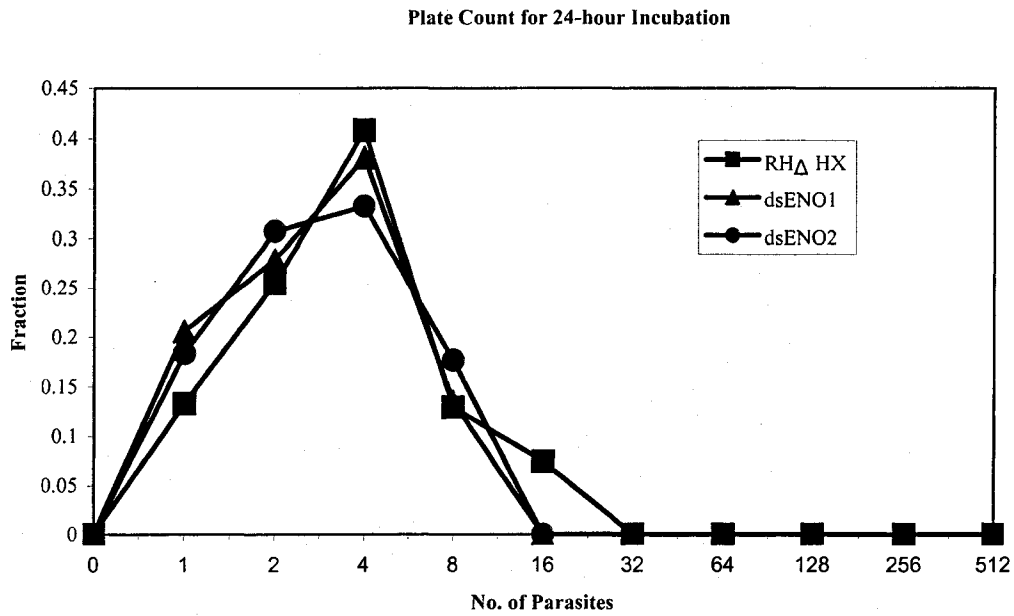
Legend

The fraction distribution of vacuole size (number of parasites in each vacuole) was determined at 24 hours (A) and 48 hours (B) after infection with the parental and transgenic parasite lines grown under tachyzoite culture conditions. About 150 vacuoles were counted for each strain at 24-hour time point, and about 200 were counted at 48-hour. The expression of ENO2 and LDH1 of the strains used at that time point were shown by western blot (C).

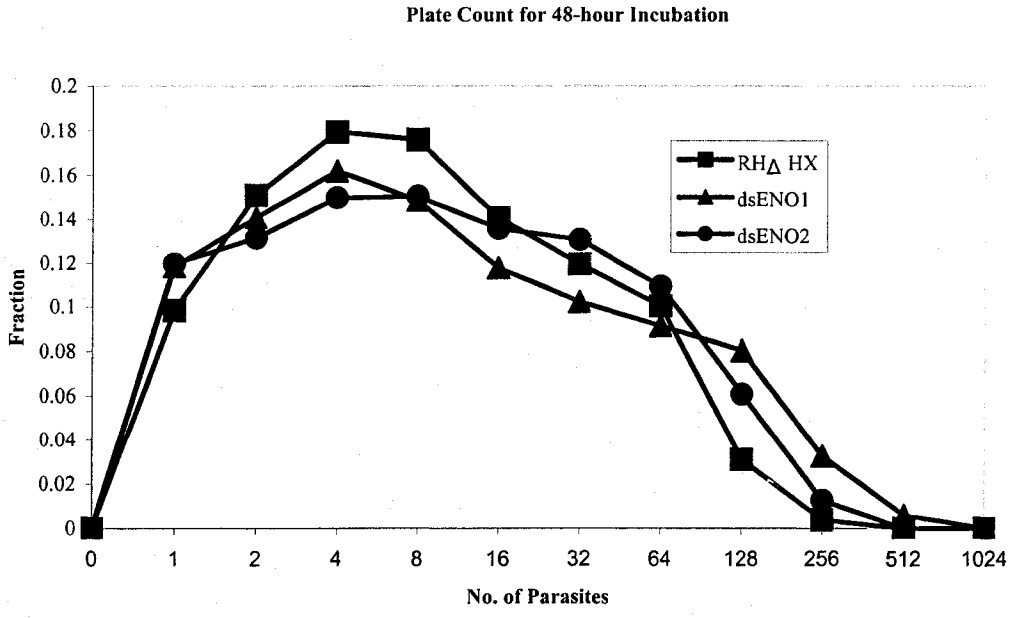
Figure 3.7

Parasite growth and replication

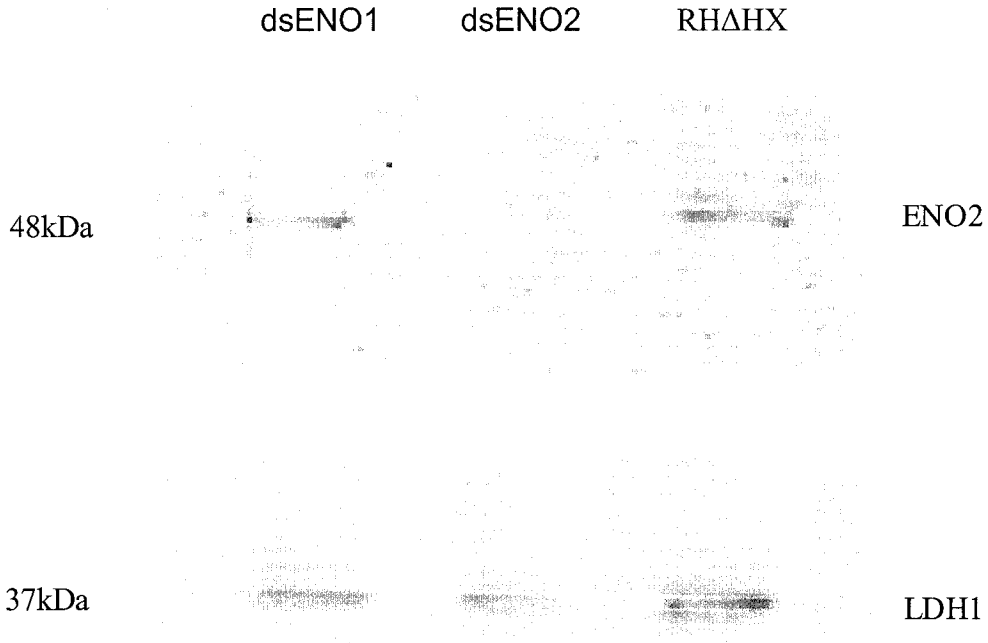
A.



B.



C.



CHAPTER 4

DISCUSSION

Enolase is one of the essential enzymes in the processes of both glycolysis and gluconeogenesis. In *T. gondii*, the stage-specific expression pattern of two isoforms of enolase (ENO1 and ENO2), suggests that the activity and effectiveness of this enzyme are important for the tachyzoite and bradyzoite. Stage differentiation and encystation of *T. gondii* involves the coordinated expression of several genes. A series of induction and suppression of gene expression are observed during the process as developmental dynamics. It is not surprising to propose the involvement of enolase expression in the process of stage differentiation between tachyzoite and bradyzoite. Although classical nuclear localization signal is not found in ENO1 and ENO2 primary sequences, their nuclear targeting can be enabled by a NLS in their tertiary structures (Sessler and Noy 2005), or be mediated by importin-independent pathways (Gorlich and Kutay, 1999). Nuclear localization of ENO1 and ENO2 proteins suggests other functions such as regulation of gene expression of this enzyme in addition to its contribution to the metabolism of the parasites (Johnstone *et al.*, 1992;Ghosh *et al.*, 1999). Thus, study on the function of this enzyme, particularly silencing of its expression, would be of great interest and be important to provide potential drug targets for the treatment to toxoplasmosis.

Transient transformation and silencing of enolase

Previous studies by our group have confirmed that dsRNA can be used as a powerful tool to repress the steady-level expression of *T. gondii* genes including *UPRT* and *LDH* (Al-Anouti *et al.*, 2003; Al-Anouti *et al.*, 2004). We first used dsRNA homologous to *ENO1* and *ENO2* genes to silence the expression of enolase in *T. gondii*. From RT-PCR and western blot analysis, I clearly demonstrated the efficiency and specificity of synthetic dsRNA in down-regulating enolase expression. Silencing of *ENO2* did not affect to the expression of *ENO1* and *GFP,* and *vice versa.* Although, we did not determine whether siRNA was produced, we suspect that siRNA produced from dsRNA can function as miRNA and can inhibit expression of non-perfect-matching genes in translational level (Doench *et al.*, 2003). Both siRNA and miRNA gene silencing pathway share similar partners such RNaseIII (Dicer) and Argonaute family proteins and are likely to be present in *T. gondii*, although miRNA pathway has not been reported in *T. gondii*. Though we cannot state definitely that miRNA as well as siRNA is responsible for the gene silencing, we can conclude that the difference between *ENO1* and *ENO2* is sufficient (65.8%) to obtain specific gene silencing.

Stable transformation and transgenic line generation

To circumvent the disadvantage of transient transformation, we used constructed plasmids and *T. gondii* strain RHΔHX to generate transgenic parasite lines. We also

attempted to generate the transgenic parasites lines using PLKΔHX. We could not produce the lines, except one expressing *ENO2* homologous dsRNA, naming dsENO2P. Parasite strains RHΔHX, PLKΔHX are different in growth and replication rate. Failure to generate transgenic parasites lines expressing ENO1 homologous dsRNA might be due to the slow proliferation ability of the PLKΔHX strain, which renders the electroporated parasites hard to propagate under selection. Another possibility is the transformation stability of this plasmid is low in PLKΔHX strain, or the expression of ENO1 is important for the growth of PLKΔHX strain.

We also found that, the expression of ENO2 in the dsENO2 parasites was gradually lowered during parasite passages and became stable at ~40% of the steady state level of the untransformed parasites. One of the possibilities is that the expression of dsRNA was increasing after transformed plasmid stably integrated into parasite genome, leading to the increasing inhibition of target gene expression induced by dsRNA. It is also possible that, after plasmid transformation, untransformed parasites and those with unstable transformation were able to survive under selection at first, but were killed gradually under selection pressure. We attempted to purify the transformed parasites by limiting dilution, but all five single clonal strains did not show any ENO2 knockdown after three passages. Probably more passages of the transformed parasites would be required to enhance the dsRNA inhibition effects. Monitoring the amount of dsRNA expressed by quantitative real-time RT-PCR may provide useful information about this question.

PCR using DNA extracted from transgenic parasite lines showed the presence of

transgenes in these generated cell lines. However, it was difficult to distinguish whether these transgenes were integrated into the genome or maintained as episomes. To have clear evidences of plasmid integration or episomal maintenance, we need to perform a plasmid rescue experiment. Briefly we would extract total DNA from the parasites and use it to transform competent *E. coli* cells. Only episomal DNA will have the transforming plasmid features to confer ampicillin resistance. Also, restriction endonuclease digestion followed by Southern blot analysis would address this question.

Phenotypic study of enolase knockdown

The ratio of vacuoles containing different numbers of *T. gondii* in total vacuoles showed the proliferation ability of the parasites, and plaque assay reflected both invasion ability and proliferate ability. Results of both methods indicated knockdown of ENO2 expression does not affect the growth of the parasites. As the glycolysis is considered to be important for the growth and development of *T. gondii*, and enolase is an essential part of glycolysis process, these results are quite surprising to some degree. One of the possibilities is that *T. gondii* has plenty amount of ENO2 expression, and the lefeover ENO2 in dsENO2 strain is sufficient for the survival of the parasites. Gene targeting of ENO2 gene by homologous recombination (knockout or knockin) or expression of inactive ENO2 to produce dominant-negative mutant may help to resolve the role of ENO2 in *T. gondii*. It is also possible that other genes, enzymes or metabolic pathways have taken over to compensate for the reduction of ENO2 expression. To have a global

view of the gene expression, we need to perform microarrays to classify affected genes. Moreover, we could use metabolic inhibitors, specific to the enzymes of the glycolysis pathway to survey the drug sensitivity in the parental and transgenic parasites. Other metabolism pathways, including TCA cycle and anaerobic respiration may provide required NADH and subsequent ATP for the survival of the parasites when glycolysis is suppressed. Additionally, fatty acids and amino acids can be oxidized to produce acetyl-CoA, pyruvate and other metabolites required in these processes instead of glycolysis pathway. Inhibition of these pathways may provide adequate insight into the metabolism system of the transgenic parasites and put forward essential information for this study.

Future work

In this study, we used dsRNA-induced gene silencing to lower the expression of enolase isoforms, ENO1 and ENO2 in *Toxoplasma gondii*. Two transgenic parasite lines with attenuated enolase expression dsENO1 and dsENO2 were successfully generated. We found that, although ENO2 expression was reduced, it did not affect the growth ability of *T. gondii* strain RH. Future work could be directed to study the effects of enolase knockdown in the stage of bradyzoite as well as the process of stage conversion. Moreover, we would like to investigate the reason for unaffected growth of *T. gondii* with lowered enolase expression. Inhibitors of Krebs cycle and anaerobic respiration as well as fatty acid and amino acid oxidation pathway will be used to elucidate the possible

pathway utilized by the parasites to compensate the suppression of glycolysis. Additionally, extra roles of *T. gondii* enolase isoforms will be another target of upcoming study. Protein and chromatin immunoprecipitation can provide useful information about proteins and DNA bound with enolase in the nuclei, and grant essential information about the functions of enolase.

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APPENDICES

Appendix I

General buffers and solutions used in the study

Buffer or solution	Constituents
Blocking solution for western blot	5 g slim milk powder in 100 ml TBS
Electroporation buffer	120 mM M KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 25 mM HEPES, pH 7.6, 2 mM EDTA, 5 mM $MgCl_2$
TBS	125 mM NaCl, 25 mM Tris
TBST	0.2% Tween 20 in TBS
6 x DNA gel loading buffer	0.25% (w/v) bromophenl blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in water
Dulbecco's phosphate-buffered saline (DPBS)	PBS buffer supplemented with 0.1 g/l Ca^{+2}
Luri Bertani broth (LB broth)	10 g/l tryptone, 5 g/l yeast extract and, 0.17 M NaCl. The broth is sterilized by autoclaving for 15 min.
Tris EDTA (TE)	10 mM Tris, pH 8.0, and 1 mM EDTA
Tris-Acetate-EDTA (TAE)	40 m Tris-acetate and 1 mM EDTA
Phosphate-buffered saline (PBS)	0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 KH_2PO_4, pH 7.4
	9% acrylamide/bisacrylamide (37.5:1), 0.39

SDS-PAGE Resolving gel	M Tris, pH 8.8, 0.1% SDS, 0.1% APS and 0.04% TEMED
SDS-PAGE Stacking gel	5% acrylamide/bisacrylamide (37.5:1), 0.125 M Tris, pH 6.8, 0.1% SDS, 0.1% APS and 0.1% TEMED
SDS-PAGE loading	25mM Tris, pH 6.8, 2% SDS, 0.1% Bromophenol Blue, 720 mM 2-mercaptoethanol and 10% glycerol
Tris-glycine	25 mM Tris, pH 8.3, 196 mM glycine and 0.1% SDS
Transfer buffer for western blot	25 mM Tris, 192 mM glycine, 20% methonal
Protein Lysis buffer	300 mM NaCl, 20 mM Tris-HCl, 1 mM DTT, 0.5 mM PMSF, pH7.5,
Genomic DNA extraction buffer	100 mM EDTA, 10 mM Tris-HCl, pH 8.0 and 1% SDS
Miniprep solution I	50 mM glucose, 25 mM Tris-HCl, pH 8.0
Miniprep solution II	0.2 N NaOH, 1% SDS
Miniprep solution III	3 M NaOAc, pH 5.2
Ponceau Staining solution	0.1% ponceau in 5% acetic acid solution

Appendix II

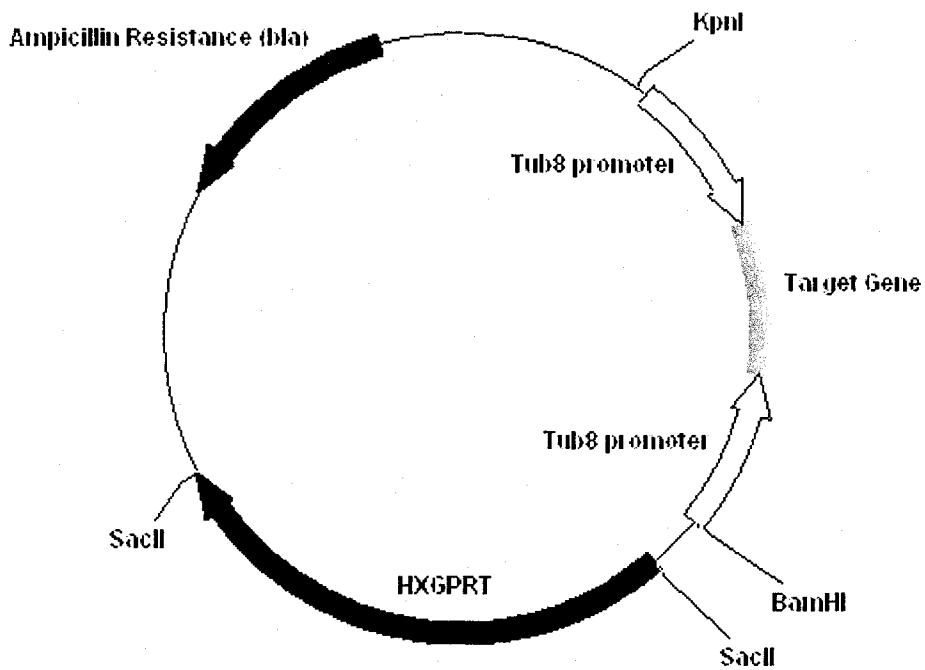
Oligonucleotide primers used in the study

Names	Sequences
BamHI 5' pTUB8	CCGGATCCGTCGACGGTATCGATAAGC
NsiI 3'pTUB8	CTCATGCATTTTGTCG
HXGPRT forward	ATGGCGTCCAAACCCATTGAA
HXGPRT reverse	ACCGGTGTCGACGTCCTC
5'HXcassatte	CCAGAATTCAAGATCCGATCTTGC
3'HXcassette	CCAGAATTCTGTCACTGTAGCCTG
5'*ENOx*	ATGGTGGTTATCAAGG
3'*ENOx*	CCAACGGGAGCGATC
EcoRITgArg5'end	TGGAATTCCCCGCAGTATTTCGCAAATG
EcoRITgArg3'end	CGGAATTCGCTTCGCCACTGAGGTGAG
OligodT	TTTTTTTTTTTTTTTT
EcoRIdTTAIL	AAGAATTCTTTTTTTTTTTTTTTT
T7on5'*ENOx*	TAATACGACTCACTATAGGATGGTGGTTATCAAGG
T7on3'*ENOx*	TAATACGACTCACTATAGGCCAACGGGAGCGATC
T7on5'*ROP*	TAATACGACTCACTATAGGGGAACATGGGCCACAGG
T7on3'*ROP*	TAATACGACTCACTATAGGCGCCGAAAGCGTCTCTG
T7-5*GFP*mut2	AAGAATTCTAATACGACTCACTATAGGAGAAGAACTTTCAC
T7-3*GFP*mut2	AAGAATTCTAATACGACTCACTATAGGTCTGCTAATTGAA

Appendix III

Nucleotide sequence of constructed plasmids used in the study

All the constructed plasmids were generated from commercial plasmid pBluescript SK+. The primers used for each DNA fragments are indicated by arrows.



p(TUB8)$_2$*ENO1-HX*

The TUB8 promoter as well as followed *ENO1* DNA fragment and the opposite TUB8 promoter were used to replace 653-719nt (*Kpn*I-*BamH*I) of pBluescript SK+. And HXGPRT expression cassette was inserted at the *Sac*II site (747 nt) of pBluescript SK+.

Nucleotide sequence of TUB8 promoter (515bp)

*Kpn*I
GGTACCGGGC CCCCCCTCGA CGGTTATCGA TAAGCTTAAC CACAAACCTT
GAGACGCGTG TTCCAACCAC GCACCCTGAC ACGCGTGTTC CAACCACGCA
CCCTGAGACA CATGTTCTAA CCACGCACCC TGAGACGCGT GTTCTAACCA
CGCACCCTGA GACGCGTGTT CAAGCTTGCC TGCATTGGGT GCGGTTGGTG
ATCCTGGTTG GACCGGTGGA GATGCGCGCG CACGAAGGGG ATGTGTCAGA
AACATTTTGT TTGTTCTCTG TGAACTTTTA GATGTGTTAA AGGAGGAGAA
TATTAGCAGA GAGTCCTCCT TGTTCCATTC TCTCTTGAAT TTCGCCCTTT
CCTTCTCTTT GCGAGTGTGG TAGAGAACAA GCACTCGTTC GCCGTCCCTG
ACGACGCAAC CCGCGCAGAA GATCCACCAA ACGGTGTTAC ACAATCACCT
TGTGTGAAGT TCTTGCGGAA AACTACTCGT TGGCATTTTT TCTTGAATTC
CCTTTTTCGA CAAAA

Nucleotide sequence of *ENO1* fragment (542bp)

ATGGTGGTTA TCAAGGACAT CGTTGCACGC GAGATTTTGG ATTCTCGCGG
CAATCCCACA ATTGAAGTTG ATGTATCAAC TGAAGGAGGT GTCTTCCGAG
CTGCGGTTCC TAGTGGAGCG TCTACGGGTA TTTATGAGGC TCTGGAGTTG
CGCGATAAAG ATCCGAAACG GTACTTGGGG AAAGGCGTGC TGAACGCTGT
AGAGATTGTC CGACAGGAAA TCAAGCCAGC ATTGCTGGGC AAAGATCCGT
GTGATCAGAA AGGTATTGAT ATGCTTATGG TGGAGCAGCT GGACGGAACA
AAAAACGAGT GGGGCTATTC CAAGTCAAAG CTAGGTGCAA ATGCCATTTT
GGGCGTGTCG ATAGCTTGCT GCCGAGCAGG AGCAGCATCA AAAGGCCTAC
CACTATACAA ATACATCGCT ACACTTGCAG GGAAAACGAT CGATAAGATG

GTTATGCCTG TACCGTTTTT CAATGTTATC AATGGAGGCG AACATGCAGG
CAATGGCTTG GCTCTTCAAG AATTCCTGAT CGCTCCCGTT GG

Nucleotide sequence of reverse TUB8 promoter (505bp)

TTTTGTCGAA AAAGGGAATT CAAGAAAAAA TGCCAACGAG TAGTTTTCCG
CAAGAACTTC ACACAAGGTG ATTGTGTAAC ACCGTTTGGT GGATCTTCTG
CGCGGGTTGC GTCGTCAGGG ACGGCGAACG AGTGCTTGTT CTCTACCAC
ACCGCAAAGA GAAGGAAAGG GCGAAATTCA AGAGAGAATG GAACAAGGAG
GACTCTCTGC TAATATTCTC CTCCTTTAAC ACATCTAAAA GTTCACAGAG
AACAAACAAA ATGTTTCTGA CACATCCCCT TCGTGCGCGC GCATCTCCAC
CGGTCCAACC AGGATCACCA ACCGCACCCA ATGCAGGCAA GCTTGAACAC
GCGTCTCAGG GTGCGTGGTT AGAACACGCG TCTCAGGGTG CGTGGTTAGA
ACATGTGTCT CAGGGTGCGT GGTTGGAACA CGCGTGTCAG GGTGCGTGGT
TGGAACACGC GTCTCAAGGT TTGTGGTTAA GCTTATCGAT AACCGTCGAC
GGATCC

*BamH*I

Nucleotide sequence of HXGPRT Cassette (1889 bp)

*Sac*II

CCGCGGAAGA TCCGATCTTG CTGCTGTTCG CGTCCCAGTA GCGTCCTGTC
GGCCGCGCCG TCTCTGTTGG TGGGCAGCCQ CTACACCTGT TATCTGACTG
CCGTGCGCGA AAATGACGCC ATTTTTGGGA AAATCGGGGA ACTTCATTCT
TTAAAAGTAT GCGGAGGTTT CCTTTTTCTT CTGTTCGTTT CTTTTTCTCG
GGTTTGATAA DCGTGTTCGA TGTAAGCACT TTCCGTCTCT CCTCCGTGCT
TTGTTCGACA TCGAGACCAG GTGTGCAGAT CCTTCGCTTG TCGATCCGGA
GACGCGTGTC TCGTAGAACC TTTTCATTTT ACCAACGGCA GTGCGGAGCA
CTGCTCTGAG TGCAGCAGGG ACGGGTGAAG TTTCGCTTTA GTAGTGCGTT
TCTGCTCTAC GGGGCGTTGT CAGATCCAAA ATGGCGTCCA AACCCATTGA
AGACTACGCC AAGGGCAACG GCCGTATTGA GCCCATGTAT ATCCCCGAGA
ACACCTTCTA GAACGCTCAT GACTTTCTTG TGCCCCCCCA CTGCAAGCCC
TACATTGACA AAATCCTCCT CCCTCGTGGA TTGGTCAAGG AGAGAGTTGA
GAAGTTGGCG TATGACATCC ACAGAACTTA GTTCGGCGAG GAGTTGCACA

TCATTTGCAT CCTGAAAGGC TCTCGCGGCT TCTTCAACCT TCTGATCGAG
TACCTTGCCA CCATACAGAA GTAGAATGGT CGTGAGTCCA GCGTGCCCCC
CTTCTTCGAG CACTATGTCC CCCTGAAGTC CTACCAGAAC GACAACAGCA
CAGGCCAGCT TACCGTCTTG AGCGACGACT TGTCAATCTT TCGCGACAAG
CACGTTCTGA TTGTTGAGGA GATCGTCGAG ACCGGTTTCA ACCTCACCGA
GTTCGGTGAG CGCCTGAAAG CCGTCGGTCC CAAGTCGATG AGAATCGCCA
CCCTCCTCGA GAAGCGCACA GATCGCTCCA ACAGCTTGAA GCCGGCCGAG
TTCGTCGGCT TCAGCATTGA AGAGGTCTGG ATCGTTGGTT GCTGCTACGA
CTTCAACGAG ATGTTCCGCG ACTTCGAGCA CGTCGCCGTC CTGAGCGACG
CCGCTCGCAA AAAGTTCGAG AAGTAAACCC TGCATAGCCC TACAGAAGCT
GCCCGTCTCT CGTTTTCCTC TCTTTTCGGA GGGATCAGGG AGAGTGCCTC
GGQTCGGAGA GAGCTGACGA GGGGGTGCCA GAGACCCCTG TGTCCTTTAT
CGAAGAAAAG GGATGACTCT TCATGTGGCA TTTCACACAG TCTCACCTCG
CCTTGTTTTC TTTTTGTCAA TCAGAACGAA AGCGAGTTGC GGGTGACGCA
GATGTGCGTG TATCCACTCG TGAATGCGTT ATCGTTCTGT ATGCCGCTAG
AGTGCTGGAC TGTTGCTGTC TGCCCACGAC AGCAGCCAAC TTTCCTTCTA
TGCACTTGCA GGATGGTGCA GCGCAAACGA CGGAGAGAAA GGAGCACCCT
CTCAGTTTCC CTACGATGTG CTGTCAGTTT CGACTCTTCA CCGCGAACGA
TTGGCGATAC GTCTCTGTTG ACTTGTTAGG CTCCGACCAC GAAGCTCCCT
TAACTAAATA AGCCGCGACA CCTAAGTGTA CACCATTTGC AGATCGATAA
TCTGCGACCG CTGAATCCGT CCAGATCAGT AAAACCGCAC CACCTAAGTG
TAAACCTTGT TTAGGTCGAT AAAATGCTAC CAACCCCCAC CCACAATCGA
GCCTTGAGCG TTTCTGCGCA CGCGTTGGCC TACGTGACTT GCTGATGCCT
GCCTCTGGCC ATTCATCCAG TCAGTGCGCA TAAAAATGTG GACACAGTCC
GGTTGACAAG TGTTCTGGCA GGCTACAGTG ACACCGCGG

*Sac*II

p(TUB8)$_2$*ENO2-HX*

Similar to p(TUB8)$_2$*ENO1-HX* except *ENO1* fragment replaced by *ENO2* fragment.

Nucleotide sequence of ENO2 fragment (542bp)

ATGGTGGCCA TCAAGGACAT CACTGCTCGT CAGATCCTCG ACTCCCGAGG
AAACCCGACC GTCGAGGTTG ACTTGTTGAC CGATGGCGGC TGCTTCCGTG
CCGCTGTCCC CAGCGGCGCA TCCACTGGCA TCTACGAGGC GCTTGAGCTC
CGTGACAAGG ACCAAACTAA GTTCATGGGC AAGGGTGTGA TGAAGGCCGT
GGAGAACATC CACAAGATTA TCAAGCCGGC GCTTATTGGC AAGGACCCGT
GCGACCAGAA GGGTATTGAC AAGCTGATGG TCGAGGAGCT CGATGGAACT
AAGAACGAGT GGGGCTGGTG CAAGTCGAAG CTCGGCGCGA ACGCGATCCT

GGCCGTCTCG ATGGCTTGCT GCCGCGCCGG CGCTGCTGCC AAGGGCATGC
CCCTGTACAA GTACATTGCC ACTTTGGCTG GAAACCCGAC AGACAAGATG
GTAATGCCCG TCCCGTTCTT CAACGTCATC AACGGCGGCT CCCACGCAGG
CAACAAGGTC GCGATGCAGG AGTTCATGAT CGCCCCCGTC GG

p(TUB8)$_2$GFP-*HX*

Similar to p(TUB8)$_2$*ENO1-HX* except *ENO1* fragment replaced by *GFP* fragment.

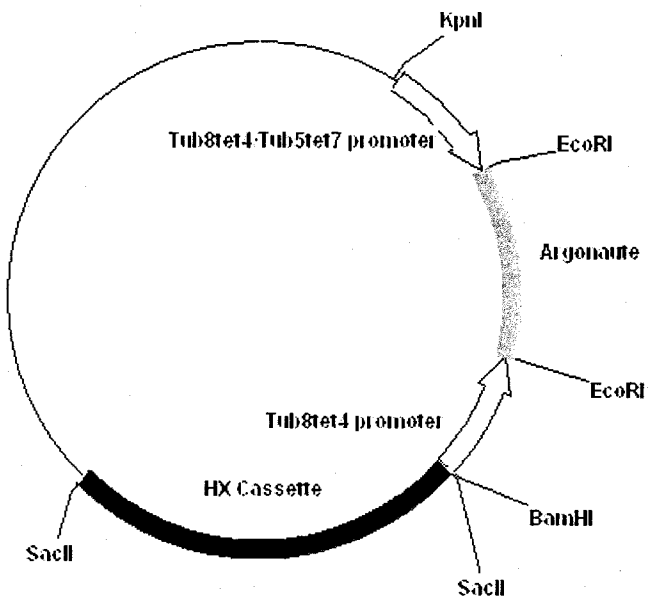
Nucleotide sequence of GFP fragment (533bp)

AGGAGAAGAA CTTTTCACTG GAGTTGTCCC AATTCTTGTT GAATTAGATG
GCGATGTTAA TGGGCAAAAA TTCTCTGTCA GTGGAGAGGG TGAAGGTGAT
GCAACATACG GAAAACTTAC CCTTAAATTT ATTTGCACTA CTGGGAAGCT
ACCTGTTCCA TGGCCAACAC TTGTCACTAC TTTCGCGTAT GGTCTTCAAT
GCTTTGCGAG ATACCCAGAT CATATGAAAC AGCATGACTT TTTCAAGAGT
GCCATGCCCG AAGGTTATGT ACAGGAAAGA ACTATATTTT ACAAAGATGA
CGGGAACTAC AAGACACGTG CTGAAGTCAA GTTTGAAGGT GATACCCTTG
TTAATAGAAT CGAGTTAAAA GGTATTGATT TTAAAGAAGA TGGAAACATT
CTTGGACACA AAATGGAATA CAACTATAAC TCACATAATG TATACATCAT
GGCAGACAAA CCAAAGAATG GAATCAAAGT TAACTTCAAA ATTAGACACA
ACATTAAAGA TGGAAGCGTT CAATTAGCAG ACC

Appendix IV

Nucleotide sequence of other constructed plasmids

All the constructed plasmids were generated from commercial plasmid pBluescript SK+. The primers for each DNA fragment are indicated by arrows.



pT8t4*Argo*T8t4-*HX*

The TUB8tet4 promoter as well as followed *Argonaute* DNA fragment and the opposite TUB8tet4 promoter were used to replace 653-719nt (*Kpn*I-*BamH*I) of pBluescript SK+. And HXGPRT expression cassette was inserted at the SacII site (747 nt)

of pBluescript SK+.

Nucleotide sequence of Tub8tet4 promoter (598 bp)

*Kpn*I
GGTACCGGGC CCCCCCTCGA CGGTATCGAT AAGCTTAACC ACAAACCTTG
AGACGCGTGT TCCAACCACG CACCCTGACA CGCGTGTTCC AACCACGCAC
CCTGAGACGC GTGTTCTAAC CACGCACCCT GAGACGCGTG TTCTAACCAC
GCACCCTGAG ACGCGTGTTC AAGCTTGCCT GCATTGGGTG CGGTTGGTGA
TCCTGGTTGG ACCGGTGGAG ATGCGCGCGC ACGAAGGGGA TGTGTCAGAT
CCCTATCAGT GATAGAGACT CGAGTCTATC ACTGATAGGG ATGTCGAGTC
TATCACTGAT AGGGATGTCG AGTCTATCAC TGATAGGGAT GTCGAGAACA
TTTTGTTTGT TCTCTGTGAA CTTTTAGATG TGTTAAAGGC GGCGAATATT
AGCAGAGAGT CCTCCTTGTT CCATTCTCTC TTGAATTTCG CCCTTTCCTT
CTCTTTGCGA GTGTGGTAGA GAACAAGCAC TCGTTCGCCG TCCCTGACGA
CGCAACCCGC GCAGAAGACA TCCACCAAAC GGTGTTACAC AATCACCTTG
TGTGAAGTTC TTGCGGAAAA CTACTCGTTG GCATTTTTTC TTGAATTC
*EcoR*I

Nucleotide sequence of Argonaute DNA fragment (861 bp)

CCCGCAGTAT TTCGCAAATG TTATGTCCAA AGTCAACATG AAGTTGCAAG
GAGTGAATCA AACCCTCGAG GCGGATATAA TAAAGCAAGA AATCGGAACG
GACAAGAGCA CATTGGTCTT GGCGGTAGAA ACATCATTCT TTGCAAATCC
GACTAAAACT TCTCCCCCGC CGACAGCGCC CATTGTGTGT GCGTGTACGG
GAAACATGGA CGATGATCTT GGCGCGTTCG GTCATGCTGT TTGCGTTGAA
TCGCGAAAGC ATCCCATAGT GACTGATATT GGGAGTATGT TTAAAACTAT
TCTCTCCTAT CGAAAGACAA CGAAAAACTG GCCGGCGCGA ATCATCTACC
TTCGATCTGC CACTACAGAA GCACACTTCC CTCTTGTATT GGCCGGCGAA
ATCCGTGCGA TCGAAGAATT ATATGTTCGT GAAAACCGTT CAAAACCTCG
AATCCTCGCC GTAGCAGTGC AAAGGCGACA GCAAACACGT TTATTCCCTA
CGAAGGAGAT GCAGGCTCAG GGAAACAATC TGCCACCAGG GTTCTTGCTT
GCGAATAGTC TGCAACATCC AGGGCACTTC CGAAATTTCT TGTTGATCTC
ACACAAAGCA CTCCAGGGAA CAGCGCGCCC AACAAGGTAT TACATTTTAA
GAGACGATGC GAACCGCGAC ATGGAAAAAG TTGCCCAGCT GATGTACTCT
CTGTGCCATG TGTACGGTCG CTGCCAGCGA GCGGTCTCCA TTCCCGCTCC
CCTGTACTAC GCCGAGCTGC TGGCGGCAAG AGCTCAAAGC TACATGAAGG
TTGGCATGCG TAGAGAAAGA AATATAGATA TTGACGATCT TTCTCACCTC

AGTGGCGAAG C

Nucleotide sequence of Reverse Tub8tet4 promoter (589 bp)

*Eco*RI
GAATTCAAGA AAAAATGCCA ACGAGTAGTT TTCCGCAAGA ACTTCACACA
AGGTGATTGT GTAACACCGT TTGGTGGATG TCTTCTGCGC GGGTTGCGTC
GTCAGGGACG GCGAACGAGT GCTTGTTCTC TACCACACTC GCAAAGAGAA
GGAAAGGGCG AAATTCAAGA GAGAATGGAA CAAGGAGGAC TCTCTGCTAA
TATTCGCCGC CTTTAACACA TCTAAAAGTT CACAGAGAAC AAACAAAATG
TTCTCGACAT CCCTATCAGT GATAGACTCG ACATCCCTAT CAGTGATAGA
CTCGACATCC CTATCAGTGA TAGACTCGAG TCTCTATCAC TGATAGGGAT
CTGACACATC CCCTTCGTGC GCGCGCATCT CCACCGGTCC AACCAGGATC
ACCAACCGCA CCCAATGCAG GCAAGCTTGA ACACGCGTCT CAGGGTGCGT
GGTTAGAACA CGCGTCTCAG GGTGCGTGGT TAGAACACGC GTCTCAGGGT
GCGTGGTTGG AACACGCGTG TCAGGGTGCG TGGTTGGAAC ACGCGTCTCA
AGGTTTGTGG TTAAGCTTAT CGATACCGTC GACGGATCC
*Bam*HI

pT5t7*Argo*T8t4-*HX*

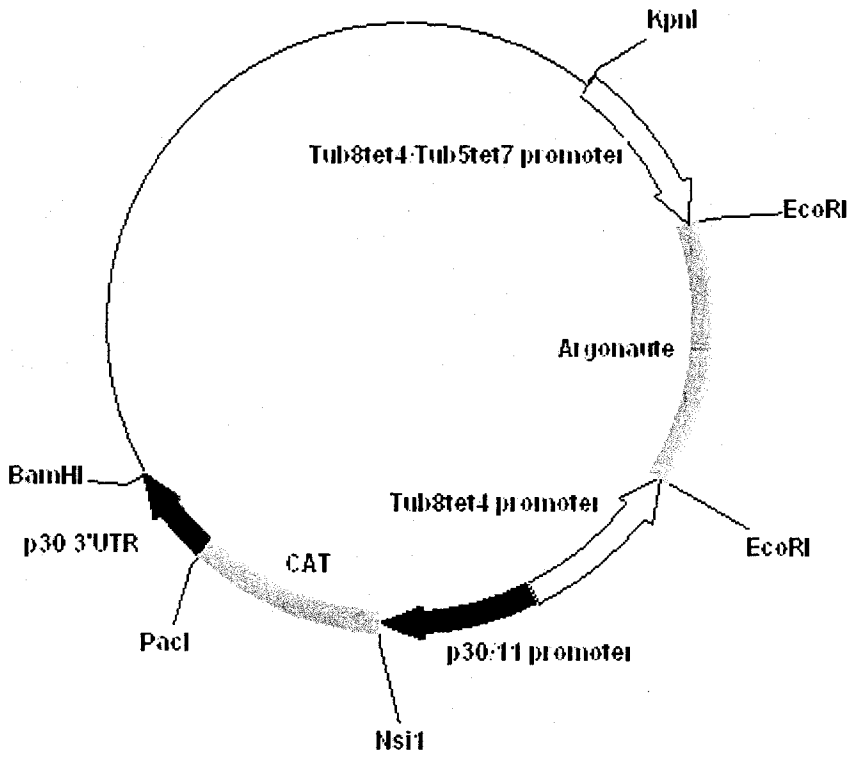
Similar to pT8t4*Argo*T8t4-*HX* except first Tub8tet4 promoter replaced by Tub5tet7 promoter.

Nucleotide sequence of Tub5tet7 (682bp)

*Kpn*I
GGTACCGGTC GACGGTATCG ATAAAGCTTG ATGGCGATGC ATGTCCCGCG
TTCGTGAAAT TCTCTGCATC AGCGGAGTGA TCAGGAATCA TCGTCTCAGC
GGGATGACGT TGCGGAGCAG GCCGGCTCGC GGTGGGCAGT CAGATGCCGA
AGGCGTAACT CAGGACGGCT TGCGCTCATC GCAGAACAGG GGTGGTGCCT
GCATTGGGTG CGGTTGGTGA TCCTGGTTGG ACCGGTGGAG ATGCGCGCGC
ACGAAGGGGA TGTGTCAGAT CCCTATCAGT GATAGAGACT CGAGTCTATC
ACTGATAGGG ATGTCGAGTC TATCACTGAT AGGGATGTCG AGTCTATCAC

TGATAGGGAT GTCGAGTCTA TCACTGATAG GGATGTCGAG TCTATCACTG
ATAGGGATGT CGAGTCTATC ACTGATAGGG ATGTCGAGAA CATTTTGTTT
GTTCTCTGTG AACTTTTAGA TGTGTTAAAG GCGGCGAATA TTAGCAGAGA
GTCCTCCTTG TTGGATTCTC TCTTGAATTT CGCCCTTTCC TTCTCTTTGC
GAGTCTCGTA GAGAACAAGC ACTCGTTCGC CGTCCCTGAC GACGCAACCC
GCGCAGAAGA CATCCACCAA ACGGTGTTAC ACAATCACCT TGTGTGAAGT
TCTTGCGGAA AACTACTCGT TGGCATTTTT TCTTGAATTC

*EcoR*I



pT8t4*Argo*T8t4-p30/11*CAT*

The TUB8tet4 promoter as well as followed *Argonaute* DNA fragment and the opposite

TUB8tet4 promoter were placed next to *Kpn*I (653 nt) of pBluescript SK+. And CAT expression cassette (p30/11 promoter + *CAT* gene + p30 3'UTR) was inserted before *BamH*I site (719 nt) of pBluescript SK+.

Nucleotide sequence of p30/11 promoter (518bp)

CTCGAGGTCG ACGGTATCGA TAAGCTTAAC CACAAACCTT GAGACGCGTG
TTCCAACCAC GCACCCTGAC ACGCGTGTTC CAACCACGCA CCCTGAGACG
CGTGTTCTAA CCACGCACCC TGAGACGCGT GTTCTAACCA CGCACCCTGA
GACGCGTGTT CAAGCTTAAC CACAAACCTT GAGACGCGTG TTCCAACCAC
GCACCCTGAC ACGCGTGTTC CAACCACGCA CCCTGAGACG CGTGTTCTAA
CCACGCACCC TGAGACGCGT GTTCTAACCA CGCACCCTGA GACGCGTGTT
CTGCCGCACA ATGTGCECCT GTAGGAAGCT GTAGTCACTG CTGATTCTCA
CTGTTCTCGG CAAGGGCCGA CGACCGGAGT ACAGTTTTTG TGGGCAGAGC
CGTTGTGCAG CTTTCCGTTC TTCTCGGTTG TGTCACATGT GTCATTGTCG
TGTAAACACA CGGTTGTATG TCGGTTTCGC TGCACCACTT CATTATTTCT
TCTGGTTTTT TGACGAGT

Nucleotide sequence of CAT gene (697bp)

*Nsi*I

ATGCATGAGA AAAAAATCAC TGGATATACC ACCGTTOATA TATCCCAATC
CCATCGTAAA GAACATTTTG AGGCATTTCA GTCAGTTGCT CAATGTACCT
ATAACCAGAC CGTTCAGCTC GATATTACGG CCTTTTTAAA GACCGTAAAG
AAAAATAAGC ACAAGTTTTA TCCGGCCTTT ATTCACATTC TTGCCCGCCT
CATGAATGCT CATCCGGAAT TCCGTATGGC AATGAAAGAC GGTGAGCTGG
TGATATGGGA TAGTGTTCAC CCTTGTTACA CCGTTTTCCA TGAGCAAACT
GAAACGTTTT CATCGCTCTG GAGTGAATAC CACGACGATT TCCGGCAGTT
TCTACACATA TATTCGCAAG ATGTGGCGTO TTACGGTGAA AACCTGGCCT
ATTTCCCTAA AGGGTTTATT GAGAATATGT TTTTCGTCTC AGCCAATCCC
TGGGTGAGTT TCACCAGTTT TGATTTAAAC GTGGCCAATA TGGACAACTT
CTTCGCCCCC GTTTTCACCA TGGGCAAATA TTATACGCAA GGCGACAAGG

TGCTGATGCC GCTGGCGATT CAGGTTCATC ATGCCGTCTG TGATGGCTTC CATGTCGGCA GAATGCTTAA TGAATTACAA CAGTACTGCG ATGAGTGGCA GGGCGGGGCT TAATTAA

*Pac*I

Nucleotide sequence of p30 3'UTR (317)

TCACCGTTGT GCTCACTTCT CAAATCGACA AAGGAAACAC ACTTCGTGCA GCATGTGCCC CATTATAAAG AAACTGAGTT GTTCCGCTGT GGCTTGCAGG TGTCACATCC ACAAAAACCG GCCGACTCTA AATAGGAGTG TTTCGCAGCA AGCAGCGAAG TTTATGACTG GGTCCGAATC TCTGAACGGA TGTGTGGCGG ACCTGGCTGA TGTTGATCGC CGTCGACACA CGCGCCACAT GGGTCAATAC ACAAGACAGC TATCAGTTGT TTTAGTCGAA CCGGTTAACA CAATTCTTGC CCCCCCGAGG GGGATCC

BamH

pT5t7*Argo*T8t4-p30/11*CAT*

Similar to pT8t4*Argo*T8t4-p30/11*CAT* except first Tub8tet4 promoter replaced by Tub5tet7 promoter.

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