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Symposium mini review

Functional Perspective of Feeder Organelle from Three-dimensional Ultrastructural Characteristics in *Cryptosporidium parvum*

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Keywords
Cryptosporidium, feeder organelle, osmium maceration, SEM, tubuloreticular formation

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Abstract

Cryptosporidium is a parasite causing extensive illness both in livestock and humans. Feeder organelle of *Cryptosporidium* is the multi-membranous structures localized on the parasite-host-cell interface that deprives nutrients from host cells. Although the feeder organelle has been summarized as a highly invaginated membranous structure, the three-dimensional fine structure remains unclear. Osmium-maceration procedure for scanning electron microscopy (OS-SEM) is one of the methods to enable visualization of the intracellular ultrastructure including depth direction information after removing soluble proteins. Recently, we investigated and assessed *C. parvum* possessed on the surface of ileal epithelial cells of mice by using transmission electron microscopy (TEM) and OS-SEM. By TEM observation, feeder organelles were recognized as aggregated structures of concentrically-, vertically- and randomly-lined bars. Correspondingly, OS-SEM observation revealed the reticulated network of stacked flat bursiform, ring-shaped bursiform and reticulated tubular membranes. These findings of the three-dimensional ultrastructural characteristics of feeder organelle, which are more intricate than expected, may potentially reinforce the limited knowledge regarding the nature of this attachment interface and the functional mechanisms around extraction of nutrients.

Introduction

Cryptosporidium is a parasite responsible for highly contagious, and cryptosporidiosis in humans and animals (Ryan, Zahedi, & Paparini, 2016). Transmission of *Cryptosporidium* is most often by the fecal-oral route via contaminated water, food or fomites, or by direct ingestion of infected feces, resulting in the intestinal diarrhea (Yoshida *et al*., 2007). Moreover, *C. parvum* is relatively resistant to chlorine at the levels used in potable water. Therefore, cryptosporidiosis infection occurs as a waterborne outbreak with the potential to affect many people at once (Efstratiou, Ongerth, & Karanis, 2017; Mac Kenzie *et al*., 1994; Yamamoto *et al*., 2000). There is no effective medical treatment for either intestinal or biliary cryptosporidiosis, and in AIDS patients, the infection is rarely spontaneously cleared (Chen *et al*., 1998; Theodos, Griffiths, D'Onfro, Fairfield, & Tzipori, 1998). In recent years, *Cryptosporidium* is a life-threatening opportunistic pathogen for children in developing countries containing Africa and Asia, who urgently need specific anti-cryptosporidial therapies (Elfadaly, Hassanain, Hassanain, Barakat, & Shaapan, 2018; HMG *et al*., 2018; Kotloff *et al*.,

2013; Liu *et al*., 2016; Platts-Mills *et al*., 2015). Thus, the infection mechanism of *Cryptosporidium* should be elucidated for the development of effective precaution or treatment of cryptosporidiosis (Ryan *et al*., 2016; Vanathy, Parija, Mandal, Hamide, & Krishnamurthy, 2017).

The infection mechanism of *cryptosporidium* to host cell

Life cycle of *Cryptosporidium* is completed in a single host, and infective sporozoites attach to and invade gastrointestinal epithelial cells to form a unique parasitophorous vacuole on top of the cells (O'Hara & Chen, 2011). The main site of contact between the maturing parasite and the host cell is an extensively folded membrane structure, called the feeder organelle (Zapata, Perkins, Riojas, Wu, & Le Blancq, 2002). The feeder organelle is peculiar and still largely uncharacterized structures (Sharling *et al*., 2010). The feeder organelle is considered as the site at which nutrient uptake from the host cell cytoplasm occurs (Clode, Koh, & Thompson, 2015; Marcial & Madara, 1986; O'Donoghue, 1995). A *Cryptosporidium*-specific ATP-binding cassette, CpABC1, involved in transportation of various molecules (e.g.: metabolites and lipids) across membranes is localized to feeder organelles, indicating that these organelles are important for selective nutrient absorption (Perkins, Riojas, Wu, & Le Blancq, 1999; Zapata *et al*., 2002). The ultrastructure of feeder organelles in *Cryptosporidium* has been examined using transmission electron microscopy (TEM) in murine models of infection and in livestock, and it has been described as "highly invaginated" (Al-Mathal & Alsalem, 2013; O'Hara & Chen, 2011; Pohlenz, Bemrick, Moon, & Cheville, 1978; Rosales, Arnedo, & Mascaró, 1998; Umemiya, Fukuda, Fujisaki, & Matsui, 2005; Valigurová, Hofmannová, Koudela, & Vávra, 2007). The feeder organelle structure was also seen in replicas as multiple membrane facets (Marcial & Madara, 1986). However, how they intake and transport molecules at an organelle level from the host cells remain uncertain.

Osmium-maceration for scanning electron microscopy to visualize 3D ultrastructures of feeder organelles

In contrast to TEM intracellular analysis, scanning electron microscopy (SEM) had been only used to analyze the three-dimensional (3D) surface structure of *Cryptosporidium* (Chen *et al*., 1998; Pohlenz *et al*., 1978; Umemiya *et al*., 2005). The osmium-maceration procedure for SEM (OS-SEM) developed by Tanaka *et al.* in 1981 enables us to make a direct observation the intracellular endomembranous organelles by removing the cytoplasmic soluble proteins selectively from the cracked surface of the cells with diluted OsO_4 solution (Hanaki, Tanaka, & Kashima, 1985; Stowe, Fukudome, & Tanaka, 1986; Tanaka & Mitsushima, 1984; Tanaka & Naguro, 1981). OS-SEM is a very useful method that enables comparative observation with TEM. Recently, we introduced this excellent method, OS-SEM, to visualize the intracellular membranous organelles, especially feeder organelles in *Cryptosporidium* (Bochimoto, Kondoh, Ishihara, Kabir, & Kato, 2019). *Cryptosporidium* oocyst remained on the surface of terminal ileum even after osmium maceration with 0.1% diluted OsO_4 (**Fig. 1A**). Moreover, we confirmed that the present OS-SEM clearly visualizes the intracellular structures of the parasite organelles including endoplasmic reticulum and some types of granules (**Fig. 1B and C**). By utilizing comparative observation of OS-SEM and TEM, we visualize the 3D ultrastructure of feeder organelle possessing three different components at the host-parasite interface: reticulated networks of stacked "dome-shaped" bursiform membranes (concentrically-spread type; cFO): networks of "ring-shaped" bursiform membranes concentrically surrounding "dome-shaped" membranes (vertically-lined type; vFO): reticulated "tube-shaped" membranes (randomly-scattered type; rFO, referred **Fig. 1C**) (Bochimoto *et al*., 2019).

Discussion and future perspectives

Feeder organelles of *Cryptosporidium* have been considered as an invaginated membrane structure that functions to secure a large surface area (Fayer, 2008). However our visualization of the 3D ultrastructural characteristics of feeder organelle indicated that feeder organelles are more intricate and organized than was previously thought (Bochimoto *et al*., 2019). Particularly, randomly tubule-reticular formation of rFO has an similarity to membrane traffic-associated organelles including trans-Golgi network and transitional ER (Hammond & Glick, 2000; Liendo & Joiner, 2000; Pelletier *et al*., 2002). This finding makes us presume that the rFO has specific functions of membrane trafficking of absorbed nutrients (**Fig.1 D**). In the future, the localization of the *Cryptosporidium*-specific transporter, like CpABC1, on each type of the feeder organelle components should be investigated to more clarify the mechanism of transport the nutrients. One of our concerns is that successful parasitism by *Cryptosporidium* needs intricate interactions between the host and the parasite (O'Hara & Chen, 2011). OS-SEM enables to visualize the intracellular 3D ultrastructure in not only *Cryptosporidium* but also the host cells (**Fig. 1C**), which may deal with this concern by comparative observation with TEM. Another concern is that the ultrastructural variation of feeder organelle components might depend on the life cycle stages of *Cryptosporidium*. Monoclonal antibodies, which recognize novel epitopes that could be recently used to define intracellular development (Wilke *et al*., 2018), may resolve this concern. Regarding the structural nature of individual stages, cell attachment and invasion processes, the future lies in 3D imaging as indicated by Clode *et al.* (2015). Ultrahigh-resolution 3D imaging methods could be adopted to ATUMTome sectioning and serial imaging by SEM, block-face serial imaging by SEM, focused ion beam SEM (FIBSEM) and electron tomography by TEM. In addition of these methods, the "long ignored" OS-SEM method is a very powerful tool of analysis of intracellular ultrastructure associated with infection of *Cryptosporidium*.

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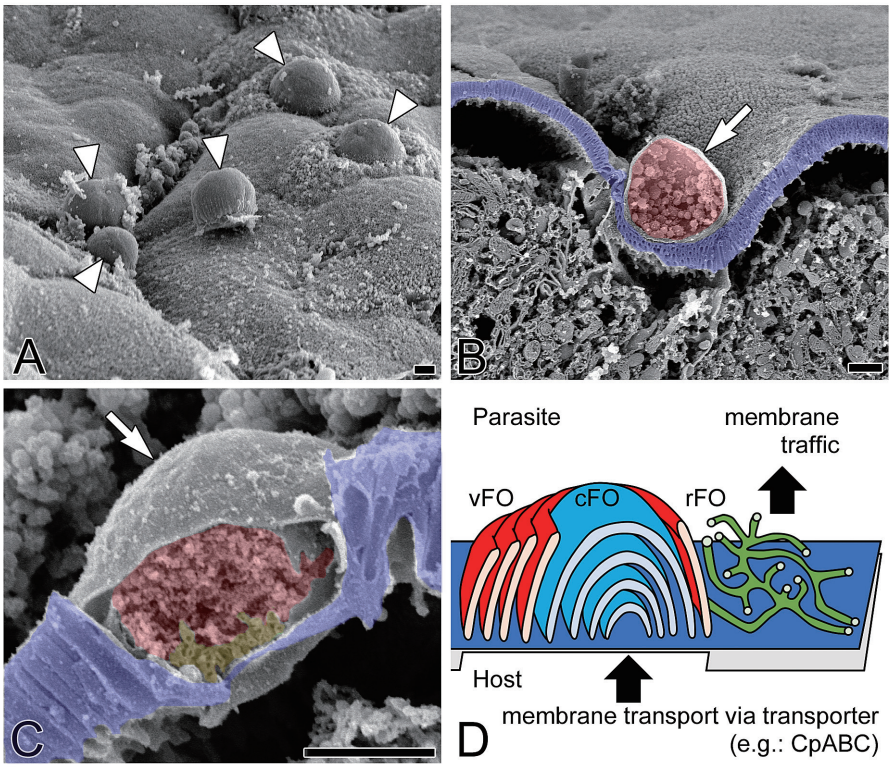

Fig. 1. Osmium-maceration scanning electron microscopic (OS-SEM) images of *Cryptosporidium parvum* attached to mouse ileal cells. [A] Surface of ileal epithelium. Arrowheads indicate outer surfaces images of parasites. [B] Higher magnified image of cross-section of ileal epithelial surface. An arrow indicates the oocyst. Intracellular structures of *Cryptosporidium* are colored red. Microvilli of host cells are colored blue. [C] OS-SEM images of feeder organelle. An arrow indicates the oocyst. Intracellular area and randomly-scattered type feeder organelle (rFO) of *Cryptosporidium* are colored red and green, respectively. Microvilli of host cells are colored blue. [D] Schematic illustration of three-dimensional ultrastructure of feeder organelle. cFO, concentrically-spread type feeder organelle; vFO, vertically-lined type feeder organelle. Bars=1 μm.

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Dedication

We dedicate this paper to the memory of the late Professor Keiichi Tanaka M.D., Ph.D. (1926-2019), who pioneered the field of biological SEM.

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Symposium mini review

Panning-based Virus Receptor Screening with Cellular cDNA Library

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Keywords
virus receptor, panning, screening, cDNA library, retroviral/lentiviral vectors, virus-like particle

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Abstract

Identification of virus receptors has been performed by various methods. We have developed panning-based, efficient cellular cDNA library screening methods to identify virus receptors. The principles of the methods are reviewed in this article.

Introduction

Among different organisms, many similar but non-identical cellular molecules exist that show the same functions. Even in a single species, the expressed molecules are dependent on cell types. This is the case with virus receptors, which are cellular components involved in viral attachment to and invasion into cells for viral replication [1, 2]. Thus, viral infections show specificity with regards to host ranges, tissues, and cell types, indicating that the identification of virus receptors is useful for the molecular explanation of the viral tropisms [3]. The identification of virus receptor has been performed by various methods, which are roughly classified into three categories: (I) speculation based on knowledge obtained from experiments [4-7]; (II) screening of libraries based on gain-of-function or loss-of-function criteria [8-10]; and (III) identification of interactive cellular molecules by peptide sequencing or mass spectrometry [11-13].

We have established an efficient, low-cost method for identification of cell surface molecules recognized by antibodies [14], in which cellular cDNA libraries were screened by a technique called “panning”. “Panning” is a classical experimental technique for separation of a specific subpopulation from a mix population [15] but can be applied to various fields with a variety of modifications. In the review, I would like to introduce our application of the panning to identify virus receptors, which belong to the category II described above with gain-of-function criteria.

1st generation panning for virus receptor identification

We have developed an efficient, low-cost cellular cDNA library screening method using a classical panning to identify virus receptors (hereafter referred to as 1st generation panning for virus receptor identification or simply 1st generation panning) [16-18]. In 1st generation panning, cellular cDNA libraries are stably expressed in non-adherent cell lines by using a retroviral vector, which integrates genes into cellular genomes with no or low cytotoxicity and do not inhibit cell growth, and screened based on colony formation of target cells on viral particle-coated dishes. From genome of colony-forming cells, cDNAs encoding molecules which confer adhesiveness onto viral particles to non-adherent cells are recovered by polymerase chain reaction (PCR) with primers targeting retroviral vector sequences flanking to cDNA library. Therefore, interaction between viral particles and surface molecules of target cells that is strong enough to trap target cells onto viral particle-coated dishes is a prerequisite for the method. The method was also applied to identify a receptor of a protozoa *Plasmodium falciparum* [19].

2nd generation panning for virus receptor identification

For viruses for which the interaction with target cells is not strong, we have developed another efficient screening method to identify virus receptors (hereafter referred to as 2nd generation panning for virus receptor identification or simply 2nd generation panning) [20, 21]. In 2nd generation panning, same as in 1st generation panning, cellular cDNA libraries are stably expressed in non-adherent cell lines by using a retroviral vector, but two additional non- or low-cytotoxic retroviral/lentiviral vectors which bear intended viral envelope proteins and whose genome encode either a membrane spanning protein or a fluorescence protein as a reporter are used for screening. Upon inoculation of library-expressing cells with a first screening vector encoding a membrane spanning protein, target cells would be specifically infected with the vector, express the reporter stably on cell surface, and form colonies

on anti-reporter antibody-coated dishes during cell culture. Because infrequent infection of non-target cells with the screening vectors would occur and result in colony formation as authentic target cells, resultant colonies are superinfected with a second screening vector whose genome encode a fluorescence protein to eliminate false positive. Fluorescent colonies are found out under a fluorescence microscope and inserted cDNA recovered by PCR like with 1st generation panning. The ability to produce moderate to high titers of retroviral/lentiviral vectors bearing intended viral envelope proteins is a perquisite for 2nd generation panning.

3rd generation panning for virus receptor identification

We have experienced cases in which some viruses did not meet the prerequisites for the above described two methods and for which virus receptors could not be identified by our methods. One of such examples was severe fever with thrombocytopenia syndrome (SFTS) virus [22]. In the current taxonomy SFTS virus is classified as follows: species, *Dabie bandavirus*; genus, *Bandavirus*; family, *Phenuiviridae*, and order, *Bunyavirales* (https://talk.ictvonline.org/taxonomy/). In infection of SFTS virus, non-evident cytopathic effects are characteristically observed in *in vitro* short cell culture [23-26]. The genome of the genus members is composed of three negative sense RNAs of large (L), middle (M), and small (S) segments, which encode viral proteins (RNA-dependent RNA polymerase, glycoprotein [GP], and nuclear and non-structural proteins, respectively). The rescue of SFTS virus with or without mutations from cDNA (reverse genetics) has been reported [27]; in that study, five plasmids expressing three anti-genome RNAs and two viral proteins (RNA-dependent RNA polymerase and nuclear protein) were used. As an application of the reverse genetics, a virus-like particle (VLP) assay was recently reported to assess the reassortment potential of SFTS virus with its related viruses [28]. By combining our 2nd generation panning for virus receptor identification [20, 21] and the reverse genetics for SFTS virus [27, 28], we recently succeeded in performing screening of SFTS virus receptors with a cellular cDNA library [22]. In the screening, infectious VLP (iVLP), which has most of SFTS virus components but is replication-incompetent due to the replacement of GP gene with a reporter gene for selection, is used like retroviral/lentiviral vector in 2nd generation panning for virus receptor identification [22]. Similar strategies might be applicable to SFTS virus-related viruses such as Heartland virus, Uukuniemi virus, and other viruses, if a prerequisite that iVLP produced shows moderate to high titers and shows no or weak cytopathic effects upon infection is fulfilled.

Conclusion

In this review, we introduced our efficient methods for identification of virus receptor. We hope the review will be useful for various viruses, while the methods are still being improved with some modifications.

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Symposium mini review

Protein Trafficking in *Plasmodium falciparum*-infected Erythrocytes

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Keywords
erythrocyte, malaria, Maurer's clefts,
Plasmodium falciparum, protein trafficking

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Abstract

Malaria remains one of the world's most important infectious diseases. Malaria parasites make modifications to host erythrocytes that are essential to their survival and pathogenesis and are facilitated by parasite proteins exported to the host cytoplasm. These exported proteins form a functional trafficking complex in the host cytoplasm to transport virulence determinants to the erythrocyte surface; this complex, termed the Maurer's cleft, is thus essential for malaria virulence. Some of these exported proteins form a large protein complex that leads to profound structural and morphological changes in the host erythrocytes. In this report, I review the exported proteins in *Plasmodium falciparum*-infected erythrocytes. Because these proteins are closely linked to malaria pathogenesis, the information provided herein is important for our understanding of the molecular mechanisms involved in the pathogenesis of *P. falciparum* infection.

Introduction

Malaria remains one of the world's most important infectious diseases, affecting approximately 200 million people worldwide annually (WHO, 2018). When *Plasmodium falciparum*, one of the most virulent forms of the human malaria parasite, establishes infection in host erythrocytes, the parasites export numerous proteins to the host cell's cytoplasm and plasma membrane to remodel the host cell (Hiller *et al*., 2004; Maier *et al*., 2009). Some of these exported proteins form a large protein complex that leads to profound structural and morphological changes in the host erythrocytes (LaCount *et al*., 2005); for example, Maurer's clefts (Lanzer *et al*., 2006) and knobs (Wickham *et al*., 2001) are established in the cytoplasm and on the surface of the erythrocytes, respectively. Consequently, the infected erythrocytes become more rigid and adhere to the vascular endothelium, which prevents their clearance by the spleen and subsequently disrupts normal blood flow, resulting in severe malaria in humans (De Niz *et al*., 2016; Maier *et al*., 2008). This mini review discusses the exported proteins that are transported to the erythrocyte surface, including those associated with parasite adherence to the erythrocyte surface and severe malaria pathogenesis.

Exported proteins in *P. falciparum*-infected erythrocytes

The adherence of infected erythrocytes to the vascular endothelium is mediated by interactions between parasite adhesins on the erythrocyte surface and host endothelial receptors (Fairhurst *et al*., 2005; Janes *et al*. 2011; Waller *et al*., 2003). *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is an antigenically variant adhesin that is transported to knobs on the erythrocyte surface (Waller *et al*., 1999). Knobs are macromolecular complexes of knob-associated histidine-rich protein (KAHRP) that anchor PfEMP1 to the membrane skeleton (Oh *et al*., 2000; Waller *et al*., 1999). Maurer's clefts are involved in the trafficking of PfEMP1 to the erythrocyte surface (Lanzer *et al*., 2006; Maier *et al*., 2008; Wickham *et al*., 2001). Many exported proteins, represented by skeleton binding protein 1 (SBP1) (Cooke *et al*., 2006; Maier *et al*., 2007), membrane-associated histidine-rich protein 1 (MAHRP1) (Spycher *et al*., 2008), ring-exported protein 1 (REX1) (Hanssen *et al*., 2008), subtelomeric variant open reading frame (STEVOR) (Przyborski *et al*., 2005), and PfEMP1 trafficking protein 1 and 5 (PTP1 and PTP5) (Maier *et al*., 2008; Rug *et al*., 2014) have been shown to reside in Maurer's clefts. Some of these exported proteins are essential for the intracellular transport of PfEMP1 to the erythrocyte surface, suggesting that they form a large protein complex in

the Maurer's clefts that serves as protein trafficking machinery to transport exported proteins to their final destination (Rug *et al*., 2014). However, essential information regarding the interactions between these exported proteins is lacking, because of the technical difficulties of studying protein-protein interactions in the cytoplasm of *P. falciparum*- infected erythrocytes (Batinovic *et al*., 2017; Rug *et al*., 2014).

Motif sequences of exported proteins

The *P. falciparum* exportome was predicted to comprise approximately 400 proteins on the basis of the discovery of a motif sequence called PEXEL (*P. falciparum* exported elements) or HT (host-targeting sequence), which is conserved in the N-terminal region of many exported proteins (Hiller *et al*., 2004; Marti *et al*., 2004). However, many proteins that lack the canonical PEXEL/HT motif have been shown to be efficiently exported to the host cytoplasm (Heiber *et al*., 2013), thereby complicating the identification of the exported proteins that comprise the *P. falciparum* exportome. These PEXEL-negative exported proteins (PNEPs) include SBP1, MAHRP1, and REX1, all of which are indispensable for malaria virulence (Cooke *et al*., 2006; Hanssen *et al*., 2008; Maier *et al*., 2007; Spycher *et al*., 2008). Given the difficulty to predict and identify PNEPs on the basis of protein sequences, an alternative approach is needed to directly identify PNEPs based on their protein-protein interactions in the host cytoplasm.

P. falciparum orthologues of exported proteins in *P. berghei*

P. falciparum orthologues of SBP1 and MAHRP1 were discovered in the rodent malaria *P. berghei* by De Niz *et al.* (De Niz *et al*., 2016) and disruption of these genes resulted in the decreased cytoadherence of *P. falciparum*-infected RBCs (iRBCs) to CD36 in a mouse model, suggesting that these genes are likely involved in the transport of an unidentified parasite ligand that allows binding of iRBCs to the vascular endothelium, similar to *P. falciparum* SBP1 and MAHRP1 (Cooke *et al*., 2006; Maier *et al*., 2007; Spycher *et al*., 2008).

Tryptophan-threonine-rich antigen

A tryptophan-threonine-rich antigen (termed TryThrA) was identified as a parasite protein that associates with SBP1 in the trafficking complex (Takano *et al*., 2019). TryThrA was previously characterized as a protein expressed on the merozoite surface that is involved in parasite invasion, a role that is supported by the inhibitory effect of synthetic peptides of TryThrA antigen on merozoite invasion of erythrocytes (Curtidor *et al*., 2006). My group found that TryThrA is expressed across the asexual cycle and localizes in Maurer's clefts. Moreover, my group demonstrated that its gene could be genetically disrupted without affecting parasite invasion, which is inconsistent with previous studies (Alam *et al*., 2015; Curtidor *et al*., 2006). This discrepancy could be explained by off-target effects of the synthetic peptides, or by alternative expression of molecules that could compensate for the loss of TryThrA in our knockout parasites. Further studies are warranted to elucidate the precise function of TryThrA in infected erythrocytes.

Membrane palmitoylated protein 1

Host-parasite protein interactions play an essential role in malaria progression and pathogenesis (Egan *et al*., 2015; Miller *et al*., 2002; Olszewski *et al*., 2009). My group has identified several host-parasite protein interactions in the host cytoplasm (Takano *et al*., 2019). Among three of the host factors my group identified (STOM, KPNB1, and MPP1), my group found that MPP1, a membrane palmitoylated protein 1 (also termed p55, 55 kDa erythrocyte membrane protein) was recruited into the Maurer's clefts (Takano *et al*., 2019). MPP1 is a member of the membrane-associated guanylate kinase (MAGUK) family and plays essential roles in the membrane organization of erythroid cells, composition of lipid rafts on erythrocyte membranes, and erythrocytopoiesis (Biernatowska *et al*., 2017; Egan *et al*., 2015; Lach *et al*., 2012; Quinn *et al*., 2009). Moreover, a previous proteomic study of microvesicles, which are secreted from the surface of *P. falciparum*-infected erythrocytes and likely bud from Maurer's clefts (Mantel *et al*., 2013), identified the presence of this protein. MPP1, therefore, likely contributes to the organization of the membranous structures of Maurer's clefts.

Plasmodium helical interspersed subtelomeric family

By using a series of knockout experiments and cytoadherence assays with potential SBP1-interacting proteins, my group identified *MAL8P1.4* as being involved in the cytoadherence of iRBCs to vascular endothelial receptors (Takano *et al*., 2019). MAL8P1.4 is a member of the *Plasmodium* helical interspersed subtelomeric (*PHIST*) family of exported proteins, which play diverse roles in parasite-infected erythrocytes (Kumar *et al*., 2018; Oberli *et al*., 2014; Oberli *et al*., 2016; Proellocks *et al*., 2014). Although the function of *PHIST* genes has not yet been fully elucidated, previous studies have revealed that specific PHIST proteins can bind to the acidic C-terminal (ATS) domain of PfEMP1, and that the depletion of genes that encode PHIST proteins results in decreased cytoadherence (Oberli *et al*., 2016; Proellocks *et al*., 2014). Moreover, the binding capacity of a PHIST protein differs for each PfEMP1 depending on the sequence of its ATS domain (Kumar *et al*., 2018; Oberli *et al*., 2016), suggesting that *PHIST* genes might have coevolved with specific ATS domains to create interaction pairs with maximum binding strength to transport a specific PfEMP1 to the erythrocyte membrane (Kumar *et al*., 2018; Oberli *et al*., 2014; Proellocks *et al*., 2014). In contrast, MAL8P1.4 does not localize to the surface of erythrocytes or a knob, and has a low binding affinity for the ATS domain of specific PfEMP1s (Oberli *et al*., 2014). Although there are many unknowns regarding the function of MAL8P1.4, given that multiple PHIST proteins are cooperatively and selectively involved in the transport of specific PfEMP1s to the erythrocyte surface (Oberli *et al*., 2016), the altered cytoadherence by *ΔMAL8P1.4* parasites may be due to the alternation of the PfEMP1 being transported and presented on the erythrocyte surface.

Conclusions

This mini review describes the functions of exported proteins in *P. falciparum*-infected erythrocytes, some of which can cause the onset of severe malaria. The information presented furthers our understanding of the molecular mechanisms for the pathogenesis of *P. falciparum* infections.

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Symposium mini review

How Does Wood-inhabiting Fungal Community Affect Forest Recovery after Deforestation Events in Subalpine Coniferous Forest?

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Keywords
dead wood, decomposition, deforestation, forest regeneration, fungi

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Abstract

Typhoon disturbance is causing large impacts on subalpine forests in Far East Asia. The decay of dead wood generated by deforestation event is important for seedling regeneration. Moreover, wood decay type, traditionally categorized into white-rot and brown-rot reflecting the decay preference for wood structural components by fungal decomposers, determines successful colonization of the seedlings on dead wood. In this article, I did a brief review of current knowledge of the effects of fungal decomposition of wood on tree seedling establishment, and discussed a possible option for forest management to stimulate forest recovery after the disturbance from mycological point of view, by comparing two case studies took place in subalpine coniferous forests dominated by *Picea jezoensis* var. *hondoensis* (Hondo spruce).

Introduction

Typhoon disturbance is causing large impacts on subalpine forests in Far East Asia (Ida, 2000; Suzuki *et al*., 2013; Guo *et al*., 2015). While blowdown disturbance have direct, short-term effects on forest dynamics, microbial communities in the ground have indirect and usually gradual—but equally pivotal—effects on the dynamics of aboveground vegetation, of which little is known (Bardgett & Wardle, 2010).

The decay of dead wood lying on the ground is important for seedling regeneration in spruce forests with developed canopies (Bače *et al*., 2012; Ando *et al*., 2017; Tsvetanov *et al*., 2018) because the majority of seedlings that germinate on shaded ground will be killed by pathogenic soil fungi within a couple of years; only seedlings on logs (or stumps), where the density of pathogens present is smaller, can survive (Cheng & Igarashi, 1987; Mori *et al*., 2004). The wood decay activity of the fungal communities that deadwood harbours has significant effects on seedling performance and density (Bače *et al*., 2012; Fukasawa, 2012, 2016, 2018; Fukasawa & Komagata, 2018; Fukasawa *et al*., 2017). The density of spruce seedlings on deadwood is negatively affected by brown-rot fungi, which decay wood holocellulose selectively and modify lignin only slightly, probably due to the acidity or fragility of brown-rotted wood (Bače *et al*., 2012). In contrast, white-rot fungi decay wood holocellulose and lignin simultaneously or lignin selectively, and have positive effects on seedling density (Bače *et al*., 2012; Ando *et al*., 2017). Such differences between wood decay types of fungi are critical determinants of complex biotic interactions on deadwood associated with seedling regeneration (Fukasawa *et al*., 2015; Ando *et al*., 2017; Fukasawa & Ando, 2018).

In this mini-review, I discussed a possible option for forest management to stimulate forest recovery after disturbance by comparing two case studies in subalpine coniferous forest dominated by *Picea jezoensis* var. *hondoensis* (Hondo spruce) from mycological point of view. Both forest sites were severely damaged by a super typhoon (Category 5), named Vera or the Isewan typhoon in September 1959: one forest has recovered whereas the other has not recovered yet.

Case study 1: Forest RECOVERED after the past disturbance

Fukasawa *et al*. (2019a) evaluate the long lasting impact of the typhoon disturbance on communities of wood-inhabiting fungi, bryophyte, wood decay and tree seedling establishment on fallen logs of Hondo spruce in Yatsugatake Mountains, central Japan (36˚00'N, 138˚23'E, ca. 2200 m a.s.l.). The mean annual temperature is 2.1 ˚C and the mean annual precipitation is 1567 mm (Japan Meteorological Agency, 1981–2010 average). Before the typhoon disturbance, forest in this area was consist of *Abies veitchii* (basal area: 10–90%), *A. mariesii* (10–40%), *Tsuga diversifolia* (0–50%), *Picea jezoensis* var.

hondoensis (0–30%), and *Betula ermanii* (0–10%) (Kimura, 1963). Although the typhoon severely damaged the forest, a large part of the disturbed area is now covered by a coniferous forest dominated by *Abies veitchii* and *A. mariesii*, which mainly originated from saplings and seedlings that existed at the time of the original canopy damage (Kimura, 1963; Kimura *et al*., 1986). In this forest, Fukasawa *et al*., (2019a) found that forest disturbance has no clear effects on current fungal communities within logs and wood decay type as well, compared to that of surrounding undisturbed stands. However, disturbance affected bryophyte communities, which had strong effects on the seedling densities on the logs. Although they recorded poor regeneration of Hondo spruce, the bryophyte species and coverage on the logs seems suitable for spruce seedlings, and will host them near future as forest succession progresses.

Case study 2: Forest DECLINED after the past disturbance

Contrast to Yatsugatake Mountains, Hondo spruce stand in Odaigahara Mountains (34˚11'N, 136˚06'E, 1600 m a.s.l.) declined after the typhoon disturbance. Fukasawa *et al*. (2019b) evaluate the effect of forest decline on communities of wood-inhabiting fungi, bryophyte, wood decay and tree seedling establishment on dead wood of Hondo spruce by comparing those variables between stands with different dieback intensity (weak, mid, heavy). They found that forest decline promoted the frequency of brown-rot fungi in spruce dead wood. The frequency of brown-rotted wood increased with dieback intensity. In this site, forest dieback had a variety of indirect effects on *Picea* seedling density via wood decay type and bryophyte cover on dead wood. First, forest dieback reduced bryophyte cover on dead wood, which was important for spruce seedling colonization. Second, brown-rotted wood dominating the dieback forest negatively affected bryophyte cover. The results suggest that the function of wood decay fungal communities and their effects on interspecific interactions between bryophytes and spruce seedlings might be a key mechanism affecting the colonization success of spruce seedlings, which could be easily modified by the forest disturbance.

Conclusions

Although Yatsugatake and Odaigahara mountains are 290 km apart from each other, data from these two forest sites represents an interesting contrast regarding the forest recovery process and possible role of wood-inhabiting fungi and wood decay on it. An obvious difference between these forest sites is the rate of forest recovery. In Yatsugatake, the canopy of the disturbed stand was already closed to the same degree as an undisturbed stand because of the rapid regrowth of *Abies* spp., which had originally coexisted with spruce (Suzuki *et al*., 2013). In contrast, the heavily disturbed stand in Odaigahara, which had been almost pure spruce stand, had not yet recovered and the canopy was completely open (Shibata *et al*., 2008). Comparison of these two sites suggests that the intensive dieback site in Odaigahara seems to be staying in an alternative semi-stable state in the forest restoration process (Klotzli & Grootjans, 2001). The collapse of causal linkages between fungal decomposition of wood, bryophyte colonization, and spruce seedling establishment could be a mechanism by which the focal ecosystem has been staying in an alternative semi-stable state for a long time after the typhoon disturbance. Slow forest recovery reduces not only the seed supply from adult trees but also bryophyte cover on dead wood, which is important for spruce seedling colonization, and enhances the dominance of brown-rot in dead wood, which is not suitable for spruces seedling colonization. Therefore, a possible option for quick recovery of Hondo spruce forest after typhoon disturbance would be to stimulate stand regeneration not necessarily by spruce, but by other coexisting rapidly growing canopy tree groups such as *Abies*.

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Symposium mini review

Detection and Epidemiological Analysis of Symbiotic Viruses from Protozoa

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Keywords
Cryptosporidium, protozoan virus, Cryptosporidium parvum virus 1, CSpV1

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Abstract

Various symbiotic viruses exist in protozoan parasite. Most species classified with the family *Totiviridae* or *Partitiviridae*, and are transmitted from cell to cell during cell division. Some symbiotic viruses have been reported to influence the pathogenicity of symbiotic parasites to their hosts. There are also reports of the use of symbiotic viruses in the epidemiological analysis of protozoan parasites. We have demonstrated that Cryptosporidium parvum virus 1 (CSpV1), a symbiotic virus of *Cryptosporidium parvum*, can be a high-resolution tool to trace *C. parvum*.

Introduction

The existence of symbiotic viruses in protozoan parasite has been suggested by electron microscopic observation. To date, protozoan viruses have been reported from various protozoa. Common features of these are having double-stranded RNA (dsRNA) and transmitting from cell to cell during cell division. *Cryptosporidium* virus belongs to family *Partitiviridae*, whereas other protozoal symbiotic viruses are classified as family *Totiviridae*. Symbiotic viruses have also been reported from plants and bacteria, and there are reports of some effects on the host of the symbiotic virus. For example, a bacteriophage symbiotic with *Pseudomonas aeruginosa* inhibits the clearance of *P. aeruginosa* from infected wounds (Sweere *et al*., 2019).

Recently, it has been reported that symbiotic viruses of *Leishmania* and *Trichomonas* affect pathogenicity and expression of surface antigens (Ives *et al*., 2011; Wang *et al*., 1987). However, the effects of other protozoan symbiotic viruses on its hosts are often unknown. Moreover, regarding protozoan symbiosis viruses, there are few reports on detection and epidemiological studies. Therefore, we conducted an epidemiological analysis of symbiotic viruses of protozoa, and also investigated the effects of symbiotic viruses on the host.

Detection of protozoal symbiotic viruses

Protozoan symbiotic viruses can be identified by electron microscopy. Since all protozoan symbiotic viruses detected at present are dsRNA viruses, dsRNA can be detected by extracting all nucleic acids from parasites, treating them with DNase and RNase, and performing electrophoresis. This is because dsRNA is resistant to DNase and RNase treatment. Alternatively, the entire nucleic acid is adsorbed on a dsRNA-specific column and electrophoresed (**Fig. 1**). However, because these methods require a large number of protozoa, they are often analyzed using next-generation sequencers. Symbiotic viruses with known sequences can be detected by PCR using primers. It is also possible to detect symbiotic viruses using dsRNA antibodies (Zangger *et al*., 2013).

Symbiotic viruses of *Cryptosporidium*

Cryptosporidium infects various animals and causes severe diarrhea. Because of the absence of effective drugs, infection in immunocompromised patients is fatal. Recently, dsRNA

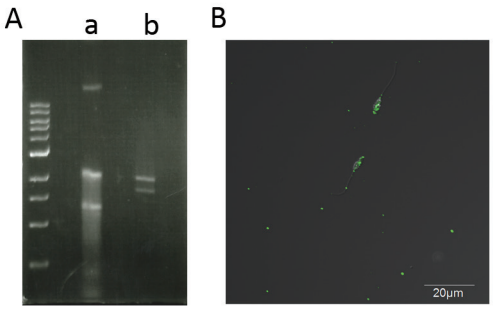

Fig. 1. Detection of dsRNA from protozoa. (A) Electrophoresis of dsRNA extracted from *Cryptosporidium parvum*. a: total nucleic acid, b: purified dsRNA. (B) Immunofluorescence assay (IFA) of *Leishmania major* using dsRNA antibody.

symbiotic virus belonging to the family Partitiviridae has also been reported in these protozoa and has been classified as *Cryspovirus* (Nibert *et al*., 2009, 2017). This virus contains two unrelated, liner dsRNA segments of 1.7 kbp (dsRNA1) and 1.4 kbp (dsRNA2) that are encapsulated separately. dsRNA 1 is RNA dependent RNA polymerase (RdRp) and dsRNA2 encodes the capsid protein (CP). This symbiotic virus has also been detected in *C. hominis*, *C. felis* and *C. meleagridis* (Leoni *et al*., 2003. 2006). *Cryptosporidium* symbiotic viruses are thought to exist as approximately 2,000 particles in an oocyst (Kniel *et al*., 2004). Therefore, studies have been conducted to detect *C. parvum* with high sensitivity using the *Cryspovirus* antibody (Tai *et al*., 2019).

The 60-kDa glycoprotein (GP60) gene is the major subtyping gene of *Cryptosporidium*. The most major subtype of *C. parvum* is the IIa subtype, which is further classified by the number of trinucleotide repeats encoding serine. However, in Japan, the GP60 subtype of *C. parvum* detected in cattle is mostly the IIaA15G2R1 subtype. Therefore, there is a problem that the subtyping by the GP 60 gene cannot be used in the investigation of the infection source. Therefore, we tested whether Cryptosporidium parvum virus 1 (CSpV1) could be used to trace *C. parvum* infection sites. Because RNA viruses show higher mutation frequencies than their hosts because they lack proofreading enzymes.

As a result, the sequence of CSpV1 detected from *C. parvum* IIaA15G2R1 collected throughout Japan reflected the infection site by phylogenetic analysis. Thus, it became clear that CSpV1 could be used as a tool for infection-site estimation and host tracking (Murakoshi *et al*., 2016).

Symbiotic viruses of *Eimeria*

Eimeria spp. infects various animals and causes diarrhea. The *Eimeria* symbiotic viruses of which sequences are reported are E. tenella RNA virus 1, E. brunetti RNA virus 1, E. necatrix RNA virus 1 and E. stiedai RNA virus 1 (Wu *et al*., 2016; unpublished; Lee and Fernando, 1998; Reets *et al*., 1989), which have been detected in each chicken *Eimeria* species. *Totiviridae* is a single-stranded dsRNA virus coding RNA dependent RNA polymerase (RdRp) and the capsid protein (CP). These are all single reports and the effect on the prevalence and pathogenicity of *Eimeria* symbiotic viruses are unknown.

We are currently conducting an epidemiological analysis of the symbiotic virus in chicken *Eimeria* in Japan, and suggested that the symbiotic virus exists at a high frequency (data not shown).

Symbiotic viruses of *Leishmania*, *Giardia*, and *Trichomonas*

Several other protozoan parasites including *Leishmania*, *Trichomonas*, and *Giardia* are also known to have symbiotic dsRNA viruses (family *Totiviridae*): Leishmania RNA viruses (LRVs) 1 and 2; Trichomonas vaginalis viruses (TVVs) 1, 2, 3, and 4; and Giardia lambia virus (GLV).

In *Trichomonas*, the presence of dsRNA has been reported to be involved in the expression of surface antigens of *Trichomonas* (Wang *et al*., 1987). In *Leishmania*, *L. guyanensis* in which LRV1 is present was reported to have significantly increased lesion size in mice (Ives *et al*., 2011).

We are currently investigating whether a similar phenomenon occurs in the presence of symbiotic viruses in *L. major*, we have obtained data that LRV2 is also involved in pathogenicity (data not shown).

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Field Science Center, Graduate School of Agricultural Science, Tohoku University

Symposium mini review

Plant Antiviral *Resistance* Genes May Have Undergone Dissimilar Selection in Nature and in Crop Fields

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Abstract

Plant genomes contain more than 100 copies of *Resistance* (*R*) genes that encode receptor proteins. Each gene product directly or indirectly recognizes pathogen infection to induce resistance against the pathogen. The product of the *Arabidopsis thaliana R* gene *RCY1* recognizes the capsid protein of cucumber mosaic virus (CMV) and induces a hypersensitive response (HR), which leads to programmed cell death of infected cells and containment of the virus in inoculated leaves. We recently demonstrated that the average number of CMV genomes that established cell infection [*i.e.*, multiplicity of infection (MOI)] after cell-to-cell movement decreased by ~23% upon induction of HR. In contrast, infection by a CMV mutant that had a smaller reduction in MOI (~10%) upon *R*-gene-mediated recognition resulted in a systemic HR in the plant, leading to plant death. This finding suggested that inefficient induction of resistance allows a virus to spread, causing the death of the infected plant. A simulation suggested that this death of an infected individual may function as a suicide strategy to protect neighboring plants that are often "kin" of the infected plant, by reducing the source of infection. Thus, systemic host death, caused by inefficient *R*-gene-mediated induction of resistance against viruses, can be positively selected in nature; this type of death serves as population-level resistance against the pathogen and the starting point for further adaptation toward more efficient resistance. During plant domestication, traits facilitating population-level resistance may have undergone negative selection, resulting in the loss of associated *R* genes in our most common crops.

Introduction

Plants are threatened by many pathogens, including fungi, bacteria, and viruses. In response to these pathogens, plants have evolved many resistance mechanisms, including the well-studied *resistance* (*R*)-gene-mediated resistance (Villena *et al*., 2018). Plant genomes carry many *R* genes, which encode receptor proteins that share nucleotide-binding (NB) and leucine-rich repeat (LRR) domains. Each *R* gene induces resistance upon recognition of specific pathogen(s), via direct or indirect interaction between the gene product (*i.e.*, receptor protein) and pathogen-derived protein(s) (reviewed in Collier and Moffett, 2009, and elsewhere). Most well-studied *R* genes induce a hypersensitive response (HR) upon pathogen recognition; HRs are characterized by containment of the pathogen in the infected leaves and the induction of programmed cell death (PCD) in infected tissue. Although many *R* genes have been found to induce HR upon virus infection, interactions between viruses and *R* genes sometimes lead to different consequences. One such consequence is the induction of extreme resistance, in which virus replication and movement to adjacent cells is suppressed in the initially infected cell without causing PCD. The *Rx* gene, an *R* gene introgressed from a wild relative to cultivated potato, induces extreme resistance against potato virus X (Bendahmane *et al*., 1999); the *RCY1* gene, an HR-inducing *R* gene of *Arabidopsis thaliana* against cucumber mosaic virus (CMV), induces extreme resistance when overexpressed in transformant plants

(Sekine *et al*., 2008). An alternative consequence of *R*-gene-mediated recognition of a virus is systemic necrosis, which has long been considered a result of aggressive virus infection; however, recent studies have suggested that, at least in some instances, systemic necrosis is caused by mechanisms that are similar to HR. Systemic necrosis is established by means of the same pathway as HR (Komatsu *et al*., 2010); notably, viruses that cause HR in certain crop cultivars can cause systemic necrosis in other cultivars (Jones and Vincent, 2018). Furthermore, viral amino-acid substitutions in the capsid protein (CP) of turnip crinkle virus have been shown to change the host response from HR to systemic necrosis (Kang *et al*., 2015). These observations suggest that systemic necrosis is sometimes a result of imperfect HR induction, when the virus infection is uncontrolled despite induction of PCD, resulting in plant death. Thus, *R*-gene-mediated systemic necrosis is sometimes regarded as systemic HR (SHR); however, several issues remain unresolved, including how and whether the strength of resistance can be compared between HR and SHR, as well as whether the fitness of plants that induce SHR is higher than that of fully susceptible plants. Our recent study enabled us to answer these questions and discuss the evolution of antiviral *R* genes.

Reduced viral MOI in the *RCY1*-CMV(Y) system

Resistance to CMV-Y (*RCY1*) is a CC-NB-LRR-type *R* gene that was originally found in *A. thaliana* ecotype C24 (Takahashi *et al*., 1994, 2002; reviewed in Ando *et al*., 2019). The RCY1 protein is believed to recognize the CP of CMV (Takahashi *et al*., 2001), although it is unclear whether RCY1 and CP interact directly or indirectly. The gene products of allelic *RCY1* homologs in *A. thaliana* ecotypes L*er* and Di-17 (i.e., *RPP8* and *HRT*, respectively) recognize distinct plant pathogens, *Hyaloperonospora parasitica* and turnip crinkle virus (Cooley *et al*., 2000). We have found that *Nicotiana benthamiana* plants transformed with a chimeric *R* gene containing the CC-NB domains from *RPP8* and an LRR domain from *RCY1* can recognize CMV and induce HR; we have also found that a CMV mutant carrying an N31T (asparagine 31 to threonine) substitution in its CP escapes recognition by RCY1 (Takahashi *et al*., unpublished). In a more recent study, we found that a T45M CP mutant caused SHR in the *RPP8-RCY1* chimeric *R*-gene transformant *N. benthamiana* and *RCY1*-carrying *A. thaliana* (Abebe *et al*., unpublished) (**Fig. 1**). Multiplicity of infection (MOI) comprises the average number of viral genomes that establish infection in a host cell. Previously, we established a method for estimation of MOI during cell-to-cell infection by plant viruses, based on fluorescent microscopy observations and statistical analysis (Miyashita and Kishino, 2010; Miyashita *et al*., 2015). Briefly, two viral derivatives that carry a *yellow-fluorescent-protein* (*YFP*) gene or a *cyan-fluorescent-protein* (*CFP*) gene are co-inoculated on plant leaves and the stochastic separation of the two derivatives is observed by fluorescent microscopy (**Fig. 2AB**). Because a smaller MOI will result in quicker separation of viral derivatives encoding *YFP* or *CFP*, MOI can be estimated from the frequency of singly infected cells after a certain number of cell-to-cell infection cycles. Using this method, we estimated that MOIs in the first cell-to-cell infections of Japanese soil-borne wheat mosaic virus and tomato mosaic virus were approximately 6 and 4, respectively (Miyashita and Kishino, 2010; Miyashita *et al*., 2015). We used this method to estimate the CMV MOI in first cell-to-cell infections in *N. benthamiana* leaf tissue, in the presence and absence of the *R* gene (**Fig. 2C**). CMV with the wild-type *CP* gene exhibited a ~23% reduction in MOI in plants with the *R* gene, compared to plants without the *R* gene; CMV with the T45M CP substitution exhibited a ~10% reduction in MOI, while CMV with the N31T CP substitution did not

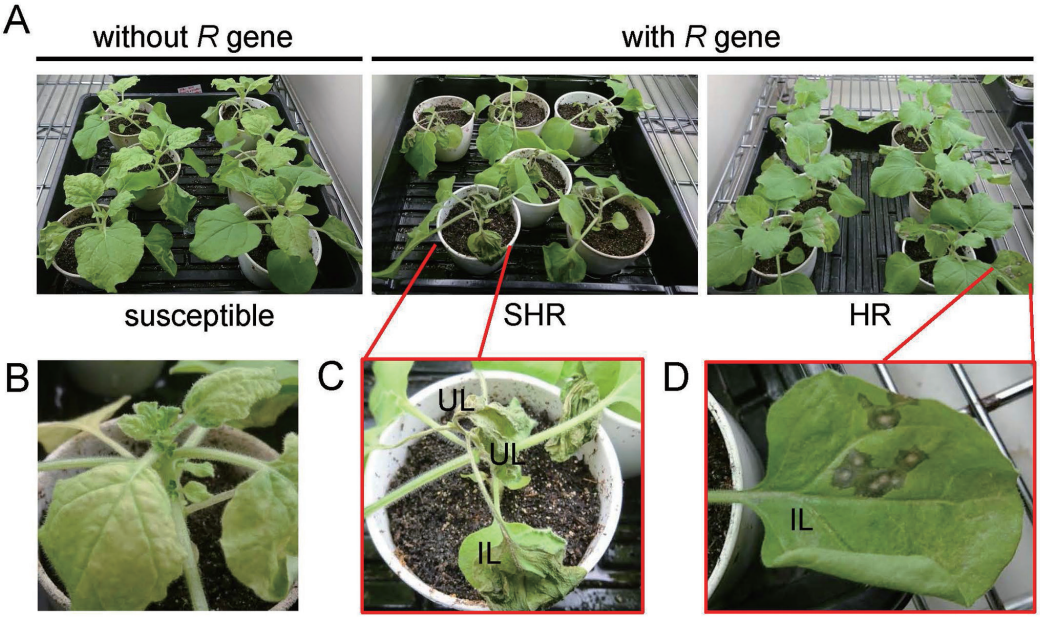

Fig. 1. Susceptible interaction and SHR/HR induction in the CMV–*N. benthamiana* system. (A) [left] Susceptible interaction between wild-type cucumber mosaic virus and wild-type *Nicotiana benthamiana*, demonstrating mosaic symptoms; [middle] SHR induction by CP T45M variant CMV in *R*-gene transformant *N. benthamiana*; [right] HR induction by wild-type CMV in *R*-gene transformant *N. benthamiana*. (B) Mosaic symptoms in a susceptible interaction. (C) Systemic necrosis caused by SHR. In addition to the inoculated leaves (IL), uninoculated upper leaves (UL) are dying. (D) HR lesions formed in an inoculated leaf. PCD did not spread beyond the leaf.

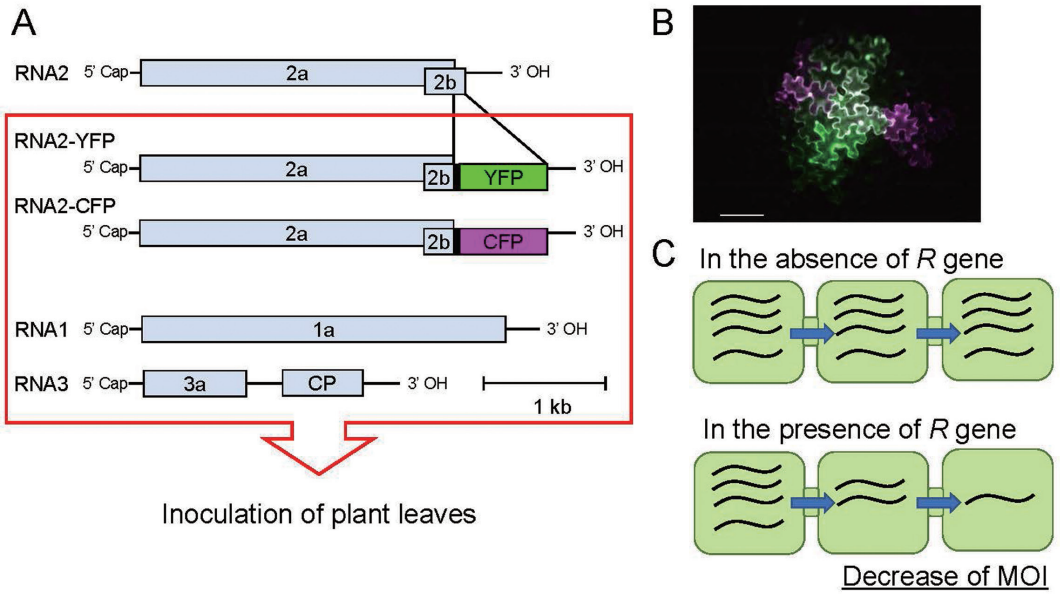

Fig. 2. Estimation of plant viral MOI during cell-to-cell movement. (A) MOI estimation for CMV. RNA2 derivatives carrying *YFP* or *CFP* genes were mixed with wild-type RNA1 and RNA3, then used to inoculate *N. benthamiana* leaves. (B) Observation of stochastic separation of the two derivatives by means of fluorescent microscopy. (C) Schematic explanation for the reduction in MOI.

exhibit a significant reduction in MOI between plants with and without the *R* gene (Abebe *et al*., unpublished). A reduction in MOI directly results in the stochastic failure of cell-to-cell infections. Because PCD is observed only after several cycles of cell-to-cell infection, a reduction in MOI in the first cell-to-cell infections suggests PCD-independent resistance against CMV. This is consistent with observations that PCD and virus containment occur via independent pathways (Komatsu *et al*., 2010; Takahashi *et al*., 2012). We previously suggested that a small MOI during cell-to-cell infection is necessary for plant viruses to enhance selection on their *trans*-acting genes or elements (Miyashita and Kishino, 2010; Miyashita 2018). In a host that recognizes a particular virus through a corresponding *R* gene, MOI may decrease to a level at which the virus cannot continue spreading through the host tissue. Furthermore, our MOI estimates provided the first direct evidence that resistance induction is weaker in SHR than in HR; in addition, HR changes to SHR due to small differences in viral MOI, which reflect small differences in the level of resistance induced by *R*-gene-mediated pathogen recognition.

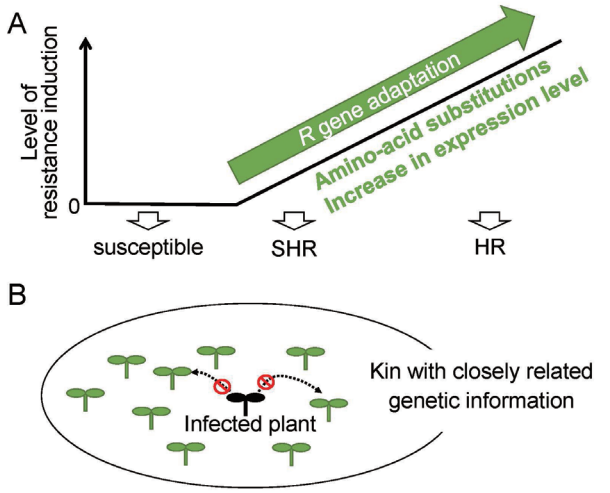

Fig. 3. Possible evolutionary trajectory of *R* genes and kin selection for SHR. (A) Possible evolutionary trajectory of *R* genes. (B) Systemic death caused by SHR can be adaptive when kin selection occurs.

Natural selection may favor SHR, while selection in crop fields may not

These findings led us to reflect on the general evolutional trajectory of antiviral *R* genes (**Fig. 3A**). When an *R* gene product recognizes a new viral protein, the level of resistance induced is not initially sufficient to contain the virus, resulting in SHR; improved recognition compatibility with amino-acid substitutions in the *R* gene and an increase in its expression gradually enhance the resistance induction level, thus changing the phenotype from SHR to HR. This concept implicitly includes the unproven assumption that SHR is more adaptive than the absence of an *R* gene to recognize the virus. However, given that SHR brings death to the plant, it is important to consider how this outcome could be adaptive. We suggest that kin selection (reviewed in Birch, 2019) can address this issue (**Fig. 3B**). Most land plants propagate by means of seeds, sometimes by means of vegetative reproduction. Thus, propagation occurs locally; individuals that are closely related genetically (i.e., "kin") are in close proximity with each other. In this context, SHR may help infected plants to avoid serving as an infection source for their kin, at the cost of the infected plant's life. Kim *et al*. (2008) mentioned the concept of kin selection for SHR; however, no study has yet analyzed whether this selection is adaptive. Accordingly, we developed a mathematical model for natural selection involving SHR, in which the locality of propagation is parameterized. Simulations based on this model revealed that SHR can be adaptive when propagation occurs locally, whereas it cannot be adaptive when propagation occurs in a more dispersed manner (Miyashita *et al*., unpublished). These results imply that individual death by SHR can serve as a suicide strategy by which plants save

their kin; however, the strategy is not applicable to organisms in which propagation does not occur locally, such as animals. SHR might not have been favored in crop domestication by our human ancestors; this might have resulted in the deletion of some *R* genes from crop genomes. Future studies focusing on the possible deletion of *R* genes may reveal the effects of human activity on crop–pathogen interactions and provide novel strategies for identification of unknown *R* genes.

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Symposium mini review

Toxoplasma Gondii Effectors TgIST and TgGRA15 Differentially Target Host IDO1 to Antagonize the IFN-γ-induced Anti-*T. Gondii* Response in Human Cells

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Keywords
Toxoplasma, IFN-γ, IDO1, TgIST, TgGRA15

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Abstract

Toxoplasma is an important intracellular pathogen that causes lethal toxoplasmosis in humans and animals. Interferon-γ (IFN-γ) is critical for anti-*T. gondii* responses in both humans and mice. Recent extensive studies using the mouse as a model organism have revealed that IFN-γ-inducible GTPases play critical roles and have revealed that virulent *T. gondii* can inhibit IFN-γ-mediated host immune responses. Thus, the relationship between host immunity and *T. gondii* virulence is well established in mice. In contrast, IFN-γ-induced anti-*T. gondii* responses in humans are not completely clear because the IFN-γ-inducible GTPase-mediated anti-*T. gondii* responses may not be notable in humans. Therefore, the *T. gondii* virulence strategy to resist IFN-γ-induced anti-*T. gondii* responses in humans also remains largely unclear. Here, we generated human cells lacking the IFN-γ-inducible gene, and showed that IDO1 is required for the IFN-γ-induced response in human cells. Then, we focused on *T. gondii* virulence mechanisms in human cells. Specifically, we focused on the distinct *T. gondii* virulence mechanisms involving TgIST and TgGRA15 to suppress IFN-γ-dependent immunity in human cells. We generated TgIST- or TgGRA15-deficient *T. gondii* by using the CRISPR/Cas9 system, and showed that IDO1 mRNA induction is inhibited in a TgIST-dependent manner in IFN-γ-stimulated human cells. We also found that the IDO1-dependent anti-*T. gondii* response is inhibited in a TgGRA15-dependent manner in secondarily infected cells. Taken together, our data show that *T. gondii* possesses at least two differential virulence mechanisms in which IDO1 is targeted by TgIST and TgGRA15 to antagonize IFN-γ-induced anti-*T. gondii* responses in human cells.

Introduction

Toxoplasma gondii is an obligatory protozoan parasite that can infect virtually all warm-blooded animals (Boothroyd, 2009; Dubey, 2010). *T. gondii* causes toxoplasmosis, the clinical signs of which include encephalitis and hepatitis, in immunocompromised people or the newborn babies of women who contracted the infection for the first time during pregnancy (Frenkel and Remington, 1980; Montoya and Remington, 2008). As humans have increased contact with domestic or wild animals, the number of *T. gondii*-infected animals and/or toxoplasmosis patients is expected to increase. In fact, we previously showed that the incidence of *T. gondii*-infected wild animals is increasing (Bando *et al*., 2015). In addition, *T. gondii* is considered one of the most important human pathogens that cause economic losses and loss of life due to food-borne illness in the United States (Batz *et al*., 2012). Thus, *T. gondii* is one of the most important zoonotic pathogens.

The host immune response plays an important role in antagonizing *T. gondii* infection. In particular, type I cytokine interferon-gamma (IFN-γ) is a critical host factor for anti-*T. gondii* responses in mammalian cells (Suzuki *et al*., 1988). IFN-γ-induced anti-*T. gondii* responses have been extensively

analyzed by using the mouse model. In mice, it is known that the accumulation of p47 immunity-related GTPases (IRGs) and p65 guanylate-binding proteins (GBPs) on parasitophorous vacuoles (PVs) of *T. gondii* lead to their destruction (Lee *et al*., 2015; Ma *et al*., 2014; MacMicking, 2012; Ohshima *et al*., 2015; Taylor *et al*., 2007; Yamamoto *et al*., 2012). In contrast, the importance of GBPs- or IRGs-mediated mechanisms in humans is less certain. For example, humans have only one IRG, which is IFN-γ-non-inducible, whereas mice have 20 IRGs (Bekpen *et al*., 2005). Thus, it remains unclear which host factors are important for anti-*T. gondii* responses in humans. Therefore, we performed a comprehensive analysis of the role of IFN-γ-inducible genes for anti-*T. gondii* responses in human cells.

T. gondii secretes various virulence factors, including rhoptry proteins (ROPs) and dense granule proteins (GRAs), into host cells to resist the IFN-γ-dependent anti-*T. gondii* host immune responses (Hakimi *et al*., 2017; Hunter and Sibley, 2012). The virulence mechanisms of parasite effector molecules against the host's immune responses have been extensively analyzed in mouse models. (Behnke *et al*., 2011; Etheridge *et al*., 2014; Fentress *et al*., 2010; Reese *et al*., 2011; Rosowski *et al*., 2014; Rosowski and Saeij, 2012; Steinfeldt *et al*., 2010). However, some previous reports suggest that IFN-γ-induced anti-*T. gondii* host immune responses differ between mouse and human cells (Ohshima *et al*., 2014; Selleck *et al*., 2015). Therefore, we also analyzed the *T. gondii* virulence mechanisms targeting IFN-γ-dependent anti-*T. gondii* molecules in human cells.

IDO1 is required for the anti-*T. gondii* response in human cells

We previously showed that Autophagy-related protein 16-1 (ATG16L1) plays an important role in the IFN-γ-dependent anti-*T. gondii* response in mouse cells (Ohshima *et al*., 2014). Subsequently, we generated ATG16L1-deficient human cells by using the CRISPR/Cas9 system and analyzed the role of ATG16L1 in IFN-γ-dependent anti-*T. gondii* responses. Interestingly, the parasite number in IFN-γ-activated ATG16L1-deficient human cells was the same as that in IFN-γ-activated wild-type human cells, suggesting an ATG16L1-independent IFN-γ-induced anti-*T. gondii* response in humans. So, we then generated various kinds of IFN-γ-inducible gene-deficient human cells by using the CRISPR/Cas9 system, and analyzed the role of the target genes in the anti-*T. gondii* responses. We found that indoleamine 2,3-dioxygenases 1 (IDO1) was strongly induced by IFN-γ and has a critical role in the anti-*T. gondii* responses in humans. Next, we analyzed the molecular mechanisms of the IDO1-dependent anti-*T. gondii* responses. We observed that IDO1-dependent degradation of tryptophan, which is an essential amino acid for *T. gondii* intracellular growth, led to restricted parasite growth, but not inhibition of parasite invasion. Taken together, our data show that IDO1 has a critical role in the IFN-γ-dependent growth inhibition of *T. gondii* in human cells (Bando *et al*., 2018b).

Toxoplasma effector TgIST directly suppresses IDO1 expression

IDO1 expression is STAT1 dependent (Sotero-Esteva *et al*., 2000), and a previous study demonstrated that TgIST is secreted from dense granules and associates with the host nucleosome remodeling and deacetylase complex to inhibit the expression of STAT1-dependent genes (Gay *et al*., 2016; Olias *et al*., 2016). Therefore, we hypothesized that TgIST has an important role in the inhibition of STAT1-mediated IDO1 expression. To examine this possibility, we generated TgIST-deficient *T. gondii* by using the CRISPR/Cas9 system, and tested whether TgIST deficiency affected *T. gondii* virulence in human cells. We found that although wild-type *T. gondii* infection led to a reduction in both IDO1 mRNA and protein, TgIST-deficient parasite infection did not. In addition, the number of TgIST-deficient parasites was significantly reduced compared to the number of wild-type parasites in IFN-γ-stimulated human cells, suggesting that TgIST has an important role in parasite growth in IFN-γ-activated human cells. Taken together, our findings demonstrate that TgIST directly suppresses the IDO1-dependent anti-*T. gondii* response by preventing STAT1 activation in human cells (Bando *et al*., 2018b).

Toxoplasma effector TgGRA15 indirectly downregulates the IDO1-dependent anti-parasitic response in human cells

Toxoplasma effector TgGRA15 influences the activation of host immune responses by inducing the host transcription factor NF-κB in mice (Gov *et al*., 2013; Jensen *et al*., 2011; Rosowski *et al*., 2011). In addition, a previous study demonstrated that TgGRA15-deficient *T. gondii* promotes parasite growth in mice (Jensen *et al*., 2013; Rosowski *et al*., 2011), suggesting that TgGRA15 supports host survival by inhibiting parasite growth. Thus, the significance of TgGRA15 as a virulence factor remains unclear. Therefore, we explored the potential role of TgGRA15 as a virulence factor in human cells. During parasite infection *in vivo*, *T. gondii* preferentially infects CD11b+ cells, such as monocytes, and spreads into various organs (Courret *et al*., 2006). To mimic this *in vivo* cell-cell interaction *in vitro* using human cells, monocyte-hepatocyte co-culture models have been developed because toxoplasmic hepatitis is a serious manifestation of *T. gondii* infection (Frenkel and Remington, 1980). First, we generated TgGRA15-deficient *T. gondii* by using the CRISPR/Cas9 system, then we infected human monocytes with the *T. gondii* and seeded these infected cells on human hepatocytes with or without IFN-γ. Surprisingly, the TgGRA15-deficient *T. gondii* infection resulted in a significant reduction in the parasite numbers compared to the wild-type parasite infection under these monocyte-hepatocyte co-culture conditions, suggesting that TgGRA15 provides a growth advantage for *T. gondii* under these co-culture conditions. Next, we analyzed the molecular mechanisms responsible for the TgGRA15-dependent pro-*T. gondii* effect. We found that TgGRA15 induced IL-1β production in *T. gondii*-infected monocytes, which was dependent on the NLRP3 inflammasome and gasdermin D, and then IFN-γ and monocyte-derived IL-

1β induced iNOS expression, which led to the inhibition of IDO1 expression in the hepatocytes. Taken together, our data demonstrate that TgGRA15 has an important role in indirectly downregulating the IDO1-dependent anti-*T. gondii* response in secondarily infected cells (Bando *et al*., 2018a).

Conclusion

In the present study, we demonstrated that IDO1 has an important role in IFN-γ-dependent anti-*T. gondii* responses in human cells. Further, we showed that the *Toxoplasma* effectors TgIST and TgGRA15 suppress IDO1 gene expression directly or indirectly to promote parasite growth in IFN-γ-activated human cells. In addition, we recently discovered new virulence mechanisms of *T. gondii* effectors by focusing on the difference between the human and mouse immune responses (Bando *et al*., 2019; Fisch *et al*., 2019). Taken together, our findings could contribute to the development of a novel therapeutic strategy for treating human toxoplasmosis.

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The 17th International Symposium on Integrated Field Science (17th IS-IFS)

Infectious Diseases and Field Science: From Pathogens to Environmental Microbes

Purpose of the symposium:

The microbes evolve uniquely in the environments, geosphere and hydrosphere. Some of them are parasitic and symbiotic in animals and plants. The pathogens which bring the problems for human beings result in their deaths ultimately. However, most of microbes are symbiotic in the environments.

In this symposium, the workshop of the infectious diseases and field science from pathogen which causes a pandemic disease to symbiotic microbes in animals or plants in the environment will be held. Poster session is open for those who are interested in field sciences.

Date: 16-17 March 2020

Venue: Tohoku University, Aobayama Commons lecture room 1 (Keynote lecture) and entrance hall (Poster session).

Organized by

Field Science Center, Graduate School of Agricultural Science, Tohoku University

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Abstract submission

Inform your name, affiliation, and poster title to the secretary office via E-mail. The office will inform a template for your abstract preparation. Guidelines for poster presentation are available on the website. **Deadline: 14th February 2020**

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The symposium will present the award, “IS-IFS Best Presentation Award” to the excellent poster presentation(s).

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Inform your name and affiliation to the secretary office via E-mail. The reception is going to be held in downtown.

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The 17th IS-IFS Program

March 16th (Mon.)

13:00-13:05 **Opening remark by Shin-ichiro OGURA (President of The Field Science Center)**

13:05-14:35 **Oral Presentation by Invited Speakers, Section 1**

O-1: Hiroki BOCHIMOTO (The Jikei University School of Medicine)
Functional perspective of feeder organelle from three-dimensional ultrastructural characteristics in *Cryptosporidium parvum*.

O-2: Masayuki SHIMOJIMA (National Institute of Infectious Diseases)
Identification of virus receptors

O-3: Kentaro KATO (Tohoku University)
Proteomic dissection of *Plasmodium falciparum* Maurer's cleft compartments using SBP1

14:35-14:50 **Break**

14:50-15:50 **Keynote lecture**

S-1: Boris STRIEPEN (The University of Pennsylvania)
The Biology of *Cryptosporidium* Infection

15:50-16:00 **Break**

16:00-17:15 **Poster Session (*3-minutes interactive presentation*, 16:00-16:42)**

March 17th (Tues.)

9:00-10:30 **Oral Presentation byInvited Speakers, Section 2**

O-4: Yu FUKASAWA (Tohoku University)
Pine (*Pinus densiflora*) deadwood act as hotspots for seedling regeneration after pine dieback caused by pine wilt disease

O-5: Richard CULLETON (Nagasaki University)
The genome of the zoonotic malaria parasite *Plasmodium simium* reveals adaptions to host-switching

O-6: Fumi MURAKOSHI (Kyoto Prefectural University of Medicine / Tohoku University)
Detection and epidemiological analysis of symbiotic viruses from protozoa using the FLDS (A Comprehensive dsRNA Sequencing Method).

10:30-10:45 **Break**

10:45-11:45 **Oral Presentation by Invited Speakers, Section 3**

O-7: Shuhei MIYASHITA (Tohoku University)
Antiviral *R* genes of plants may have experienced different "selections" in nature and in crop fields

O-8: Hironori BANDO (Tohoku University)
Toxoplasma gondii effectors TgIST and TgGRA15 differentially target host IDO1 to antagonize the IFN-γ-induced anti-*T. gondii* response in human cells

11:45-12:00 **Closing remark by Kentaro KATO (The 17th IS-IFS Organizer)**

Keynote Lecture

Boris Striepin, Ph.D.

Professor of Microbiology and Immnology

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

Boris grew up in Germany in an industrial area then dominated by coal and steel. He studied zoology, botany and cell biology at the universities of Bonn and Marburg and conducted undergrad research on liver flukes in Bonn and trypanosomes in Bobo Dioulasso, Burkina Faso. Boris earned a PhD for work on parasite biochemistry with Ralph Schwarz in 1995, was a postdoc with David Roos studying parasite cell biology, and joined the faculty of the University of Georgia in 2000. In 2017, Boris and his lab moved to the University of Pennsylvania in Philadelphia. Boris studies the biology of apicomplexan parasites and how they interact with their hosts, his current research focus is the parasite Cryptosporidium, a leading global cause of diarrhea and mortality in young children. Boris is also engaged in education and training. He taught undergraduate and graduate classes, directed an NIH training grant program in parasitology, and served as faculty and director of the Biology of Parasitism summer research course at the Marine Biology Laboratories in Woods Hole. Boris is married to a social worker with remarkable patience for scientists and has three children, two are scientists – all are awesome.

S-1. The Biology of *Cryptosporidium* Infection

Boris STRIEPEN

Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania

The protozoan parasite *Cryptosporidium* is a leading cause of severe diarrhea in young children and an important contributor to early childhood mortality. Effective drugs and vaccines are lacking, in part due to the overall poor tractability of this parasite. To overcome this, we established molecular genetics for *Cryptosporidium* and a natural mouse model of infection. This seminar will focus on recent and largely unpublished work to understand the cell biology of *Cryptosporidium* infection. First, I will discuss how the parasite manipulates the host cell by injecting proteins using two independent delivery systems. Then I will describe what we are learning about the parasite's lifecycle. The entire cycle, including an asexual and a sexual phase unfolds over three days and is tractable in culture and in animals for a significant portion. This is a remarkably simple model that can be interrogated by live cell imaging, single cell sequencing and genetic manipulation.

O-1. Functional Perspective of Feeder Organelle from Three-dimensional Ultrastructural Characteristics in *Cryptosporidium parvum*

Hiroki BOCHIMOTO[1,2], Daisuke KONDOH[3], Yo ISHIHARA[4], Mohammad Hazzaz Bin KABIR[2] and Kentaro KATO[2,5]

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[3]Laboratory of Veterinary Anatomy, Obihiro University of Agriculture and Veterinary Medicine
[4]Shonan Kamakura General Hospital
[5]Laboratory of Sustainable Animal Environment, Graduate School of Agricultural Science, Tohoku University

Cryptosporidium is a parasite causing extensive illness both in livestock and humans. Feeder organelle of *Cryptosporidium* is the multi-membranous structures localized on the parasite-host-cell interface that deprive nutrients from host cells. Although the feeder organelle has been summarized as being highly invaginated membranous structure, the three-dimensional fine structure remains unclear. Osmium-maceration procedure for scanning electron microscopy (OS-SEM) is one of the methods to enable visualization of the intracellular ultrastructure including depth direction information after removing soluble proteins. Recently, we investigated and assessed *C. parvum* possessed on the surface of ileal epithelial cells of SCID mouse by using transmission electron microscopy (TEM) and OS-SEM. By TEM observation, feeder organelles were recognized as aggregated structures of concentrically-, vertically- and randomly-lined bars. Correspondingly, OS-SEM observation revealed the reticulated network of stacked flat bursiform, ring-shaped bursiform and reticulated tubular membranes. These findings of the three-dimensional ultrastructural characteristics of feeder organelle, which are more intricate than expected, may potentially reinforce the limited knowledge regarding the nature of this attachment interface and the functional mechanisms around extraction of nutrients.

O-2. Identification of Virus Receptors

Masayuki SHIMOJIMA

Special Pathogens Laboratory, Department of Virology I, National Institute of Infectious Diseases

Among different organisms, many similar but non-identical cellular molecules exist that show the same functions. Even in a single species, the expressed molecules are dependent on cell types. This is the case with virus receptors, which are cellular components involved in viral attachment to cells and invasion into cells for viral replication. Thus, viral infections show specificity with regards to host ranges, tissues, and cell types, indicating that the identification of virus receptors is useful for the molecular explanation of viral tropisms. Because target tissues or cell types of viruses are strongly associated with virus-induced diseases, the identification of virus receptors often leads to a more profound understanding of the associated diseases. Furthermore, the identification of virus receptors might aid the efficient development of countermeasures against the induced diseases.

The identification of virus receptors has been performed by various methods, which are roughly classified into three categories: (I) speculation based on knowledge obtained from experiments; (II) screening of libraries based on gain-of-function or loss-of-function criteria; and (III) identification of interactive cellular molecules by peptide sequencing or mass spectrometry. We have developed efficient, low-cost cellular cDNA library screening methods (classified into category II) to identify virus receptors. In the symposium, I would like to introduce the developed methods and also their applications.

O-3. Proteomic Dissection of *Plasmodium falciparum* Maurer's cleft Compartments Using SBP1

Kentaro KATO[1,2], Ryo TAKANO[1], Hiroko KOZUKA-HATA[3], Daisuke KONDOH[1], Hiroki BOCHIMOTO[1,4] and Masaaki OYAMA[3]

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Host erythrocyte modifications by malaria parasites, which are essential to their survival and pathogenesis, are facilitated by parasite proteins exported to the host cytoplasm. These exported proteins form a functional trafficking complex in the host cytoplasm to transport virulence determinants to the erythrocyte surface; this complex, Maurer's cleft, is thus essential for malaria virulence. Here, we report a comprehensive map of the interaction network of this trafficking complex. We developed authentic, unbiased, highly sensitive proteomic approaches and systematically determined the proteins that interact with a core component of the complex, SBP1 (skeleton-binding protein 1). SBP1 interactomes revealed numerous exported proteins that have not previously been linked to the protein complex, as well as potential interactors associated with the intracellular trafficking of SBP1. We further identified several host-parasite protein interactions and identified the exported protein MAL8P1.4 as being linked to the virulence of *Plasmodium falciparum* in infected erythrocytes. Our study sheds light on the highly complicated interplay between parasite and host proteins in the host cytoplasm and provides a reliable interaction dataset connecting dozens of exported proteins that are required for the virulence of *P. falciparum*.

O-4. Pine (*Pinus densiflora*) Deadwood Act as Hotspots for Seedling Regeneration after Pine Dieback Caused by Pine Wilt Disease

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Forest dieback caused by tree diseases often generate huge amounts of deadwood. The decay process of deadwood is crucial for biodiversity in forest ecosystems. Wood decay types, traditionally categorized into white and brown rots, are the consequences of fungal decay activities and strongly affect biotic communities inhabiting deadwood, including tree seedlings. Given that fungal community is affected by climatic conditions, it is important to evaluate the occurrence patterns of the decay types along a geographical range to understand forest dynamics in wide spatial scale. In 30 sites covering a latitudinal gradient in Japan, I examined the effects of environmental variables on the occurrence of wood decay types in logs of *Pinus densiflora*, which was severely damaged by Pine Wilt Disease in last century. Among the wood decay types, the frequency of brown rot was negatively correlated with latitudinal gradient, whereas white rot was negatively correlated with MAT. These results suggested that activity of brown rot fungi is more prominent in the warmer lower-latitude areas than in the cooler higher-latitude areas in pine log decomposition. I also examined the effects of wood decay type on seedling densities of 14 tree species growing on pine logs and found that responses to brown rotted wood was considerably different among tree species. These results suggested that pine deadwood act as hot spots for variety of tree seedlings and that functional diversity of wood decay fungi is important to prepare diverse regeneration sites for seedlings after forest dieback.

O-5. The Genome of the Zoonotic Malaria Parasite *Plasmodium simium* Reveals Adaptions to Host-switching

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Plasmodium simium, a malaria parasite of non-human primates in the Atlantic forest region of Brazil was recently shown to cause zoonotic infections in humans. Phylogenetic analyses based on of six *P. simium* isolates from humans and two isolates from brown howler monkeys revealed that *P. simium* is monophyletic within the broader diversity of South American *Plasmodium vivax*, consistent with the hypothesis that *P. simium* first infected non-human primates as a result of a host-switch of *P. vivax* from humans. Very low levels of genetic diversity within *P. simium* and the absence of *P. simium*-*P. vivax* hybrids suggest that the *P. simium* population emerged recently with a subsequent period of independent evolution in Platyrrhini monkeys. We find that *Plasmodium* Interspersed Repeat (PIR) genes, *Plasmodium* Helical Interspersed Subtelomeric (PHIST) genes and Tryptophan-Rich Antigen (TRAg) genes in *P. simium* are divergent from *P. vivax* orthologues and are enriched for non-synonymous single nucleotide polymorphisms, consistent with the rapid evolution of these genes. Analysis of genes involved in erythrocyte invasion revealed several notable differences between *P. vivax* and *P. simium*, including large deletions within the coding region of the Duffy Binding Protein 1 (DBP1) and Reticulocyte Binding Protein 2a (RBP2a) genes of *P. simium*. Sequence analysis of *P. simium* isolates from non-human primates (NHPs) and zoonotic human infections revealed a deletion of 38 amino acids in DBP1 present in all human-derived isolates, whereas NHP isolates were multi-allelic at this locus. We speculate that these deletions in key erythrocyte invasion ligands along with other significant genetic changes may have facilitated zoonotic transfer to humans. NHPs are a reservoir of parasites potentially infectious to humans that must be considered in malaria eradication efforts. The *P. simium* genome is an important resource for understanding the mechanisms of malaria parasite zoonoses.

O-6. Detection and Epidemiological Analysis of Symbiotic Viruses from Protozoa Using the FLDS (A Comprehensive dsRNA Sequencing Method)

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We have detected the symbiotic virus of *Eimeria* that infects chickens by next-generation sequencing (NGS) analysis using the FLDS method (Fragmented and primer Ligated dsRNA Sequencing). Total nucleic acid was extracted from *Eimeria* oocysts and dsRNA was purified using a cellulose column that specifically adsorbed dsRNA. Subsequent analysis with NGS using the FLDS method yielded various dsRNA contigs. Of these, the percentage of total leads indicates that there is a high probability that three types of contigs are present in chicken *Eimeria* or chickens. As a result of BLAST analysis, 1 contig showed more than 80% homology to Eimeria brunetti RNA virus 1, and this contig was considered to be the sequence of the symbiotic virus of *Eimeria*. The remaining two species were suggested to be novel dsRNA viruses.

Next, we designed primers using the analyzed sequences and carried out epidemiological analysis of Eimeria brunetti RNA virus 1 by PCR. As a result, viral sequences were detected from some chicken *Eimeria* in Japan.

O-7. Antiviral *R* Genes of Plants May Have Experienced Different "Selections" in Nature and in Crop Fields

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Plant genomes carry more than 100 copies of *R* (*Resistance*) genes that encode receptor proteins. Each of the gene products directly or indirectly recognizes a pathogen-derived protein to induce resistance against the pathogen. An *R* gene of *Arabidopsis thaliana*, *RCY1*, recognizes the capsid protein (CP) of cucumber mosaic virus (CMV) and induces hypersensitive reaction (HR), which is characterized by programmed-cell death of the infected cells and inclusion of the virus in the inoculated leaves. Our recent study showed that the average number of CMV genomes that established cell infection (i.e., MOI, multiplicity of infection) after cell-to-cell movement decreased from 3.60±0.26 to 2.76±0.28 upon resistance induction. A CMV mutant that shows smaller decrease of MOI against *R*-gene recognition caused systemic HR (SHR) of the plants, which results in systemic death of the plant individuals. This result suggests that inefficient recognition allow expansion of the virus and cause death of the plant individual. A simulation suggested that such an individual death can function as a suicide strategy to protect neighboring plants, that are often "kin" of an infected plant, by diminishing the source of infection. Thus, systemic death caused by inefficient virus recognition by *R* genes can be positively selected in nature, serving as a starting point for further adaptation toward more efficient recognition. On the other hand, systemic death may have been negatively selected in crop field during the history of crop-plant domestication. Based on this idea, I will discuss possible new strategies for hunting *R* genes.

O-8. *Toxoplasma Gondii* Effectors TgIST and TgGRA15 Differentially Target Host IDO1 to Antagonize the IFN-γ-induced Anti-*T. Gondii* Response in Human Cells

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Toxoplasma is an important intracellular pathogen that causes lethal toxoplasmosis in humans and animals. Interferon-γ (IFN-γ) is critical for anti-*T. gondii* responses in both human and mice. Recent extensive studies using the mouse as a model organism have revealed that IFN-γ-inducible GTPases play critical roles, and also revealed that virulent *T. gondii* can inhibit IFN-γ-mediated host immune response. Thus, the relation between host immunity and *T. gondii* virulence is well established in mice. On the other hand, IFN-γ-induced anti-*T. gondii* responses in human is not completely clear because the IFN-γ-inducible GTPase-mediated anti-*T. gondii* responses may not be major in human. Therefore, *T. gondii* virulence strategy to resist IFN-γ-induced anti-*T. gondii* responses in human also largely remains unclear. Here, at first, we generate various human cells lacking IFN-γ-inducible gene, and show that IDO1 is required for IFN-γ-induced response in various types of human cells. Then, we focus on *T. gondii* virulence mechanisms in human cell. In this study, we focus on distinct *T. gondii* virulence mechanisms involving TgIST and TgGRA15 to suppress IFN-γ-dependent immunity in human cells. We generate TgIST or TgGRA15-deficient *T. gondii* by CRISPR/Cas9 system, and show that IDO1 mRNA induction is inhibited TgIST-dependently in various types of IFN-γ-stimulated human cells, and also show that IDO1-dependent anti-*T. gondii* response is inhibited TgGRA15-dependently in secondly infected cells. Taken together, we demonstrate that *T. gondii* possesses at least two differential virulence mechanisms targeting IDO1 by TgIST and TgGRA15 to antagonize IFN-γ-induced anti-*T. gondii* responses in human cells.

P-1. The Influence of Stroking Way on the Establishment of Human-cattle Relationships

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The influence of stroking way on the establishment of human-cattle relationships was examined. In experiment 1, 16 newborn calves were used. Six newborn calves were stroked by a trainer for 6 minutes once daily for 5 consecutive days after birth (F1), and six newborn calves were stroked by a trainer for 3 minutes twice daily for 5 consecutive days after birth (F2). For four newborn calves, the trainer stared without stroking as controls (C1). In experiment 2, 12 newborn calves were used. Four newborn calves were stroked by a trainer for 3 minutes twice daily for 5 consecutive days, and four newborn calves were stroked by a trainer for 5 minutes twice daily for 3 consecutive days after birth. For four newborn calves, the trainer stared without stroking as controls (C2). In both experiments, calves were assessed human-cattle relationships in a novel arena at 1 and 3 months. In experiment 1, both stroking groups approached significantly closer to the trainer than C1 at 1 month old ($P<0.001$). F2 still approached significantly closer to the trainer than C1 ($P<0.001$) at 3 months old, indicating that more frequent stroking establishes effectively a positive human-cattle relationships. In experiment 2, there was no difference in the closest approach distance to the trainer among treatments. However, the calves in both stroking groups stayed longer within a closer area to the trainer than C2 at 1 month old. From these results, it is concluded that frequent stroking per day promote the establishment of positive human-cattle relationships.

P-2. Development of a Highly Sensitive Method for the Detection of *Cryptosporidium parvum* Virus Type 1 (CSpV1)

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Cryptosporidium is one of the most important zoonotic parasites that causes cryptosporidiosis. Although, infection occurs throughout the world in high prevalence rates, none completely effective drugs are available. Therefore, the development of a rapid and accurate diagnostic method and genetic surveillance of *Cryptosporidium* are critically required to predict and prevent the spread of infection. Recently, some studies have been focused on *Cryptosporidium parvum* virus type 1 (CSpV1), the first member within Partitiviridae family to infect protozoan host, as a new tool for detection and genetic surveillance of *Cryptosporidium*. However, these studies followed different molecular detection methods, therefore the relationship between PCR-based CSpV1 detection, the target site of the virus genome, and detection sensitivity remains unclear. In this study, we show that the second half of the coding region of dsRNA2 is effectively detected from various types of clinical samples without the need for oocyst purification by using a nested PCR technique. Importantly, our method showed higher sensitivity in field fecal samples compared with *Cryptosporidium* 18S rRNA-based detection method. Moreover, this targeted short sequence reveals a high level of genetic polymorphism compared with the *Cryptosporidium* GP60 gene. Taken together, these results suggest that our method might be good strategy for *Cryptosporidium* and/or CSpV1 analysis.

P-3. Development of Optimal Continuous Culture Method for Rumen Fluid Using Ferrite Particles

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In continuous culture of rumen fluid, the outflow of microorganisms from the reactor makes reduce the efficiency of cellulose degradation. Therefore, it was investigated to use ferrite particles (FP) to capture suspended particles and microorganisms and prevent them from flowing out of the reactor. The movement of FP is controlled by magnetically because it made of ferromagnetic iron oxide particle. The effects of addition of FB on rumen microorganisms were investigated by adding 0 to 10 vol% FP to the rumen solution. As a result, concentration of dissolved chemical oxygen demand (D-COD) and volatile fatty acid (VFA) increased in all conditions. In particular, D-COD and VFA concentrations increased rapidly with the addition of FP, which was more effective at higher concentrations of FP. These results suggest that addition of FP promoted the solubilization of paper by rumen microorganisms and took a step forward in the development of a continuous culture method.

P-4. Comparative Study of Bacterial Populations in the Feces of Pasture-kept and Stabled Horses and Ponies

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Social interactions and access to natural environments can affect the composition of animal intestinal microbiota, which plays important roles in host nutrition and immunity. Previous studies have shown that turnout and stabling may affect the behavior of horses, while it is not known how they can affect microbial composition in the intestinal microbiota. In this study, we collected fecal samples from horses and ponies kept in different environments (stabled or un-stabled, group or single turnout, pasture or paddock turnout) and compared the bacterial community structure by 16S rRNA gene next-generation sequencing. While the phylum-level bacterial composition was similar between the fecal samples, significant variations were observed at the genus-level composition. The genus *Carnobacterium*, which is commonly found in dairy and meat products, was more common in stabled horses than the pasture-turnout group. The pasture-turnout horses possessed unique bacterial groups that been found in gravy zebra's feces, which suggests that the 24h pasture-turnout may have shifted the gut microbiota composition to more "wild" composition. Besides, *Bacillus*-related bacterial groups were also common in turnout horses, which may reflect the close contact to the soil. Interestingly, horses from the same farm or facility shared similar microbiota regardless of single or group-turnout, which implies inter-host dispersal is not restricted to the group-turnout. Our findings suggest that turnout and stabling as well as inter-host dispersal are important factors shaping bacterial composition of horse intestinal microbiota.

P-5. Change Detection Using Multi-Temporal Optical Satellite Imagery for Grassland Area in Kawatabi Filed Science Center, Tohoku University

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The Great East Japan Earthquake on March 11, 2011 caused the Fukushima Daiichi nuclear disaster. The spread of radioactive materials due to this disaster affected many areas of eastern Japan. Some pasture in the polluted area have suspended their grazing use. To clarify the effect of the absence of cattle grazing to the pastures, we performed the land cover change detection using time series optical satellite images. We compared two test sites: a unused for grazing pasture since 2011 and a continuously grazed area even after the earthquake disaster for experiments. We analyzed both high and medium spatial resolution satellite images obtained after the Great East Japan Earthquake. The maximum likelihood supervised classification method was used to generate land cover maps. We extracted grassland form the land cover classification map. We calculated the grassland area, and the temporal change of the areas on the two test sites were compared. The result shows that the area of the unused pasture decreased from 66.0 ha on July 19, 2012 to 50.0 ha on September 26, 2018 based on the analysis of high-resolution satellite images. And the area obtained by the medium resolution satellite images analysis decreased from 69.6 ha on May 31, 2011 to 44.6 ha on May 26, 2018. On the other hand, there was no significant change in the continuously grazed area. It is recognized that the grassland area of the unused pasture has greatly decreased since the voluntary suspension of grazing from 2011.

P-6. Usefulness of Environmental DNA for Surveillance and Control of Schistosomiasis: Detection and Tracking *Schistosoma mansoni* and Its Intermediate Host *Biomphalaria glabrata* in Low Endemic Areas of Minas Gerais, Brazil

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Schistosomiasis is one of the most important parasite infections around the world with 3 main species of medical importance: *Schistosoma haematobium*, *Schistosoma japonicum* and *Schistosoma mansoni*. In Brazil, schistosomiasis mansoni is endemic in many areas, and uses the snail *Biomphalaria glabata* as the intermediate host. Ecoepidemiologic approach using the environmental DNA (eDNA) has been increasing over the years, because its high sensitivity and specificity and easiness of sampling. In this way, this study aimed to introduce eDNA detection for *B. glabata* and *S. mansoni* in municipalities of Minas Gerais, Brazil. Five cities were selected (Arceburgo, Comercinho, Guaranésia, Perdigão and Simão Pereira), and 18 water sources were used for eDNA detection. Tests *in vitro* using laboratory strain of *B. glabata* was performed. eDNA of single snail could detected in water even exposed in natural conditions after 96 hours. For the field samples collected, eDNA of *S. mansoni* were detected in 10 sites (Arceburgo - Farm; Comercinho - Point 4, 6, 7, 8 and 9; Guaranésia - Ribeirão; Simão Pereira - Paraibuna River - Point 1 and 2 and Perdigão - Lake); and *B. glabata* could be detected in 3 sites (Arceburgo - Farm; Comercinho - Point 5 and Perdigão - Lake). Parasite and snails eDNA could be detected in field samples showing the usefulness of the designed system for determination of active transmission sites. The application of the technique in schistosomiasis surveillance can be useful in endemic areas, for monitoring and prevention of schistosomiasis transmission.

P-7. Evaluation of Drainage Performance by Reduction of Excess Soil Moisture on Corn Fields Converted from Rice Paddy Using Satellite Remote Sensing

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Satellite remote sensing data observing three corn fields which attempted different methods of reducing soil-moisture damage were analyzed in this study. Test fields were located in Shinchi Town, Fukushima prefecture. The near-infrared band was extracted from Sentinel-2 satellite data obtained on 9 March, 13 April, 18 April, and 23 April in 2019 to estimate soil moisture. These data were acquired one to two days after the heavy rainfalls. GeoEye-1 satellite images obtained on 29 July, corn growing season, were used for calculation of Normalized Difference Vegetation Index (NDVI). Comparison between the near-infrared band reflectance by Sentinel-2 and the NDVI from GeoEye-1, a relationship between the estimated drainage performance and crop growth was investigated. In addition, the plant height and SPAD values obtained by field surveys and positions of the survey points were used for the analysis. As the result, the near-infrared band reflectance shows the effectiveness of the ridge-farm method and cut-drain method to reduce soil-moisture damage. The NDVI on crop growing stage shows a response to the observed drainage performance in the same way as the actual plant height and SPAD value. In some cases, the NDVI disagrees with the plant height and the SPAD value. However, improvement of the analysis method, as the change of a calculating buffer size could solve this disagreement. Calculation of other types of vegetation indices also could solve the discrepancy. The result of this study shows the usefulness of satellite remote sensing data for the evaluation of the soil drainage performance and the crop growth for the response to the wet damage control measures.

P-8. Characterizing the Key Factors in Potential Suppressive Anaerobic Digester Effluent on Bacterial Wilt Disease (*Ralstonia solanacearum*)

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Anaerobic digestion is a promising method for treating organic wastes, but it results in the discharge of huge amounts of effluent. Anaerobic digester effluent was reported to have the potential to suppress plant disease. However, the mechanisms of suppressive effects on plant pathogens are not yet well understood. In our study, the suppressive effect of anaerobic digester effluent on the typical soil-born plant disease, bacterial wilt (*Ralstonia solanacearum*), were investigated by screening the antagonistic bacteria from six types of anaerobic digester effluent. From the results, antagonistic bacteria were isolated from anaerobic digester effluent of vegetables, dairy manure, sludge and cattle manure, while no antagonist was detected from anaerobic digester effluent from mixed organic wastes. All of the isolated antagonists from anaerobic digester effluent were identified as *Bacillus* spp. and showed strong antagonistic activity against *R. solanacearum* in vitro. Higher Mg2+ concentrations positively affected the antagonistic activity of antagonist from the effluent. Furthermore, anaerobic digester effluent of cattle manure was applied in a pot experiment and showed a reduction trend on the disease incidence of plants. These results indicated the possibility that antagonistic bacteria contained in digested effluent of cattle manure may have been effective in inhibiting bacterial wilt. Anaerobic digester effluent with higher Mg2+ content may have a higher disease-suppressive effect by increasing the antagonistic activity of antagonists present within it. Thus, our study characterized the dominant disease-inhibiting factor in anaerobic digester effluent and provided new information for the effective use of such effluent as a bio-control agent.

P-9. Economic Evaluation of Full-Head Test for Enzootic Bovine Leukosis in Japan

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In recent years, the number of bovine leukemia cases has increased and economic loss is also increasing. Prevention of infection, early detection and prompt initial response should be put on emphasis. Eliminating positive cows can cause significant economic loss to farm management, so only diseased cows can be removed. Therefore, a possible cleaning process for Japan is to take measures to reduce the positive rate by separating positive cows detected by ELISA or PCR and negative cows. We collected statistical data from the Ministry of Agriculture, Forestry and Fisheries and Miyagi Ken and estimated the economic loss (H30) due to bovine leukemia in Japan. To clarify the economic burden of sample farmer who performed a full-head test, a simulation was performed through four cases; (1) loss for 10 years without any measures. (2) loss to achieve cleaning in a short period of time basing on eliminating of all positive cows. (3) loss to achieve cleaning in a short period of time basing on the early shipment of all positive breeding cows. (4) loss for 10 years basing on implying a full-head test and separation. The result shows that the economic loss caused by not performing a full-head test was three times that of performing full-head test. Therefore, it is considered necessary to conduct a full-head test. However, the cost of testing costs to small farmers for breeding scale of 10-49 is higher than other farmers. Larger farmers are more able to bear the cost of full-head test.

P-10. Promoting Methane Gasification in Anaerobic Scum Degradation Using Microbial Community Adapted to Scum

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To promote anaerobic scum decomposition and methane fermentation, microbial communities adapted to scum and addition of activated carbon (AC) to the reactor were investigated. In this study, two experiments were conducted using batch cultures. Microbial communities were analyzed by metagenomic analysis of 16S rRNA amplicons in each experiment. At first, it was compared degradation of scum using adapted sludge (AS) to that using unacclimated sludge (US). Methane yield under the AS condition was 15% higher than that under the US condition. Different scum loading rates on AS were also examined, and the AS condition was tolerable to 17.3 g COD/L loading. According to High-throughput sequencing data analysis, Methanosaetaceae, protein and sugar degrading bacteria have high relative abundance in AS compared that in US. Acetate was accumulated in the US condition on the 8th-15th day. On the other hand, there were no accumulation of acetate in the AS conditions. Secondly, whether the contact with scum changes microbial community on AC was verified. Floating AC had been contacted to the scum, but precipitated AC had been not. As a result, the big difference had observed in microbial communities between floating AC and precipitated AC. *Syntrophomonas* and *Acinetobacter* which degradable LCFA were much more abundant in floating AC. Adding the floating AC to the reactor, approximately twice methane yield was observed than that without AC. These results can contribute to apply to methane fermentation for lipid-rich waste.

P-11. Conspecific Distance-dependent Seedling Performance and Replacement of Conspecific- by Heterospecific Seedlings in Five Hardwood Species in a Temperate Forest

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The mechanisms creating species diversity in the context of Janzen-Connell model would be well understood by evaluating not only the conspecific distance dependent (CDD) seedling performance but also replacement effects (RE: extent of the replacement of conspecific- by heterospecific seedlings beneath adults). We evaluated CDD and RE as a log response ratio of seedling performance (height, age) between beneath and far from conspecific adults and a log response ratio of that between conspecifics and heterospecifics beneath adults, respectively, for five hardwood species with different ecological traits (i.e., seed size, mycorrhizal type, relative abundance) in a temperate forest. CDD was greater in three small-seeded species associated with arbuscular mycorrhizae (AM) than two large-seeded species associated with ectomycorrhizae (EM), probably due to that higher local conspecific densities of small-seeded species would amplify the pathogens beneath adults and AM fungi would have lower defensive ability against pathogens. As a result, small-seeded AM species showed smaller relative abundance compared to large-seeded EM species. RE was also greater for small-seeded and AM species compared to large-seeded and EM species, resulting in close positive relationship between CDD and RE. The traits indicate that replacement of conspecific by heterospecific seedlings was easily occurred in the small-seeded AM species with stronger CDD, suggesting that the small-seeded species would be attacked by strong virulence of specialized pathogens (e.g., soil pathogens, leaf diseases). The study demonstrates that the process and mechanisms creating species diversity is well understood by analysing RE effects in addition to CDD.

P-12. Effect of Rumen Fermentation Characteristics on Stress-related Hormone Concentration and Behavior of Sheep

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It is well known that behavioral stress response is related to stress-related hormones. In ruminants, the relationships between rumen fermentation and endocrine system are also suggested. We examined the effect of rumen fermentation characteristics on stress-related hormones and behavior of sheep under different diet conditions. Eight Suffolk lambs were allocated into high concentrate group (HC; 80% of concentrate and 20% of bermudagrass hay, n=4) and roughage group (RH; 100% of bermudagrass hay, n=4). The experiment consisted of 8-10 days of acclamation and 4-days measurement periods, and repeated twice. Blood samples (10 mL) were collected from jugular vein on day1 of each measurement period for plasma growth hormone (GH) and cortisol were measured. On day 2, rumen fluid was collected orally and the concentration of acetic acid, propionic acid, and butyric acid was measured. The open-field test (OFT) was conducted on day 4 of each period, and behavior of lambs were recorded for 20 minutes. In HC, the concentration of butyric acid and GH concentration tended to be higher than in RH ($P<0.1$). Frequency of escape attempt in OFT was also higher in HC than RH ($P<0.05$). Correlation analysis showed a positive relationship between sniffing frequency to the novel object and concentration of acetic acid/propionic acid ratio in the rumen ($\rho=0.545$, $P<0.05$), and negative relationship between environmental exploration frequency and blood cortisol concentration ($\rho=-0.528$, $P<0.05$). From these results, it was suggested that concentration of volatile fatty acids in the rumen affected behavioral stress response of sheep through changes in plasma hormone concentration.

P-13. Estimation of the Contribution of Salt-block Feeding to Mineral Intake of Cattle Under Grazing and Indoor Conditions

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We investigated salt-block intake of cattle under grazing and indoor conditions to evaluate how salt-block feeding contributes to fulfillment of mineral requirements. Three commercial salt-blocks were used in this study. KOEN® SELENICS TZ (ZENOAQ Co., Ltd.) was offered to two grazing herds (a sown pasture and mountainous pasture-forest combining area) and two indoor herds (breeding cows and calves). COWSTONE® 100 TZ (ZENOAQ Co., Ltd.) was offered to early and late fattening beef steers and heifers in shed. SALTICK® (Shiraishi Calcium Co., Ltd.) was offered to dry and lactating dairy cows in free stall shed. The recording of salt licking behavior (duration and number of licking), to estimate salt intake of the animals, and amount of salt-block intake during three consecutive days was repeated three times in summer and autumn, respectively. The contents of thirteen elements (Na, Cl, S, P, Ca, Mg, K, Fe, Zn, Cu, Mn, Co, Se) in the diets (grazing forages and compound rations) and salt-blocks were determined. The amount of salt-block intake ranged from 0.04±0.01 g/day/kg $BW^{0.75}$ of dry dairy cows in autumn to 0.63±0.12 g/day/kg $BW^{0.75}$ of calves in summer. The mineral intake from diets did not fullfill cattle requirements of Na, P, Ca, Zn, Cu and Se. The salt-block contribution ratios were high in Na (6.4-85.8%), Cl (2.2-45.7%), Zn (2.8-25.8%), Cu (0.2-22.7%), Co (7.3-65.0%) and Se (0.0-94.5%), and Na met requirement in most of the herds. However, salt-block intake could not make up for the deficiencies of Zn (beef, dry and lactating cow), Cu (dry and lactating cow) and Se (beef cow in summer and dry cow).

P-14. A Multifaceted Analyses of the Effects of Medicinal Plant *Phyllanthus ninuri* on the Improvement of Severe Malaria

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Malaria is one of the most prevalent infectious diseases caused by *Plasmodium* parasites. *Plasmodium falciparum* is the major cause of this disease in Africa region, and it can lead to cerebral malaria (CM) which is one of the features of severe malaria. CM induces Interferon-gamma (IFN-γ)-dependent neurological damage by affecting several central nervous system (CNS). For a long ago, *Phyllanthus* species, medicinal plant, have got expansive uses in traditional methods for improvement of severe malaria in Africa region. However, little is known about the effect of this plant on CM. In this study, we analyzed the effect of *Phyllanthus ninuri* extracts (PNE) on IFN-γ expression, brain cell damage or parasitic growth. At first, we investigated the effect of the PNE on IFN-γ expression level in human T cell. As a result, IFN-γ expression level in the PNE treated cells inhibited compare to non-treated cells. This result suggests that, the PNE potentially inhibit the expression of inflammatory cytokine expression which coursed CM. Next, we investigated the effect of the PNE on apoptosis of human brain cells. As a result, activation of apoptotic caspase in the PNE treated cells was suppressed compared to non-treated cells. This result suggests that the PNE potentially prevents the apoptotic damage of CNS. Finally, the investigation of the effect of the PNE on parasite growth is ongoing. This presentation will discuss our current progress in the research about the multifaceted effects of PNE on the improvement of severe malaria.

List of scientific papers in 2019 published by field science group in Graduate School of Agricultural Science, Tohoku University

The Forest-Andisols Group

Baba, Y., Y. Matsuki, Y. Mori, S. Takizawa, Y. Suyama, C. Tada, Y. Fukuda, M. Saito and Y. Nakai (2019) Pretreatment of lignocellulosic biomass with cattle rumen fluid for methane production: fate of added rumen microbes and indigenous microbes of methane seed sludge. Microbes and Environments, 34(4): 421-428.

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Information to Authors from Journal of Integrated Field Science (JIFS)

The Journal publishes articles in all areas of field science in Agricultural science. The journal is an English magazine started in 2003 fiscal year when Integrative Field Science Center, Graduate School of Agricultural Science, Tohoku University, has started. Our journal places the edit committee. Under the committee, an original paper including short paper, proceedings, a review, description, and data are published. An original paper will be peer reviewed by two referees. Our journal has published one volume in principle every year and we will start to publish it as an on-line journal from volume 15 (JIFS: http://www.agri.tohoku.ac.jp/jp/about/field/jifs/index.html). We will also publish all the manuscripts on the web site as e-journal (Tohoku University Repository: https://tohoku.repo.nii.ac.jp/).

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Abstract

An informative, self-explanatory abstract should be about 250 words as a single paragraph. In the abstract you must not use subtitles such as Methods and Results. Do not cite tables, figures or literatures.

Introduction

The “author and year” style of literature citation should be in e.g., Sano (2008) or (Sano, 2008) and Sano and Ito (2008) or (Sano and Ito, 2008) or Sano et al. (2008) or (Sano et al., 2008) for literature by one, two, three or more authors, respectively.

Materials and Methods

Results

Discussion

(or Results and Discussion)

(Acknowledgement(s))

References

When citing references in the text, give the name of the authors and the year of publication in parentheses. Examples for literature citation are given below. Do not number the references listed.

If a literature is non-English language is cited, note the language at the end of the literature. Examples for literature citation are given below.

Lauenroth, W. K. and R. Gill (2003) Turnover of root systems, H. de Kroon and E.J.W. Visser (Eds.), Root Ecology, Springer, Berlin, pp. 61-89.

Sakaigaichi, T., Y. Terajima, S. Irei, S. Fukuhara, K. Ujihara, A. Sugimoto, J. Abe, R. Tajima and M. Matsuoka (2006) Estimation of root direction based on growth direction of shoot roots in sugarcane (*Saccharum* spp. hybrid). International Society of Sugar Cane Technologists: Agronomy Workshop Abstract Book. p40.

Sano, O., T. Ito and M. Saigusa (2008) Measurement of organic phosphorus mineralization in non-allophanic Andosols using anion exchange resin. Journal of Integrated Field Science, 6: 109-115.

The case articles from journals, give the name of the author, the year of publication in parentheses, the title of the paper, the name of the journal, volume and the page, in that order. The case from books, give the name of the author, the year of publication in parentheses, the title of the articles, the name of the book, the name of the editors, the page (inclusive), and the name and city of the publisher, in that order.

Tables

Using the same software as used for the text, create tables on A4 size and number them sequentially with Arabic numerals (e.g. Table 1.) in the order which they are cited in the manuscript. Give the caption on the top of each table, and footnotes, legends etc. should be under the table.

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Use figures directly created as camera-ready copy. Place each figure on A4 size and number them with Arabic numerals (e.g. Fig. 1.) in the order which they are cited in the manuscript.

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