

ASSESSMENT OF PRION UPTAKE BY PLANTS USING PROTEIN MISFOLDING
CYCLIC AMPLIFICATION

by

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DEDICATION

To my family, whose guidance installed the belief that there was no limit to my success, and who taught me all you need is a thumbs up to express the utmost encouragement.

And to my girlfriend, who has shown me what it feels like to be infinite.

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ABBREVIATIONS

BH	brain homogenate
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob disease
CWD	chronic wasting disease
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
GPI	glycosyl phosphatidyl inositol
HY	“hyper” strain of hamster-passaged transmissible mink encephalopathy
i.c.	intracerebral
IgG	immunoglobulin G
LS	Linsmaier and Skoog plant growth medium
mb-PMCA	miniaturized bead protein misfolding cyclic amplification
MS	Murashige and Skoog plant growth medium
NBH	normal brain homogenate
PBS	phosphate buffered saline
PMCA	protein misfolding cyclic amplification
PMCAb	protein misfolding cyclic amplification with beads
PrP	prion protein without distinction for different isomers
PrP ^C	normal cellular prion protein
PrP ^{TSE}	disease-associated misfolded prion protein; also abbreviated PrP ^{Sc} , PrP ^d , or PrP ^{BSE,CJD,CWD} , etc.

PrP ^{res}	proteinase K-resistant core of prion protein which could be identical to PrP ^{TSE} , also known as PrP ²⁷⁻³⁰
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TME	transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
UK	United Kingdom
vCJD	variant Creutzfeldt-Jakob disease

Assessment of Prion Uptake by Plants Using Protein Misfolding Cyclic Amplification

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Prion diseases (also known as transmissible spongiform encephalopathies, or TSEs) are infectious neurodegenerative disorders that affect a variety of mammals. Typical of all TSEs is the misfolding of the normal prion protein isoform (PrP^{C}) into an abnormal, β -sheet rich conformation (PrP^{TSE}), which is capable of propagating itself by recruiting and converting PrP^{C} . The increasing prevalence of two prion diseases, chronic wasting disease (CWD) and scrapie, across North America in captive and free-ranging animals indicates involvement of horizontal transmission throughout contaminated environments. Studies have shown that prions can enter the environment through shedding of bodily fluids, including urine, saliva, blood, and feces, from infected animals in addition to decomposition of diseased animal carcasses. Prions also bind tightly to soil particles and remain infectious years after initial introduction to the environment, suggesting that soil may serve as an environmental disease reservoir. Vegetation is ubiquitous in prion-contaminated environments and, because plants have demonstrated the capability of absorbing whole proteins and other pollutants, plants may interact with prions and provide an additional route of transmission in natural habitats. To investigate the potential uptake

of prions, we analyzed stem and leaf tissues that had been exposed to prion-infected brain homogenate for the presence of prion protein using protein misfolding cyclic amplification (PMCA). Our results show evidence of prions in these aerial tissues from the model plant *Arabidopsis thaliana* as well as the crop plants alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*), and corn (*Zea mays*) grown in sterile hydroponic and soil cultures. A quantitative PMCA procedure was utilized to calculate the concentrations of prion protein in the stem and leaves from these species, before and after taking the inhibitory effects of plant material during PMCA into consideration. Our results demonstrate that various species of plants are capable of taking up prion protein upon root exposure to prion-contaminated media and suggest that plants may play a currently unrecognized role in horizontal transmission of the CWD and scrapie agents.

CHAPTER ONE:

Introduction

1.1 Overview and history of transmissible spongiform encephalopathies

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a unique class of neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE, or “mad cow” disease) in cattle, and chronic wasting disease (CWD) in deer, elk, and moose. Human forms of the disease include Creutzfeldt-Jakob disease (CJD), fatal familial insomnia, Gerstmann-Sträussler-Scheinker syndrome, and kuru. In each case, the etiological agent is proposed to be an abnormal isomer (PrP^{TSE}) of the host encoded prion protein (PrP^{C}) (Prusiner, 1998). TSEs are characterized by prolonged incubation periods (months to years) during which host PrP^{C} is converted into PrP^{TSE} that accumulates predominantly in the central nervous system prior to the onset of clinical disease. This extended preclinical phase of disease progression currently prevents identification of infected humans and animals prior to neural damage (Prusiner, 1998). Clinical signs of disease include tremors, ataxia or myoclonus, behavioral changes, hyperexcitability, and emaciation (Center for Food Security and Public Health, 2008; Detwiler, 1992). Although rare, TSEs are capable of causing widespread epidemics with devastating effects on human, animal, and environmental health.

Evidence suggests that scrapie, the archetypal prion disease, was first observed by sheep farmers and breeders in the United Kingdom in 1732 (McGowan, 1922) and spread rapidly during the next few decades as a result of inbreeding of certain flocks to improve the quality of wool (Brown and Bradley, 1998). Early descriptions indicated a lengthy clinical phase that comprised of “shaking in its hind-quarters... rubbing themselves very much, and reeling about as if intoxicated” (Liberski, 2012). Additionally, loss of appetite led to emaciation and eventual death of the infected animal (Animal Health and Veterinary Laboratories Agency, 2013; Scottish Agricultural College, 1998). In the late 19th century, the scientific study of scrapie commenced in

Europe to identify the etiological agent. Besnoit and colleagues identified neuronal vacuolization as a characteristic feature of the disease, but their attempt to show transmissibility failed to produce any infected sheep, most likely resulting from short observation periods (Besnoit, 1899; Besnoit and Morel, 1898). However, after taking note of epidemiological studies that observed incubation periods of 18 months or longer in naturally-occurring scrapie (Brown and Bradley, 1998), Cuillé and Chelle successfully demonstrated the transmissibility of the disease in 1936 by inoculating brain and spinal cord tissue from scrapie-diseased sheep into healthy sheep that subsequently developed scrapie after up to two-year incubation periods (Cuillé and Chelle, 1936; Cuillé and Chelle, 1938; Cuillé and Chelle, 1939). The transmissible nature was confirmed in a separate event when sheep immunized against louping ill with a vaccine prepared from scrapie-infected brain, spinal cord, and spleen tissues resulted in an outbreak of scrapie in England (Gordon, 1946). While available laboratory techniques could not identify the infectious agent, several lessons about its unusual properties were learned – scrapie is transmissible, the agent is found in the central nervous system of diseased animals, and prolonged incubation periods occur before the onset of clinical signs.

Around this same time, human TSEs were becoming more prominent as Alfons Jakob and Hans-Gerhard Creutzfeldt both independently observed a number of patients displaying clinical signs mildly suggestive of multiple sclerosis, but with a deranged mental state (Creutzfeldt, 1920; Creutzfeldt, 1921; Jakob, 1921a; Jakob, 1921b). They believed the patients displayed a condition distinct from all other known mental diseases and soon called it Creutzfeldt-Jakob disease (CJD). While the spongiform degeneration in brain tissues from terminal patients was present (Jakob, 1923), CJD was not believed to be transmissible because no evidence of a host immune response existed (Creutzfeldt, 1920; Gerstmann et al., 1936). In

1959, William Hadlow recognized similar neuropathological findings between scrapie and kuru (Hadlow, 1959), a fatal neurodegenerative disorder in humans restricted to cannibalistic tribes from regions of Papua New Guinea (Gajdusek and Zigas, 1957; Zigas and Gajdusek, 1957), that implied kuru might also be transmissible. Successful laboratory transmission of kuru (Gajdusek et al., 1966; Gajdusek et al., 1968) and CJD (Gajdusek and Gibbs, 1971) to chimpanzees led to observations of clinical signs similar to those of the two human TSEs. Scientific interest in these diseases increased dramatically upon identification that animal and human TSEs were related and that transmissibility was one of the defining hallmarks.

In addition, another TSE that emerged in the 20th century in North America was chronic wasting disease (CWD), the only known prion disease to affect free-ranging deer, elk and moose species (cervids) (Baeten et al., 2007; Williams and Miller, 2002). CWD was first detected in deer held in captivity at wildlife research facilities in Colorado and Wyoming in the 1960s (Williams and Young, 1980), and later discovered in free-ranging populations in 1981 (Williams and Young, 1982). The origin of CWD is still unknown, but two main theories have been suggested: 1) a cross-species transmission event occurred where mule deer and white-tailed deer developed CWD from sharing pastures and ranges with scrapie-infected sheep (Race et al., 2002), or 2) spontaneous mutation of the prion protein gene in a single mule deer started the subsequent transmission to other deer and elk (Williams and Miller, 2002). Difficulties in controlling CWD are attributed to lack of effective ante-mortem diagnostic tests (Miller and Williams, 2002; Monello et al., 2013; Wolfe et al., 2002), varying incubation periods before clinical signs are present (Center for Food Security and Public Health, 2008; Johnson et al., 2011; USGS National Wildlife Health Center, 2013), and environmental persistence of the causative agent (summarized in later sections of this report). These factors have contributed to

the geographical expansion of CWD across North America with confirmation of disease in 22 U.S. states, two Canadian provinces, and South Korea (Kim et al., 2005; USGS National Wildlife Health Center, 2014) (Figure 1-1). In areas where CWD has become endemic, the prevalence has increased to upwards of 90% in captive herds (Keane et al., 2008; Williams and Young, 1980) and 40-50% in free-ranging herds (Saunders et al., 2012; Wyoming Game and Fish Department, 2011).

Major public interest in prion diseases did not come about until after identification of bovine spongiform encephalopathy (BSE), or “mad cow” disease, as an emerging epizootic in the United Kingdom (UK) in the 1980s and 1990s (Wells et al., 1987). Epidemiological studies identified meat-and-bone meal, a supplement high in protein created from tissues from various species, as a common feature in all UK farms with cattle infected with BSE (Wilesmith et al., 1988). One hypothesis for the origin of BSE suggested, similar to CWD, that a sporadic case of BSE developed and was accidentally incorporated into meat-and-bone meal, leading to the epidemic (Weissmann and Aguzzi, 1997). Another hypothesis stated that, because neuropathological findings in BSE are similar to those seen in scrapie, BSE may have resulted from cattle infected with a scrapie-like agent (Smith and Bradley, 2003; Wilesmith et al., 1991; Wilesmith et al., 1988). Even though the BSE strain was found to be molecularly and biologically different from the scrapie strain (Bruce et al., 1994), widespread concern was raised about the possibility that TSEs may be able to circumvent the species barrier and infect other mammals, including humans. (Bruce et al., 1997; Lennon, 1996). In 1996, a new type of CJD, designated variant CJD (vCJD) was reported in the UK (Will et al., 1996), and the geographical

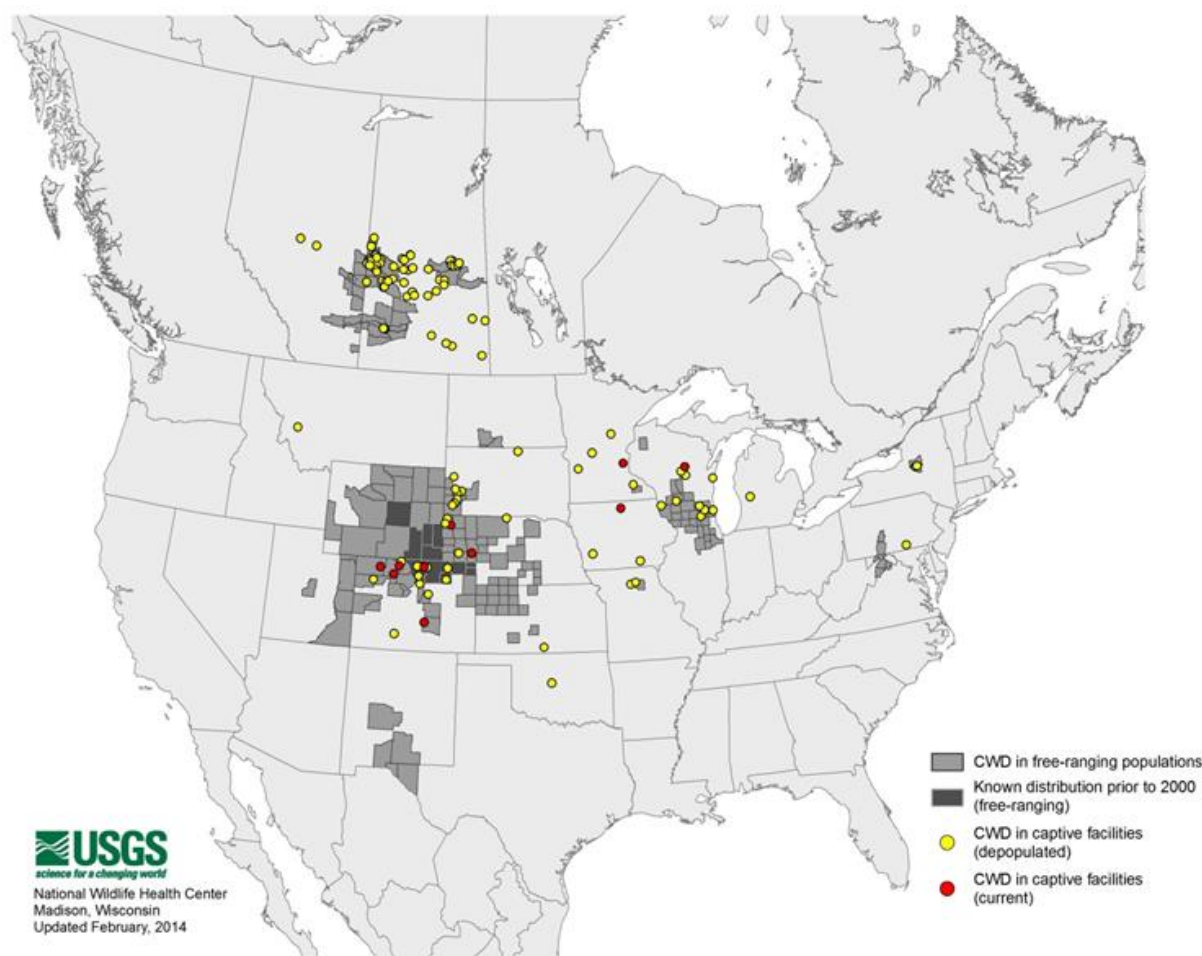


Figure 1-1. Known distribution of chronic wasting disease in North America. Since its known distribution prior to 2000 (dark gray shading), CWD has spread from Colorado and Wyoming to 20 additional U.S. states, 2 Canadian provinces, and South Korea (not shown), as of February 2014. All locations are approximations based on best-available information (USGS National Wildlife Health Center, 2014).

association to BSE raised concerns that there may be a causal relationship. Scientific evidence supported BSE as the likely cause of vCJD (Belay and Schonberger, 2005; Bruce et al., 1997; Hill et al., 1997; Scott et al., 1999), highlighting the disease risk posed to humans consuming infected bovine tissue (Brown et al., 2001). As of June 2014, 229 worldwide cases of vCJD (Centers for Disease Control and Prevention, 2014) and more than 180,000 indigenous cases of BSE in the UK alone have been reported while one to three million cattle may have been infected with BSE and entered the food chain in a subclinical phase (Donnelly et al., 2002).

Many countries throughout Europe had imported cattle and meat-and-bone meal from the UK, leading to the detection of BSE in 19 other European countries (Belay and Schonberger, 2005; Matthews, 2003). Additionally, preventative measures were attempted by Canada, the United States, and Mexico to collectively ban the importation of cattle from countries already affected by or were at risk of possessing BSE-infected livestock; nevertheless, there have been 23 cases of BSE identified in North America (four in the United States) as of April 2012 (Centers for Disease Control and Prevention, 2013). In total, the direct cost of the BSE crisis has been estimated at €3.7 billion (Beck et al., 2005; Richey, 2006) while indirect effects in regards to public trust and the surveillance of farmed animals are still being measured today.

Even though research has been ongoing for centuries, much remains unknown about TSEs including their etiology, transmission, and pathogenesis. Moreover, these diseases are invariably fatal, and no effective treatments or cures are presently available. While no evidence of cross-species transmission to humans has been demonstrated for CWD or scrapie (Beringue et al., 2008; Chatelain et al., 1981; Sigurdson, 2008), limited epidemiological studies cannot rule out the possibility that these TSEs harbor the potential to become zoonotic agents similar to the cross-species “jump” of the BSE agent from cattle into humans. Prion diseases remain threats to human and animal health and can have destructive wildlife and economic impacts; therefore, further understanding of prion transmission and its interactions in natural environments are of the utmost importance.

1.2 Neuropathology and diagnosis of TSEs

After manifestation of clinical symptoms, prion disease is confirmed post-mortem by histopathological examination of brain tissue (Kubler et al., 2003). TSEs in humans and animals

are characterized by a number of pathogenic events that occur in the central nervous system (CNS) of the host including spongiform vacuolization, which leads to the “sponge-like” appearance of CNS tissue, propagation of astrocytes and microglia, and neuronal degeneration (Budka, 2003; Collinge, 2001; Prusiner, 1998). This classical neuropathological triad may also be accompanied by the deposition of amyloid plaques comprised of aggregates of the abnormal PrP^{TSE} isomer (Beck and Daniel, 1987), as seen in Figure 1-2. Immunohistochemical detection relies on the accumulation of PrP^{TSE} aggregates as a diagnostic marker for confirmation of prion disease in infected hosts (Kubler et al., 2003), although negative PrP staining in clinically-diseased hosts can occur during neurological assessment (Brown et al., 1994).

Spongiosis and vacuolization within the brain are the most consistent characteristics of TSEs during the onset of disease and is distinguished by dispersed and clustered vacuoles in the cerebellum or grey matter (Voigtlander et al., 2001). The severity of spongiform change varies depending on the size of PrP^{TSE} fragments and can even be ambiguous or absent (Almer et al., 1999), but lesion profiles may also be influenced by age of host at infection, route of infection, and the duration of clinical signs (Begara-McGorum et al., 2002; Ligios et al., 2006). Importantly, the distribution and localization of PrP^{TSE} does not always take place in similar regions of the brain. For sporadic CJD (sCJD) and iatrogenic CJD (iCJD), genetic prion diseases in humans, and BSE in cattle, PrP^{TSE} and infectivity is mainly found in the CNS in contrast to vCJD, scrapie, and CWD where peripheral tissues are also affected, most notably the lymphoid system (Hilton et al., 2002; Schreuder et al., 1996). Therefore, species of the infected mammal should be deemed an important factor when considering the potential for disease transmission.

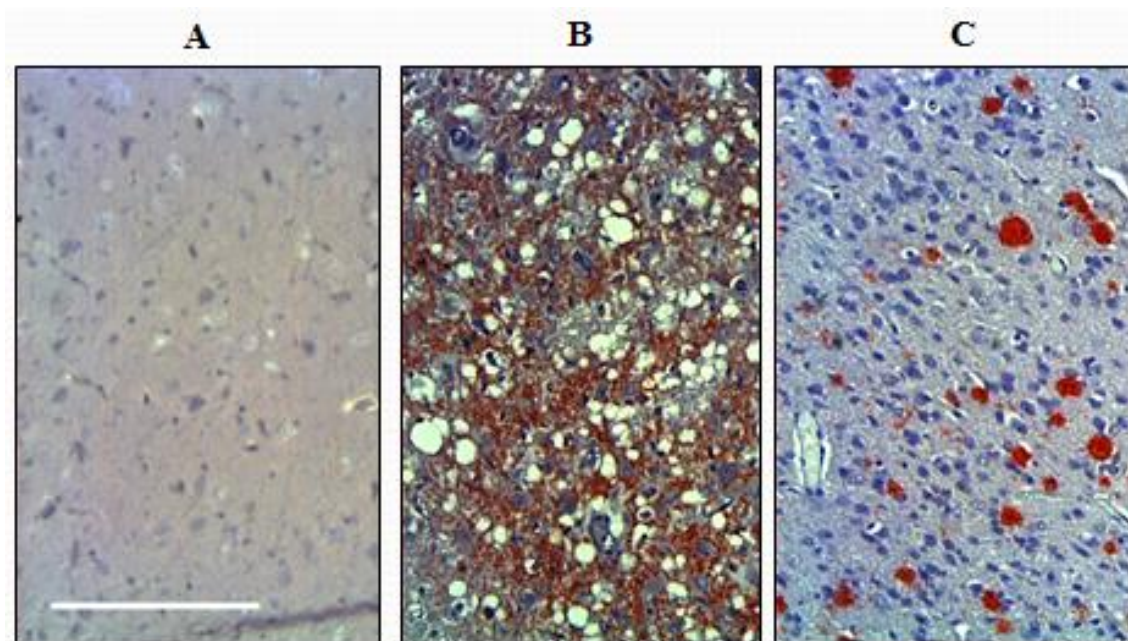


Figure 1-2. Characteristics of TSE-infected and non-infected brain tissue as assessed by immunohistochemistry (IHC). Healthy, non-infected meadow vole brain tissue (A) is compared with prion-infected meadow vole brain from the cerebral cortex (B and C). Brains display spongiform vacuolization (B) and accumulation of PrP^{TSE} (C) stained red following IHC using anti-PrP monoclonal antibody SAF 83 and counterstained with hematoxylin. Scale bar indicates 10 μ m (Images courtesy of Christina Carlson, USGS National Wildlife Health Center).

1.3 Prion protein and the TSE disease agent

While experimental evidence indicated that both kuru and CJD were transmissible diseases (Gajdusek et al., 1966; Gajdusek and Gibbs, 1971; Gajdusek et al., 1968), the infectious agent could not be identified because of the limitations of laboratory techniques available at the time. In light of exceptionally slow disease progression exhibited by TSE-infected hosts, led to the hypothesis that TSEs were caused by a “slow virus” too small to purify, but able to be filtered and capable of transmitting from infected host to naïve host (Alper et al., 1967). However, remarkable resistance to procedures which modify nucleic acids, including UV and ionizing radiation, nucleases, high temperatures, and high pressures, ruled out known bacteria and viruses as the causative agent (Alper et al., 1967; Alper et al., 1978; Griffith, 1967). Other characteristics such as hydrophobicity and a propensity for aggregation further complicated

identification of the infectious agent of prion diseases (Prusiner et al., 1980; Prusiner et al., 1978).

In the 1960s, Griffith and colleagues hypothesized that the disease agent might be composed of a protein able to replicate in the body (Griffith, 1967). After a number of years, purification and isolation of the major component of infective fractions from scrapie-infected hamster brain homogenate (BH) was accomplished (Prusiner et al., 1982), and the term “prion” was created to distinguish the infectious agent in TSEs from bacteria, parasites, and viruses. Stanley Prusiner received the 1997 Nobel Prize in Medicine for the identification and characterization of prions, which he defined as “small proteinaceous infectious particles which are resistant to inactivation by most procedures that modify nucleic acids” (Prusiner, 1982; Prusiner, 1998). The protein-only prion hypothesis postulates that the infectious agent in human and animal TSEs is composed largely, if not solely, of an aberrant protein, designated PrP^{TSE} (for TSE-associated prion protein) (Prusiner, 1982). PrP^{TSE} is a misfolded isomer of the host prion protein (PrP) that is capable of self-propagation by converting the normal cellular form of PrP, designated PrP^{C} , into the infectious, disease-associated form (PrP^{TSE}). Subsequent experiments using TSE-infected hamster BH led to the isolation and discovery of a proteinase K (PK) resistant form of PrP^{TSE} , designated PrP^{res} (Bolton et al., 1982; Prusiner et al., 1982). PrP^{TSE} was confirmed as the major component of the infective fraction through correlations between infectivity and PrP^{TSE} concentration (Bolton et al., 1982; Hope et al., 1986; Prusiner et al., 1982). Purification of PrP^{TSE} determined that it is encoded by a single host gene while a protein of similar size and identical amino acid sequence was identified in non-infected brains that, unlike PrP^{TSE} , was completely digested by protease treatment (Basler et al., 1986; Bolton et al., 1982;

Stahl and Prusiner, 1991). Thus, instead of a novel protein, the disease-associated PrP^{TSE} proved to be a modified isomer of a normal brain protein, PrP^C (Westaway et al., 1987).

1.3.1 Structure of the prion protein

The PrP gene (PRNP) is highly conserved in mammalian species, suggesting a wide-ranging function and important evolutionary role (Windl et al., 1995; Wopfner et al., 1999). PrP encoded by PRNP was the first protein recognized to exist in two different conformations despite having the same primary structure (Prusiner, 1998). One conformation, the normal cellular isomer of the prion protein, PrP^C, is a glycoprotein of about 250 amino acids that is expressed predominantly on the cell surface of neurons within the CNS (Stahl et al., 1987). PrP^C is also widely expressed throughout the body and regulated in skeletal muscle, the peripheral nervous system, and organs such as the lymph nodes, spleen, liver, lungs, kidney, and heart, (Aguzzi and Heikenwalder, 2006; Aguzzi and Polymenidou, 2004; Bendheim et al., 1992; Caughey et al., 1988). PrP^C is synthesized in the endoplasmic reticulum (ER) and passes through the Golgi on its way to the cell surface, where PrP^C is located mainly in lipid rafts and attached to the cellular membrane via a glycosyl phosphatidyl inositol (GPI) anchor. Throughout its biosynthesis in the ER, PrP^C is subject to a number of post-translational modifications, including cleavage of the N-terminal signal peptide upon entry into the ER, N-linked glycosylation, formation of a single disulfide bond, and cleavage of the C-terminal signal sequence for the attachment of the GPI anchor (Haraguchi et al., 1989; Harris, 2003; Stahl et al., 1987; Turk et al., 1988) (Figure 1-3).

Structural information about the N-terminal domain of PrP^C is incomplete because of the highly flexible nature of this segment (Donne et al., 1997; Riek et al., 1997); nevertheless, the N-

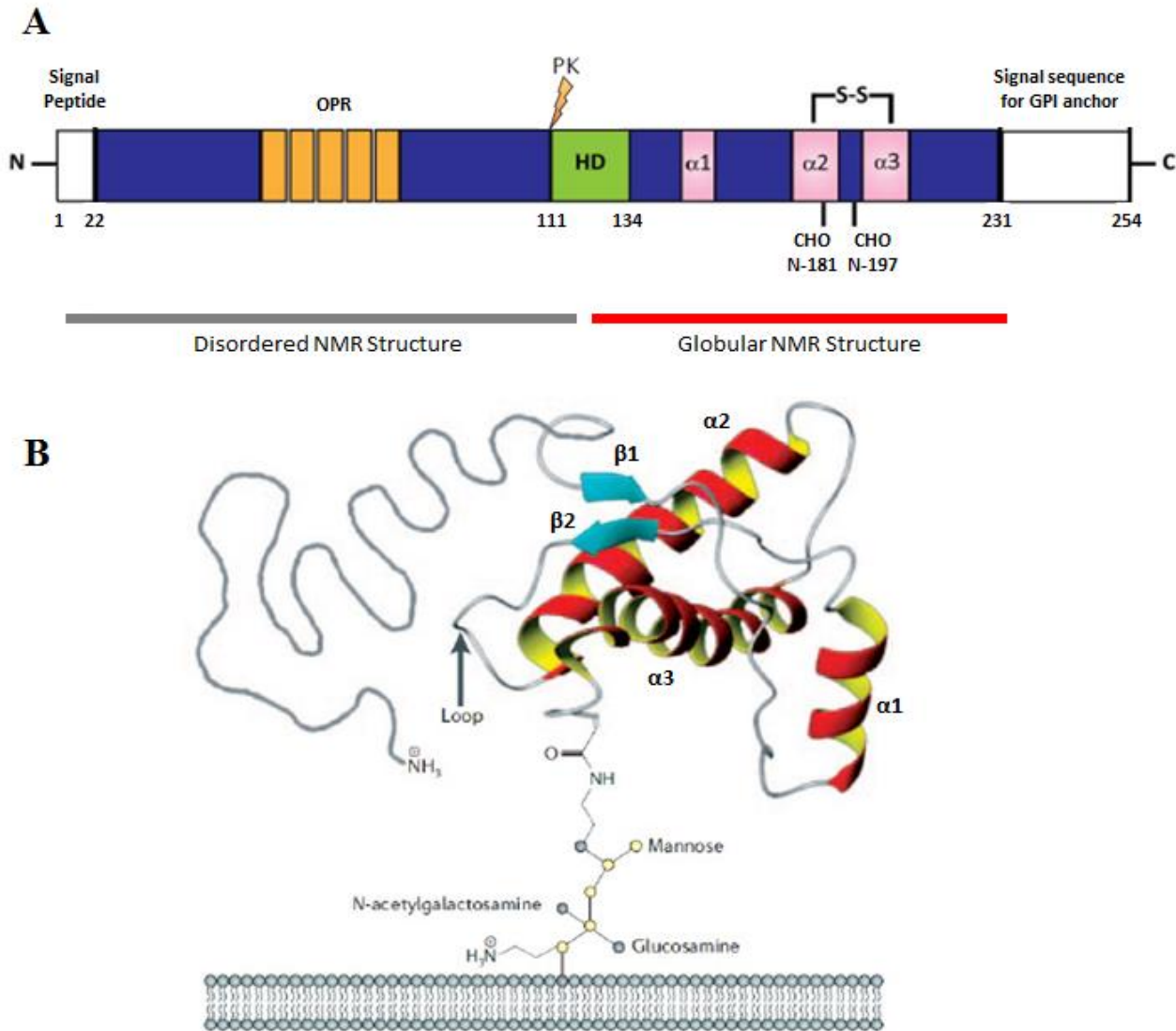


Figure 1-3. Primary and tertiary structural features of the cellular prion protein (PrP^C). (A) An outline of the primary structure of PrP^C including post-translational modifications. The numbers beneath the bar diagram describe the position of the respective amino acids. Human PrP is comprised of a secretory signal peptide at the extreme N-terminus and another signal sequence at the extreme C-terminus (white), OPR (orange) defines the octapeptide repeat region, HD (green) defines the hydrophobic domain, three α -helices (pink), one disulfide bond (S-S) between residues 179 and 214, and two potential N-glycosylation sites. The PK-resistant core of PrP^{TSE} is depicted between residuals 134 and 231, and the approximate cutting site of PK within PrP^{TSE} is indicated by the lightning bolt. (B) Tertiary structure of PrP^C including the unstructured N-terminal tail (gray), the locations of the three α -helices and two β -sheets, and the GPI anchor attached to the lipid bilayer. The loop connecting β 2 and α 2 is indicated by the black arrow. Image adapted from (Aguzzi and Heikenwalder, 2006).

terminus does contain two defined, conserved regions. One region is a set of five octapeptide repeats, shown to have binding sites for copper and zinc (Hosszu et al., 1999; Riek et al., 1998), and the other is comprised of a hydrophobic core that may function as a transmembrane domain at the cell surface (Forloni et al., 1993; Glover et al., 2001). Unlike the N-terminus, the C-terminal domain of PrP^C is structured consisting of three α -helices and two short β -sheets (Knaus et al., 2001; Riek et al., 1996). Two *N*-linked glycosylation sites indicate PrP can exist in three glycoforms, di-, mono-, or unglycosylated states, and are conserved in all mammalian PrP genes analyzed to date, suggesting functional significance for prion protein glycosylation (Lawson et al., 2005).

While the three-dimensional structure of PrP^C has been established by nuclear magnetic resonance (NMR) spectroscopy (Figure 1-4a), there is little known about the structure of PrP^{TSE} because of its highly insoluble and aggregated nature in non-denaturing detergents. PrP^{TSE} has the same primary sequence and post-translational modifications as PrP^C (Basler et al., 1986), but their different properties could be attributed to variations in secondary, tertiary, and quaternary structures. Analysis through multiple computational techniques revealed the secondary structure of PrP^{TSE} contains a higher β -sheet and little-to-no α -helical content compared to PrP^C (Caughey et al., 1991; DeMarco et al., 2006; Pan et al., 1993; Smirnovas et al., 2011), implying that the formation of PrP^{TSE} may involve transformation of α -helices from PrP^C into β -sheets for PrP^{TSE}. This conformational change alters the biophysical properties of PrP^{TSE}, producing a number of distinguishing characteristics between the two isomers. PrP^{TSE} forms insoluble amyloid aggregates in water and detergents while PrP^C is soluble in both solutions (Pandeya et al., 2010; Prusiner et al., 1982). Furthermore, PrP^C is fully degradable by PK treatment while PrP^{TSE} is

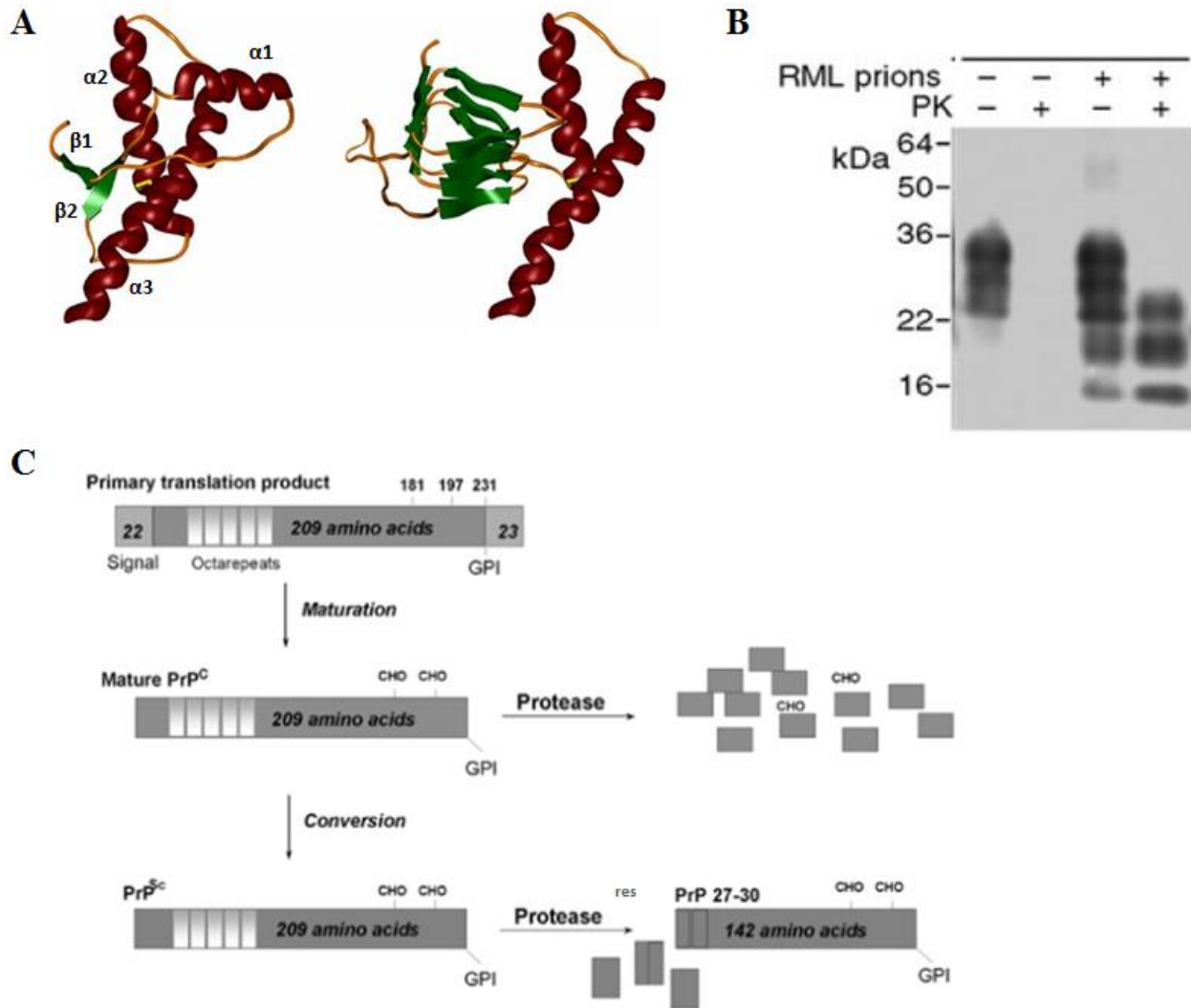


Figure 1-4. Differences in prion protein isomers. (A) NMR structure of PrP^{C} (left) and PrP^{TSE} (right) minus the N-terminal regions. The globular C-terminal domain of PrP^{C} contains high α -helix content (red) while one hypothetical structure for PrP^{TSE} deduced from electron microscopic analyses of purified material indicates a much higher β -sheet content (green) (Images courtesy of Cedric Govaerts, UCSF). (B) Western blot of 10% (w/v) non-infected (lanes 1 and 2) and prion-infected (lanes 3 and 4) mouse brain homogenates. Samples in lanes 2 and 4 were digested with 50 $\mu\text{g/mL}$ PK for 30 minutes at 37°C. PK treatment completely hydrolyzes PrP^{C} while cleaving only about 90 amino acids from the N-terminus of PrP^{TSE} to generate PrP^{res} , or PrP^{27-30} . Image adapted from (Goold et al., 2011). (C) Bar diagrams describing the post-translational processing of PrP. Upon reaching its destination on the cell surface, the N-terminal signal peptide is cleaved as well as the C-terminal signal sequence during the addition of the GPI anchor. Once modifications are completed, mature PrP^{C} and PrP^{TSE} both contain 209 amino acids and have the same primary sequence. The normal isomer PrP^{C} is protease-sensitive while the pathogenic isomer PrP^{TSE} is partially protease-resistant and produces PrP^{27-30} . Image adapted from (Aguzzi and Heppner, 2000).

partially resistant to PK and leaves the truncated form of the protein, PrP^{res} (Parchi et al., 1998) (Figure 1-4b and c).

1.3.2 Function of PrP^C

Even though PrP^C is expressed throughout the body, its main function has yet to be determined. PrP knockout mice display no abnormalities in development with only slight irregularities in sleep patterns (Bueler et al., 1992; Mallucci et al., 2002; Tobler et al., 1996); in fact, the only overt phenotype is resistance to infection after inoculation with prions whereas susceptibility is restored after introducing PrP^C back into the host (Bueler et al., 1993), providing further support for the “protein-only” prion hypothesis. Studies have identified numerous roles PrP^C actively participates in once matured. The observation that PrP^C is a copper-binding protein is the most widely agreed upon function (Brown et al., 1997a; Kramer et al., 2001; Stockel et al., 1998; Westergard et al., 2007). Defects in copper metabolism are linked to the pathogenesis of TSEs, leading researchers to believe PrP^C may play a part in regulating copper release at the neuronal synapse (Brown, 2001a; Cerpa et al., 2005; Herms et al., 1999; Waggoner et al., 1999), and copper may be essential for the function of PrP^C (Jackson et al., 2001). The cell surface location of PrP^C could also activate transmembrane signaling pathways and initiate signal transduction (Kanaani et al., 2005; Santuccione et al., 2005) in addition to being involved with cell adhesion (Graner et al., 2000; Mange et al., 2002; Schmitt-Ulms et al., 2001) and cellular trafficking (Forloni et al., 1993; Glover et al., 2001). Furthermore, PrP^C may be involved with neuroprotective functions against internal or environmental stresses (Roucou et al., 2004; Roucou and LeBlanc, 2005), protection of cells from oxidative stress (Brown et al., 2002; Brown et al., 1997b; Milhavet and Lehmann, 2002), synaptic structure and transmission (Carleton et al.,

2001; Collinge et al., 1994), cell-to-cell communication (Fevrier et al., 2004), and circadian rhythms (Lasmezas, 2003; Martins et al., 2002), among others. However, to date, no definitive consensus as to the function of PrP^C has been determined.

1.3.3 Conversion of PrP^C to PrP^{TSE}

Prion replication requires direct interaction between the PrP^{TSE} template and endogenous PrP^C that drives the formation of infectious prions (Prusiner et al., 1998). The central feature in prion disease pathogenesis is the conversion of PrP^C to PrP^{TSE} that occurs post-translationally after PrP^C has reached its normal extracellular location. While evidence exists implicating other proteins or molecular chaperones participate in the refolding event as cofactors (Deleault et al., 2003; Telling et al., 1995; Wang et al., 2010), PrP^C is the one component required for replication as PrP knockout mice fail to develop prion disease (Bueler et al., 1993). Nevertheless, the mechanism by which this conversion takes place remains to be elucidated.

Two competing models have been proposed to describe the transformation between the PrP isomers. In the template-assisted refolding model, monomeric PrP^{TSE} interacts with monomeric PrP^C to form a heterodimer with the potential dependence of an unidentified protein, designated protein X, capable of binding to PrP and assisting in this conversion (Cohen et al., 1994; Prusiner, 1998). This conversion causes the unfolding and restructuring of the normal PrP^C molecule into PrP^{TSE}, and, after separation of the newly-formed homodimer, the two new PrP^{TSE} molecules can bind more PrP^C molecules to start the replication process over again (Figure 1-5a). However, protein X has never been isolated during *in vitro* replication. Alternatively, the seeded-nucleation model suggests PrP^C and PrP^{TSE} are in a reversible thermodynamic equilibrium that strongly favors PrP^C, and conversion takes place only when several monomeric PrP^{TSE} molecules

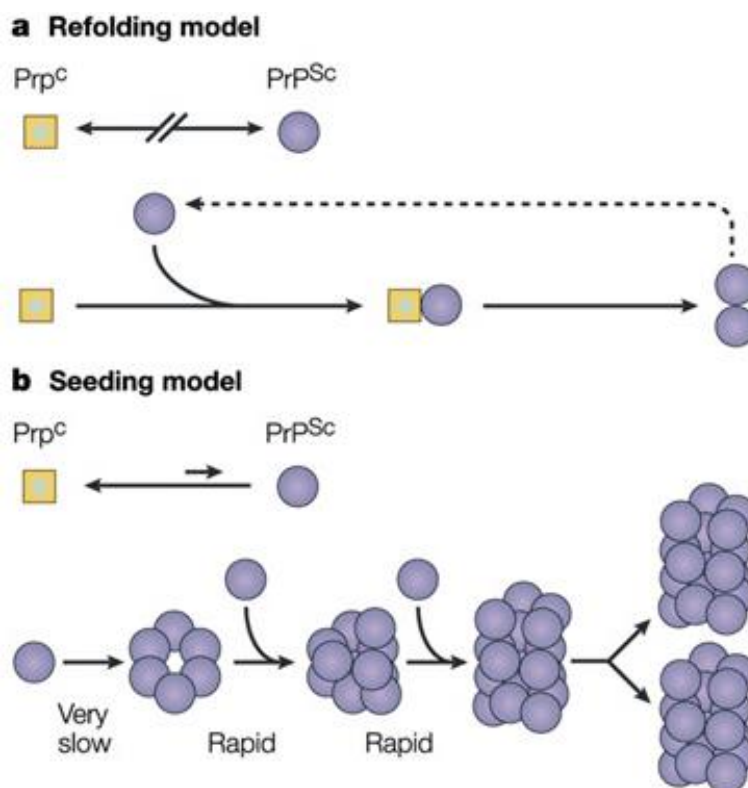


Figure 1-5. Models for the conformational conversion of PrP^C to PrP^{TSE}. (A) Template-assisted conversion, or refolding, model proposed by (Prusiner, 1991). While a high activation energy barrier preventing spontaneous conversion, exogenous PrP^{TSE} (blue circles) causes PrP^C (yellow squares) to undergo an induced conformational change to produce PrP^{TSE}, which may be facilitated by an enzyme or molecular chaperone. (B) Seeded-nucleation conversion, or seeding, model proposed by (Jarrett and Lansbury, 1993). PrP^C and PrP^{TSE} are in equilibrium, with PrP^C strongly favored, where PrP^C is only converted after direct interaction with seeds or aggregates of PrP^{TSE}. Adapted from (Weissmann, 2004).

fixed in a highly-ordered seed directly interact with monomeric PrP^C. The formation of the seed would be rare, but, once present, it will recruit PrP^C quickly and cause aggregation into an amyloid structure (Weissmann, 1999) (Figure 1-5b). The seed must also contain a minimum number of molecules to initiate PrP^{TSE} formation, reported as few as 2-7 PrP molecules (Bellinger-Kawahara et al., 1988; Deleault et al., 2007), whereas oligomers of 14-28 PrP molecules were reported as the most infectious entities (Silveira et al., 2005). At some point, the amyloids break into smaller pieces by an unknown process where fragmentation increases the

number of seeds and enables further aggregation (Soto, 2011; Weissmann, 2004). Consistent with this model, cell-free conversion and protein misfolding cyclic amplification (PMCA) studies indicate PrP^{TSE} aggregates can convert PrP^{C} into the infectious isomer (Caughey et al., 1995; Kocisko et al., 1994; Kocisko et al., 1995; Saborio et al., 2001).

1.4 Prion transmission and persistence in the environment

One of the unique characteristics of prion diseases is the host-to-host transmissibility of the TSE agent. While intracerebral (i.c.) inoculation is the most efficient route to generate infection within a host, a major route of prion transmission in the environment is through oral ingestion (Belay et al., 2004; Bruce et al., 1997) where accumulation and amplification of the disease agent begins in the lymphoid tissues associated with the gut (Andreoletti et al., 2000; Safar et al., 2008). Not only did the BSE epizootic transmit by cattle consuming contaminated meat-and-bone meal (Wilesmith et al., 1988), but human TSEs such as kuru and vCJD were also direct consequences of either ritualistic cannibalism of infected tissues (Gajdusek and Zigas, 1957) or ingestion of BSE-contaminated beef (Brown, 2001b; Hill et al., 1997). The nasal cavity, scarified skin, and conjunctiva have also been suggested as alternative routes of infection (Hamir et al., 2008; Kincaid and Bartz, 2007; Mohan et al., 2004).

Among the known TSEs, only CWD and scrapie are communicable within a population and show horizontal transmission under natural conditions. Because CWD is the only known TSE to affect free-ranging animals (Williams and Young, 1982), coupled with the observance of scrapie regularly occurring in previously affected areas (Pallson, 1979), the idea that CWD and scrapie prions remain infectious in the environment is likely (Williams, 2005). As the molecular mechanisms behind this natural transmission remain largely unknown (Gough and Maddison,

2010), routes of secretion and excretion and the presence of prion agents within environmental samples have been studied extensively. Not only does vertical transmission from mother to lamb or fawn contribute to disease spread (Detwiler and Baylis, 2003; Nalls et al., 2013), but horizontal transmission through direct or indirect contact can occur as well (Dexter et al., 2009; Schramm et al., 2006). TSE infectivity may be released into the environment from infected animals in a variety of bodily fluids, including saliva, urine, feces, blood, and milk (Castilla et al., 2005b; Gonzalez-Romero et al., 2008; Haley et al., 2009b; Kruger et al., 2009; Lacroux et al., 2008; Maddison et al., 2009; Maddison et al., 2010b; Mathiason et al., 2006; Saa et al., 2006a; Terry et al., 2011), and prion shedding can occur many months prior to clinical manifestation of the disease (Haley et al., 2009a; John et al., 2013; Saa et al., 2006a; Tamguney et al., 2009).

Because the TSE agent is also present throughout a diseased host, including skeletal muscle, cardiac muscle, peripheral nervous tissue, fat, and the central nervous system (Race et al., 2009; Sigurdson, 2008), CWD and scrapie prions will enter the environment through decomposition of infected animal carcasses (Miller et al., 2004). Once deposited in the environment, TSE infectivity can accumulate in soil and near environmental fomites, including building surfaces and fence posts, (Pedersen and Somerville, 2011). Burying large numbers of animal carcasses could also foster a dissemination of the disease agents in surrounding soils or groundwater (Scudamore, 2001). Furthermore, the total CWD titers shed in saliva, urine, and feces from an infected animal over the course of its lifespan may be approximately equal to the titer contained in the CNS of an infected carcass (Gregori et al., 2008b; Tamguney et al., 2009; Tamguney et al., 2012). Naïve cervids can become exposed to the CWD disease agent in the environment a number of ways: orally, through soil ingestion while feeding or grooming on contaminated pastures (Weeks, 1978) or contact with infected surfaces (Mathiason et al., 2009);

through the nasal cavity in the absence of consumption (Kincaid and Bartz, 2007); or through scarification, by scratching or rubbing on fomites where the TSE agent had been previously deposited (Pedersen and Somerville, 2011). Figure 1-6 summarizes horizontal transmission of CWD while also emphasizing a potential role for “hot spots” of concentrated prion infectivity, including areas of communal activity where shedding occurs, animal mortality sites, mineral licks, and scrapes (Denkers et al., 2011; Jennelle et al., 2009).

One factor influencing environmental transmission of CWD and scrapie prions is their long-term survival and stability in the environment. Healthy sheep and deer can become infected with scrapie or CWD while grazing on pastures where infected animals had been previously housed (Georgsson et al., 2006; Miller and Williams, 2002). TSE infectivity is known to persist in soil for a number of years. The scrapie agent was found to remain infectious after burial in soil for three years (Brown and Gajdusek, 1991; Seidel et al., 2007) while evidence exists that the scrapie agent may be preserved under environmental conditions for at least 16 years (Georgsson et al., 2006). Similarly, Miller and colleagues demonstrated the CWD agent could transmit from infected deer carcasses to naïve animals after decomposition of the carcasses occurred two years earlier (Miller et al., 2004). Exposing naïve deer to water, feed buckets, and bedding used by CWD-infected deer also led to acquisition of the disease (Mathiason et al., 2009). Finally, prions can survive typical wastewater process and bind to biosolids (Hinckley et al., 2008) while transport by surface water (Nichols et al., 2009) and predators and various scavengers, such as lions, coyotes, and vultures may also contribute to the spread of the CWD agent (Jennelle et al., 2009; Miller et al., 2008).

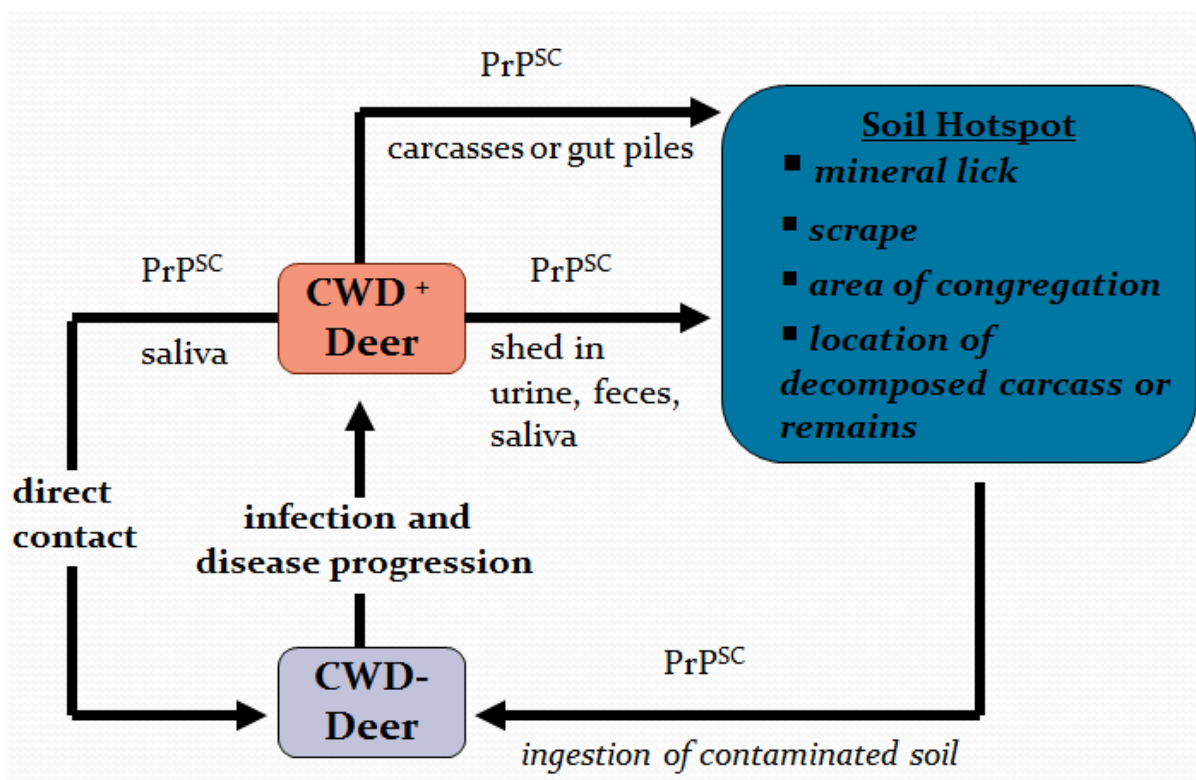


Figure 1-6. Potential routes of horizontal chronic wasting disease transmission. Conceptual model of direct (via host-to-host contact or physical interaction in bodily fluids like saliva and urine) and indirect (via contaminated environmental fomites or soil “hot spots”) environmental transmission of CWD agent. Diagram adapted from (Schramm et al., 2006).

1.5 Soil as a potential environmental reservoir

The studies cited above suggest that, once prions are shed from infected animals, formation of a reservoir within the environment occurs that can maintain prion infectivity for long periods of time. While other reservoirs have been hypothesized (Fitzsimmons and Pattison, 1968; Post et al., 1999), soils appear to be a likely source of prion infection in the horizontal transmission of scrapie and CWD. As seen in Figure 1-6, ingestion or inhalation of contaminated soil takes place for indirect exposure of prions to naïve animals. Grazing animals can ingest soil intentionally for the acquisition of nutrients and incidentally when eating leafy plants (Beyer et al., 1994; Hui, 2004; Wiggins, 2009). Deer may consume anywhere from dozens (Arthur III and

Allredge, 1979) to hundreds of grams of soil per day (Mayland et al., 1977; Weeks and Kirkpatrick, 1976), providing the potential pathway for uptake of TSE infectivity into new hosts.

According to Schramm and colleagues, six things must remain true for soil to serve as an environmental reservoir: “1) prions must enter the environment; 2) prions must persist in soil; 3) prions in soil must retain infectivity; 4) prions must remain near the soil surface where they can be accessed by animals; 5) naïve animals must be exposed; and 6) the dose must be sufficient to cause infection” (Schramm et al., 2006). A variety of plausible routes describing prion introduction into and evidence of persistence in the environment were detailed in the previous section. Once deposited in the environment, PrP^{TSE} has demonstrated the ability to strongly adsorb to soil and soil minerals (Cooke et al., 2007; Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007; Wiggins, 2009), remain infectious once bound to soil minerals (Johnson et al., 2007), and maintain that infectivity for years (Brown and Gajdusek, 1991; Georgsson et al., 2006; Miller et al., 2004; Seidel et al., 2007). In fact, the binding between soil and PrP^{TSE} is essentially irreversible and only exposure to harsh chemicals, like anionic surfactants not present in natural environments, can elute PrP^{TSE} (Cooke et al., 2007). Experimental evidence suggests clay particles, specifically montmorillonite, are responsible for this strong adsorption of prions in the environment because of their high binding capacity (Johnson et al., 2006). Other soil minerals and compounds have also been associated with prion adsorption, including quartz, organic material, and tannins excreted from trees (Cooke et al., 2007; Jacobson et al., 2013; Polano et al., 2008; Rigou et al., 2006), indicating clay particles may not be the only factor determining prion binding capacity in soils (Wyckoff et al., 2013),

The mobility of prions in soil has not been thoroughly investigated; nevertheless, saturated column experiments were utilized to observe any migration of PrP^{TSE} through soils and

porous landfill materials. Jacobson and colleagues demonstrated no elution of PrP^{TSE} occurred using artificial rainwater as an eluent in columns of five varying soil types in addition to minimal elution when landfill leachate was used as the eluent for fine quartz sand, municipal solid waste, natural soils, and green waste residual (Jacobson et al., 2009; Jacobson et al., 2010). Limited transport in column experiments was also reported under varying water content and microbial activity (Cooke and Shaw, 2007). Taken together, these results suggest that, consistent with the strong attachment of PrP^{TSE} to soil particles, extended washing of soils with rainwater or landfill leachate alone is inefficient to promote migration of PrP^{TSE} to depths lower than the soil surface, resulting in TSE infectivity to remain at or near the site of dissemination (Smith et al., 2011).

Supporting the hypothesis that soil represents a plausible environmental reservoir, prions bound to soil and mineral particles retained infectivity when i.c. inoculated into hamsters (Johnson et al., 2006). Moreover, Johnson and colleagues later discovered that prions not only maintain oral infectivity when bound to soil particles, but PrP^{TSE} binding to montmorillonite resulted in an increase in infectivity compared to that of the unbound agent (Johnson et al., 2007). The formation of soil-prion complexes are believed to enhance prion transmission by protecting prions from inactivation during intestinal uptake, and this impact on oral TSE transmission warrants additional research (Smith et al., 2011). Much remains to be understood about the mechanisms of PrP^{TSE} adsorption to soil particles and how infectivity is increased by adsorption. Once bound to soil and immobilized, prions may persist at the surface and become accessible to grazing animals (Bartelt-Hunt and Bartz, 2013). A study in northern Colorado suggested that elevated levels of clay minerals in certain regions corresponds to an increased incidence of CWD infection, which may explain recent rises in CWD prevalence in free-ranging cervids (Walter et al., 2011). It should be noted that prion detection in soil has been successful

only in laboratory settings, and, to date, no studies have demonstrated the presence of PrP^{TSE} or prion infectivity in naturally-contaminated soils (Gough and Maddison, 2010). However, soil is likely an environmental reservoir for prion infectivity and plays a significant role in horizontal transmission of CWD and scrapie.

1.6 Role of plants in environmental transmission of prions

Because plants are ubiquitous in contaminated environments, they may be affected by the deposition of prions into the soil. Plants can absorb a variety of substances from soil, including water, metals, and inorganic and organic compounds for proper growth and development. Nitrogen is one of the most important primary macromolecules that plants acquire from the environment. Even though inorganic nitrogen (NO_3^- and NH_4^+) is easier for plants to acquire and is readily assimilated (Nasholm et al., 2009), soil microorganisms outcompete plants for these simple inorganic compounds (Johnson, 1992; Kaye and Hart, 1997; Nadelhoffer et al., 1985). Therefore, organic matter, predominantly as protein, released from the remains of animals, bacteria, and other plants (McEwen, 2011; Read, 1991) is an alternative nitrogen source for plants (Chapin et al., 1993; Kielland, 1994; Qualls et al., 1991), especially in harsh arctic (Kielland, 1997; Nordin et al., 2004) and alpine (Lipson and Monson, 1998; Miller and Bowman, 2002; Raab et al., 1996) ecosystems where inorganic nitrogen content is low.

Evidence as far back as 1909 suggests plants take up proteins through root hairs while studies performed in the 1960s and 1970s indicate absorbed proteins perform relevant biological functions in numerous species (Drew et al., 1970; Jones, 1909; McLaren et al., 1960; Seear et al., 1968; Sung, 1974; Ulrich et al., 1964). Absorption of amino acids from test solutions was demonstrated in various plants (Bright et al., 1983; Kielland, 1994; Shobert and Komor, 1987;

Soldal and Nissen, 1978), and better growth was observed on mixtures of amino acids and inorganic nitrogen than on inorganic nitrogen only (Joy, 1969). Whole exogenous proteins are also taken up by numerous plant species, including barley, rice, birch trees, pearl millet, and sorghum, as a sole source of nitrogen (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980; Okamoto and Okada, 2004; Yamagata and Ae, 1999). Recently, the woody plant *Hakea actites* and the herbaceous model plant *Arabidopsis thaliana* were shown to uptake whole bovine serum albumin (BSA) and green fluorescent protein (GFP) in root cells and use BSA as the lone nitrogen source for growth without assistance from other organisms (Paungfoo-Lonhienne et al., 2008). Similarly, barley roots exposed to various amino acids displayed the capacity to absorb them all from solutions containing amino acid concentrations lower than those found in the environment (Jamtgard et al., 2008).

While *A. thaliana* was discovered to preferentially take up certain bacterial species that are beneficial for its growth (Bulgarelli et al., 2012), pollutants can still enter root hairs. Soils amended with sewage sludge contain elevated concentrations of heavy metals such as zinc, copper, nickel, and lead (Alloway and Jackson, 1991; McBride, 2003; Sposito et al., 1982), and metal contamination was found in turnip, carrot, and potato roots (Ali and Al-Qahtani, 2012). In addition, the insecticidal Cry1Ab protein was present in aerial tissues of crops grown on fields previously used to grow corn (Icoz et al., 2009). Taken together, the previous reports support the concept that plants take up proteins and exogenous contaminants, demonstrating that plants may serve as a currently unrecognized prion transmission route in the environment.

1.7 Protein misfolding cyclic amplification

While common diagnostic tools such as western blotting and enzyme-linked immunosorbent assay (ELISA) can reliably detect prion infection in tissues, these assays are not sensitive enough to detect the low levels of prions in bodily fluids and various non-neural tissues (Head et al., 2005; Mathiason et al., 2006; Saa et al., 2006b). After recognizing that the BSE agent led to vCJD in humans (Bruce et al., 1997; Hill et al., 1997), the development of screening tests for TSEs during the pre-clinical phase became even more paramount. In 2001, Saborio and colleagues identified a highly sensitive technique where minute amounts of PrP^{TSE} present in a sample can be amplified to levels which can be readily detected using the assays listed above (Saborio et al., 2001). This technology, known as protein misfolding cyclic amplification (PMCA), was the first *in vitro* amplification assay developed for prions and has already been used to detect prions from a number of TSEs, including scrapie in sheep, BSE in cattle, CWD in deer, vCJD and sCJD in humans, and scrapie in rodent, hamster, and vole models (Castilla et al., 2005b; Haley et al., 2009a; Johnson et al., 2012; Jones et al., 2007; Murayama et al., 2007; Soto et al., 2005).

PMCA reactions are initiated by combining seeds of PrP^{TSE} from a suspected infected sample with uninfected normal brain homogenate (NBH) expressing a surplus of PrP^{C} , which acts as a substrate. Samples are then subjected to incubation and sonication cycles to promote conversion of PrP^{C} to PrP^{TSE} and amplify total PrP^{TSE} in each sample (Saborio et al., 2001) (Figure 1-7). Previous studies have considered one cycle of amplification as about thirty minutes of incubation followed by a 40-second pulse of sonication (Castilla et al., 2005b; Haley et al., 2009a; Saa et al., 2006b; Saborio et al., 2001); however, lengths of incubation and sonication can

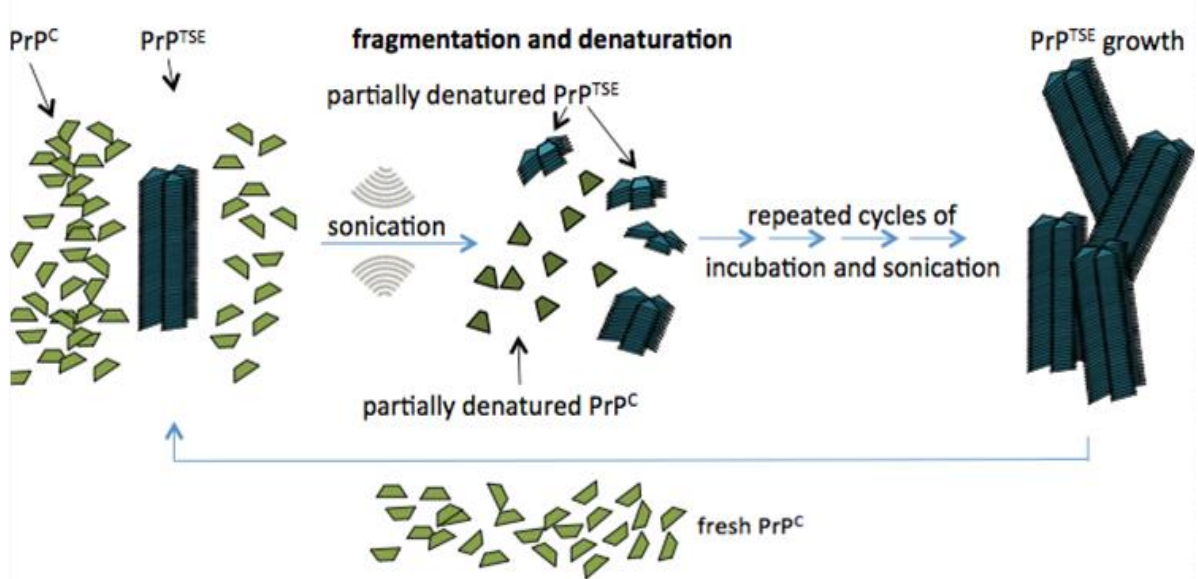


Figure 1-7. Schematic representation of the serial PMCA process. Cyclic amplification subjects a sample containing relatively small quantities of PrP^{TSE} in an excess of PrP^{C} to repeated cycles of incubation and sonication. Incubation assists the growth of PrP^{TSE} aggregates as they convert and incorporate PrP^{C} into the polymer while sonication breaks down large aggregates into many smaller pieces to multiply the number of potential converting units. PMCA rounds can be repeated by combining products from previous rounds with fresh PrP^{C} to amplify undetectable quantities of PrP^{TSE} (Saborio et al., 2001; Soto et al., 2002).

vary depending on the prion strain (Castilla et al., 2008; Daus et al., 2013; Murayama et al., 2006; Pritzkow et al., 2011). Normally, PMCA samples undergo 96 cycles of amplification over a 48-hour period, representing one round of PMCA. Once completed, the products can be either analyzed by western blotting or serially diluted in fresh NBH substrate for multiple PMCA rounds (Castilla et al., 2005a; Soto et al., 2002).

Typical western blotting cannot detect PrP^{TSE} in dilutions lower than 1,000-fold without the assistance of PMCA (Castilla et al., 2005b; Saa et al., 2006b). Castilla and colleagues demonstrated one round of amplification can increase sensitivity of detection by approximately 6,600-fold compared to western blotting (Castilla et al., 2005b) while seven PMCA rounds increases sensitivity by 3 billion-fold and can induce PrP^{C} conversion with only a single particle

of oligomeric infectious PrP^{TSE} present in the sample (Saa et al., 2006b). This ultrasensitive technique has led to the detection of PrP^{TSE}-affected tissues and bodily fluids previously unrecognized for prion accumulation (Castilla et al., 2005b; Chen et al., 2010; Gonzalez-Romero et al., 2008; Haley et al., 2009a; Mathiason et al., 2006; Pulford et al., 2012; Saa et al., 2006a). Even compared to animal bioassays, thought to be the gold standard for TSE diagnosis and prion detection, PMCA has proven to be greater than 4,000 times more sensitive (Johnson et al., 2012; Makarava et al., 2012; Saa et al., 2006b) and can be completed in weeks whereas bioassays can take up to two years. As appropriate controls and necessary precautions are put in place to avoid false-positives and *de novo* generation of prions, PMCA provides faithful *in vitro* amplification and, when combined with existing detection systems, offers the opportunity to investigate diverse samples that might be contaminated with prions, including plant tissues.

1.8 Importance of thesis work to prion biology

The previous information presented on TSEs and the ability of prions to transmit horizontally through direct and indirect contact in contaminated environments contributed to the overarching goals of my thesis work. Evidence for the considerable longevity of prions in natural environments has been recognized for decades (Brown and Gajdusek, 1991; Georgsson et al., 2006; Seidel et al., 2007) while soil appears to be a highly plausible environmental reservoir for prions resulting from the maintenance of infectivity and enhanced oral transmission of soil-bound prions (Johnson et al., 2007; Johnson et al., 2006), the lack of migration of prions from the initial deposition site (Jacobson et al., 2009; Jacobson et al., 2010; Seidel et al., 2007), and the consumption of soil by herbivores (Arthur III and Alldredge, 1979; Weeks and Kirkpatrick, 1976). Despite this knowledge, routes of natural transmission remain incompletely understood.

Prions likely accumulate in soil once deposited into the environment, and, because plants are ubiquitous in CWD-contaminated environments, it seems plausible that plants could be interact with prions and serve as a route for environmental prion transmission.

The aims of this work were twofold: firstly, to determine the capacity for prion uptake by various plant species, and secondly, to quantify the prion protein load taken up by plants and determine the disease risk associated with prion contamination of plants. In order to detect the uptake of prions by plants, the model plant *A. thaliana* and a variety of crop species were first grown hydroponically in prion-containing media for various lengths of time. Leaf and stem tissues were harvested and homogenized, then subjected to PMCA for prion amplification and detection. Additionally, *A. thaliana* was grown in a variety of soils with differing compositions rendered with an enriched prion media preparation, and plant tissues were also subjected to PMCA. We found that plants are able to take up prions in both hydroponic and soil culture environments, and each plant absorbed varying amounts of PrP^{TSE}, indicating that prion uptake is dependent on plant type and substrate composition. To quantify the amount of prion protein in exposed plants, a method was adapted from Chen and colleagues that calculated concentrations of PrP^{TSE} in various bodily fluids and tissues using PMCA (Chen et al., 2010). Quantifying prion concentrations from plant tissues suggest that consumption of infected plants can lead to prion infectivity sufficient to cause illness through the oral route.

Overall, this work was intended to provide additional understanding of CWD and scrapie transmission in the environment and demonstrate another potential route of TSE infectivity. Resulting from the fact that many reports already imply soil is a potential natural reservoir for CWD and scrapie (Cooke et al., 2007; Johnson et al., 2007; Johnson et al., 2006; Schramm et al., 2006), any components found in natural conditions, including plants, may represent a risk of

TSE exposure to naïve animals. While CWD surveillance programs have predominantly relied on voluntary submission of deer brain samples after hunting and removal of animals in infected areas to slow disease transmission (Chronic Wasting Disease Alliance, 2014; Samuel et al., 2003; United States Department of Agriculture, 2014), these efforts may be hindered by environmental reservoirs and unknown CWD foci. The mechanisms behind prion attachment to soil particles and their uptake by plants remain to be elucidated, and further studies are needed to determine these implications on the transmission of CWD and scrapie in the environment.

CHAPTER TWO:
Materials and Methods

2.1 Animals

The animal work was conducted at the United States Geological Survey National Wildlife Health Center under the following approved controls through the Animal Care and Use Committee: #EP080716-A3 and #EP090616B. The Syrian golden hamsters used exclusively for this study were purchased from Harlan Laboratories (Madison, WI).

2.2 Source of brains containing infectious prion protein

Hamster PrP^{TSE} was produced by experimentally infecting Syrian golden hamsters with the hyper (HY) strain derived from hamster-passaged transmissible mink encephalopathy (TME) (Bessen and Marsh, 1992; Marsh and Kimberlin, 1975). Brains were harvested from animals displaying clinical signs of disease consistent with TSEs and stored at -80°C until used. Non-infected brains were never experimentally challenged or placed in areas where prion-challenged animals were housed or exposed to tools previously used to handle prion-contaminated materials.

2.3 Enrichment of PrP^{res} by enzymatic degradation of TSE-affected brain tissue

Brain tissue was treated according to a novel prion enrichment method developed for this study that more closely mimics environmental degradation of prion-infected tissues than currently available protocols (Bolton et al., 1991; Raymond and Chabry, 2004). HY-infected or non-infected brain tissue was made into a 10-20% (w/v) homogenate in either a 1:1 ratio of deionized and tap water at pH 5.7 for soil cultures or 1× PBS or 10 mM HEPES at pH 7.0 for hydroponic cultures. Because all major biomolecules – nucleic acids, lipids, proteins, etc. – are found in mammalian tissue, BHs were subjected to a series of sequential enzyme treatments that aimed to degrade these biomolecules and enrich exclusively for PrP^{res}. Before use, all enzyme stock

solutions were prepared and stored according to the manufacturer's instructions. To begin, 10 µg/mL of DNase I (Worthington Biochemical Corporation #LS002139, lot S0N12281A) and 10 µg/mL RNase A (AppliChem #A2760, lot 8M003055) were simultaneously added to HY-infected or non-infected BHs and incubated for 24 hours at room temperature (RT) on an end-over-end mixer. Following nuclease treatment, 100 µg/mL of AY30 lipase (Acros Organics #2947, lot A0269035) was added to BHs and incubated for 24 hours on an end-over-end mixer at RT. Finally, 50 µg/mL of PK (Sigma #P9290, lot 037K5159) was added to BHs and incubated for 24 hours on an end-over-end mixer at RT. Protease digestion was halted by either 1 mM Pefabloc SC (Roche Diagnostics #11836170001, lot 14493900) that was added to BHs and incubated for 1 hour on an end-over-end mixer at RT for the soil culture or low speed centrifugation (≤ 0.2 RCF) and separation of BH preparations from PK-agarose pellets by pipette transfer for the liquid culture.

Immediately following enzymatic digestion, lipid extraction was performed on BHs using three parts Freon-113 (1,1,2-trichloro-1,2,2-trifluoroethane; ChemNet #CN1099.9) and two parts BH with incubation on an end-over-end mixer for 30 min at RT. The aqueous upper phase containing PrP^{res} was separated from the denser Freon-113 phase by pipette transfer to a new sterile micro-centrifuge tube. BHs were then dialyzed using Slide-A-Lyzer G2 dialysis cassettes (10 kDa MWCO; Thermo Scientific #66380) where deionized water (pH 7.0) was changed 1-3 times per day over a five-day period at 4°C to remove small molecular weight contaminants and products of the enzymatic digestion process. Dialyzed BH samples were diluted to a 4-10% working concentration for all subsequent analyses, placed in a new sterile micro-centrifuge tube, and stored at -80°C until needed.

2.4 Plant growth and culture conditions

Arabidopsis thaliana ecotype Columbia [Col-0] was used as the model plant in this study in addition to alfalfa (*Medicago sativa*), barley (*Hordeum vulgare*), and corn (*Zea mays*) crop species. To meet the specific requirements of individual experiments, various plant growth and culture systems were employed, described in the following sections:

2.4.1 Vertical plate agar culture

Surface-sterilized seeds were grown on square (100 mm x 15 mm) or circular (surface growth area of 60.1 cm²) petri dishes containing 40-50 mL of either nitrogen-free 0.5× Murashige and Skoog (MS) (Murashige and Skoog, 1962) or nitrogen-free 0.5× Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1965) basal salt solution (Caisson Labs, North Logan, UT) supplemented with 15 g/L sucrose, 0.4-0.5% phytoagar (MIDSCI, St. Louis, MO), and 2.5 mM MES buffer at pH 5.7 (Thermo Fisher Scientific, Inc., Waltham, MA). Autoclaving of agar solution first occurred for 20 minutes at 121°C and 15 psi to ensure a sterile growth media before the addition of seeds. Once plated, seeds were incubated at 4°C for 3-5 days, then transferred to a growth rack (RT with constant 24 hour fluorescent lighting at intensity of 150 μmol/m² per second) and braced in a vertical position.

2.4.2 Horizontal plate agar culture

Plate and seed preparation as well as growth conditions for horizontal agar cultures were identical to those for vertical plate agar cultures (described above), with the exception that plates were placed in a horizontal position for plant growth. Both agar cultures were utilized for growth in the absence of prion-containing media.

2.4.3 *Ice-Cap liquid culture*

Developed by Krysan and colleagues, most of the plants used in these studies were grown using the Ice-Cap system, which provided a method for high throughput plant growth and tissue sampling in a shortened time period (Clark and Krysan, 2007; Krysan, 2004; Su et al., 2011). Briefly, surface-sterilized plant seeds were placed onto the surface of sterile agar plugs (agar composition identical to that used in vertical plate agar culture above; 200 - 400 μL agar per well, depending on plant species) in 96-well unfritted deep well plates (Fisher catalog no. 278012). Once seeded, plates were covered with clear plastic lids (e.g. lids taken from Falcon Microtest flat bottom 96-well polystyrene plates; Falcon catalog no. 351172), wrapped in aluminum foil, and incubated at 4°C for four days to synchronize germination. Following incubation, foil was removed and seedling plates were transferred to a growth rack (RT with constant 24 hour fluorescent lighting at intensity of 150 $\mu\text{mol}/\text{m}^2$ per second) for 3-5 days, or until seedlings germinated.

Once germination occurred, seedling plates were transferred to the Ice-Cap continuous watering system developed by and maintained in the laboratory of Dr. Patrick Krysan at the University of Wisconsin-Madison (Clark and Krysan, 2007). Unlike the classical Ice-Cap approach, our seedling plates were floated in the water-filled upper metal pan reservoir without lower root plates, and plants were allowed to grow at RT under constant lighting until root tissue was observed emerging from the holes in the bottom of each well of the 96-well seedling plates. These plates were then removed from the watering system and each placed onto a tight-fitting, inverted pipette tip box lid that contained 30-50 mL of 0.1-2.0% (w/v) HY-infected or non-infected BH in plant buffer solution (20 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM MES at pH 5.7), such that only emerging plant roots on the bottom of seedling plates came into direct

contact with BH-containing liquid media in the reservoir. Media-containing reservoirs were adhered to seedling plates with tape and plants were grown on a growth rack (RT with constant 24 hour fluorescent lighting at intensity of $150 \mu\text{mol}/\text{m}^2$ per second) and cultures were aerated daily by gentle manual shaking.

The modified 96-well plate Ice-Cap method worked well for growth of *A. thaliana*, alfalfa, and barley, but was not large enough to accommodate corn plants. The corn plants were grown in a modified, scaled-up version of the Ice-Cap method in which sterilized corn seeds were sown on the surface of agar-filled 50-mL conical tubes that had their bottoms cut off after agar had solidified. Seeded topless and bottomless conical tubes were placed into sterile 250-mL glass Erlenmeyer flasks containing 200 mL of 0.1% (w/v) HY-infected or non-infected BH in plant buffer solution, previously described, such that only plant roots emerging from the bottom of conical tubes came into direct contact with BH-containing media in the flask. Conical tubes were secured to Erlenmeyer flasks using parafilm, and the plants were placed on a growth rack (RT with constant 24 hour fluorescent lighting at intensity of $150 \mu\text{mol}/\text{m}^2$ per second) where cultures were aerated daily by gentle manual shaking.

2.4.4 Soil culture

Using the novel prion enrichment method previously described, enriched HY-infected or non-infected hamster BH was dialyzed against a 1:1 mixture of tap and distilled water and added at a final working concentration of 2.0% (w/v) to 15 mL of each of the following soils in sterilized glass vials: potting soil (Miracle-Gro® Organic Choice Potting Mix), Plainfield A soil (kind gift of Dr. Matilde Urrutia, University of Wisconsin-Madison), Bluestem clay (kind gift of Dr. Joel Pedersen, University of Wisconsin-Madison), and Ottawa sand (Fisher catalog no. S23-

3). Surface-sterilized *A. thaliana* seeds were placed on the surface of agar-filled 5-mL pipette tips (Gilson reference no. F161571) that had their tops trimmed to approximately 1-2 centimeters above the agar fill line and were incubated at 4°C for four days to synchronize germination. Following incubation, agar tips were transferred to a growth rack (RT with constant 24 hour fluorescent lighting at intensity of 150 $\mu\text{mol}/\text{m}^2$ per second) for 5-7 days or until seedlings germinated, then the bottoms were also trimmed to create greater surface area for roots to access each soil type. The shortened agar tips were positioned on the open tops of glass vials containing soil-BH mixtures such that plant roots would grow downward through the agar plug and eventually into the soil medium below. Seeded agar tips were secured to glass vials with parafilm and were placed into a humidified chamber constructed by inserting the cultures in a clear plastic container filled with 1-2 inches of tap water and enclosing the entire system in plastic wrap while on the growth rack (RT with constant 24 hour fluorescent lighting at intensity of 150 $\mu\text{mol}/\text{m}^2$ per second). All soil cultures were aerated daily by gentle manual shaking.

2.5 Prion uptake by plant roots for PrP^{res} detection

Each plant type used in these experiments was grown using the Ice-Cap or soil culture systems previously described. Duration of growth on prion-containing media (or non-infected media for controls) varied by plant species: *A. thaliana* for 4 days grown via Ice-Cap method and 22 days grown via soil culture, Alfalfa for 25 days grown via Ice-Cap method, Barley for 25 days grown via Ice-Cap method, and Corn for 50 days grown via Ice-Cap method. Following these incubation periods, aerial tissues of plants were harvested by cutting them away from the top surface of either the agar plugs in 96-well plates, the 5 mL agar tips for soil-grown *A. thaliana*,

or the 50 mL conical tubes for corn plants using scissors or blades never before used for prion work. Leaf and stem tissues were immediately placed in -80°C freezer until assayed.

2.6 Protein misfolding cyclic amplification (PMCA)

Non-infected Golden Syrian hamsters, age 3-4 weeks, were euthanized by carbon dioxide asphyxiation and immediately perfused with Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} or Mg^{2+} (Thermo Scientific #SH30028.02) modified with 5 mM EDTA. After perfusion, hamster brains were immediately removed, flash frozen in liquid nitrogen, and stored at -80°C until use. Brains were homogenized on ice to a 10% (w/v) concentration using one of three methods: 1) pestle grinder with disposable attachment (Fisher catalog no. 03-392-106), 2) Bullet Blender Storm 24 bead beater (Next Advance, Averill Park, NY), or 3) Dounce homogenization (Kimble Chase, Rockwood, TN) in ice-cold PMCA conversion buffer (DPBS supplemented with 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 7 tablets of Roche Complete Mini, EDTA-free protease inhibitors cocktail [Roche catalog no. 11836170001] per 50 mL of conversion buffer). NBH was clarified by centrifugation for either 2 minutes at 2,000×g or 3 minutes at 1,500×g. After separation of solid and liquid phases, the supernatant was transferred to pre-chilled micro-centrifuge tubes and stored at -80°C until use while the pellet was discarded. NBH was used as the substrate for *in-vitro* PMCA conversion.

Control or spiked seeds for PMCA were prepared from HY-positive hamster brain tissue and homogenized in DPBS with the conversion buffer described above, and the resulting 10% (w/v) HY-infected BH was serially diluted 10-fold in NBH to generate a dilution series ranging from 10^{-1} to 10^{-17} . Plant inoculums for PMCA were prepared from aerial tissues harvested from Ice-Cap grown or soil-grown plants where stem and/or leaf tissues were homogenized to 40-50%

(w/v) in conversion buffer by flash freezing tissues with liquid nitrogen and grinding them into a fine powder using mortar and pestle, then using the Bullet Blender Storm 24 bead beater to create the homogenates.

2.6.1 PMCA with beads

Control or plant seeds (10 μ L) were used to seed 90 μ L of NBH in 0.2 mL thin-walled PCR tubes with two 2.38 millimeter Teflon® beads (McMaster-Carr, #9660K12). Experimental tubes were placed on a Plexiglas® rack inside either the Misonix S-4000 (Misonix, Farmingdale, NY) or Qsonica Q700 (Qsonica, LLC, Newton, CT) microplate horn, and the reservoir was filled with 300 mL of fresh deionized water. In one round of PMCA with beads (PMCAb), the sonicator was set to following conditions: 96 cycles where each cycle consisted of 10 seconds of sonication (80% or 70% amplitude for Misonix S-4000 and 80% or 35% for Qsonica Q700) followed by 29 minutes 50 seconds of incubation at 37°C. Subsequent rounds of PMCAb were generated by combining 10 μ L of the PMCAb products from the previous round with 90 μ L of fresh NBH and two new Teflon® beads and subjected new samples to another 96-cycle round.

2.6.2 Miniaturized bead PMCA

For all assays after optimization of PMCA settings, we employed the high throughput “miniaturized bead-PMCA” (mb-PMCA) method recently developed for ultra-efficient PrP^{res} amplification (Moudjou et al., 2013). Briefly, control or plant seeds (4 μ L) were suspended in 36 μ L of NBH per well in a 96-well polymerase chain reaction (PCR) microplate (Axygen, #10011-220) supplemented with one 2.38 millimeter Teflon® bead (McMaster-Carr, #9660K12). Microplates were covered with a sealing mat (Axyamat, #14-222-024) and placed on a Plexiglas®

rack inside either the Misonix S-4000 or Qsonica Q700 microplate horn, and the reservoir was filled with 300 mL of fresh deionized water. Each sonicator was maintained in an enclosed incubator with the temperature adjusted to 37°C.

In one round of mb-PMCA, the sonicators were set to the following conditions: 96 cycles where each cycle consisted of 10 seconds of sonication at 220-260 Watts (70% amplitude for the S-4000 sonicator or 35% amplitude of the Q700 sonicator) followed by 29 minutes 50 seconds of incubation. Subsequent rounds of mb-PMCA were generated by combining 4 µL of the mb-PMCA products from the previous round with 36 µL of fresh NBH and a new Teflon® bead and subjected new samples to another 96-cycle round of mb-PMCA. Once completed, mb-PMCA products were analyzed for PrP^{res} content by PK treatment and subsequent western blotting (see Section 2.8).

2.7 Detection of PrP^{res} by western blotting

Aliquots of 20 µL from each PMCA reaction or 15 µL from each mb-PMCA reaction were transferred into thin-walled PCR tubes and treated with 100 µg/mL PK (Mo Bio Laboratories Inc, Carlsbad, CA) and incubated for 1 hour at 37°C with 400 RPM shaking. Following PK digestion, samples were treated with 4× LDS sample buffer and 10× NuPAGE reducing agent (Life Technologies, Grand Island, NY), each reduced to final concentrations of 1×, and heated for 5 minutes at 95°C. Each sample was completely resolved on 12% NuPAGE Bis-Tris gels (Life Technologies, Grand Island, NY) for 65-75 minutes at 200 Volts and then transferred to a polyvinyl difluoride (PVDF) membrane for 60 minutes at 25 Volts. PVDF membranes were blocked for 30 minutes in 5% (w/v) powdered milk in Tris-buffered saline plus Tween-20 (TBST), followed by incubation of the primary antibody 3F4 for hamster samples,

(Millipore, Billerica, MA) diluted 1:5,000 or 1:10,000 in a second 5% powdered milk and TBST mixture, for 90 minutes. PVDF membranes were rinsed 3 times with TBST for 3 minutes each, then incubated for 60 minutes with the secondary antibody goat anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (1:10,000 dilution in 5% milk in TBST). Immunoreactivity was detected using an enhanced chemiluminescent detection system (Thermo Scientific, Rockford, IL) in the EC3 imaging system (UVP Bioimaging Systems, Upland, California). Densitometry was performed using VisionWorks LS software version 6.6a (UVP Bioimaging Systems, Upland, California) once PrP^{res} was detected.

2.8 Data analysis

The PMCA₅₀ value was determined by regression analysis through the Solver function in Microsoft Excel using non-linear least-squares fitting of the endpoint titration data to the sigmoidal equation:

$$Y = A - [(B + A)/(1 + 10^{(L-X)(S)})]$$

where Y is the percent of positive mb-PMCA reactions, A is the maximum detection percentage at a given dilution level (100%), B is the minimum detection percentage at a given dilution level (0%), X is the logarithm of the dilution level, and L and S were fitted parameters that defined the logarithm of the PMCA₅₀ value and the slope of the sigmoidal curve, respectively. The least-squares value was determined by adjusting the L and S parameters to find the minimum difference between calculated and observed percentages of positive mb-PMCA reactions.

CHAPTER THREE:

Results

3.1 Experimental optimization of PMCA settings for Hyper (HY) prion strain

Before attempting to detect PrP^{TSE} from various plant types and cultures, we first ensured highly-specific amplification was observed in the PMCA technique. The procedure was performed by incubating 90 μ L of NBH substrate expressing PrP^C with 10 μ L of HY-infected BH acting as PrP^{TSE} seed and running 96 incubation and sonication cycles for one round of PMCA. To evaluate the cyclic amplification procedure, standard PMCA with beads (PMCAb) protocols (Castilla et al., 2006) were initially executed within individual PCR tubes supplemented with two Teflon® beads in each tube, previously demonstrated to significantly improve the level and the reproducibility of amplification (Gonzalez-Montalban et al., 2011). However, poor amplification was consistently observed using two different sonicator horns: a Misonix S-4000 model and a Qsonica Q700 model. Under these standard conditions, PrP^{res} was detected until the brain homogenate was diluted approximately 10⁻⁴-fold in two independent experiments (Figure 3-1a).

With the basic PMCAb protocol providing substandard results, a couple of technical changes were employed to improve the sensitivity of the technique. Recent publications recommended using a power level that had an average output of 200-260 W per sonication cycle (Barria et al., 2012; Morales et al., 2012); however, our group was using higher power setting based on previous results that were recommended and were successful in amplifying other prion strains (Castilla et al., 2006; Meyerett et al., 2008; Morawski et al., 2013). Thus, the settings were altered to reflect this enhancement: the Misonix S-4000 sonication level was decreased from 80% to 70% amplitude whereas the Qsonica Q700 decreased from 80% to 35% amplitude. These power settings have previously been applied for the hamster-passaged HY prion strain

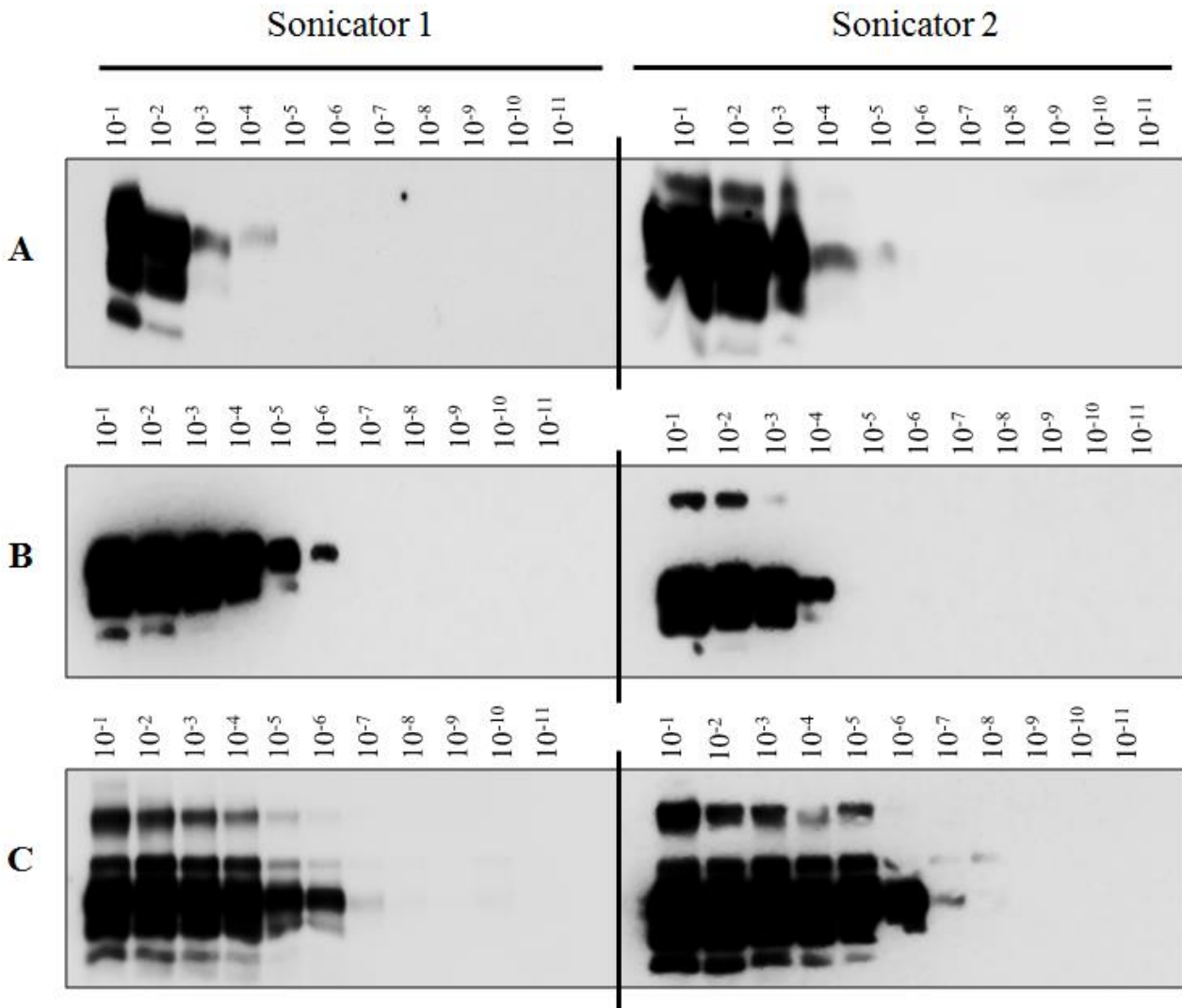


Figure 3-1. PCR microplate improves the sensitivity of PrP^{TSE} detection. 10% (w/v) HY brain homogenate was diluted in tenfold serial steps in hamster NBH and subjected to a single round of PMCA. Amplification from two independent experiments is shown where one endpoint titration was run using the Misonix S-400 (Sonicator 1) and the other with Qsonica Q700 (Sonicator 2). Each series was subjected to a single round of PMCA using different experimental conditions: (A) 80% amplitude in both sonicators using individual PCR tubes containing 2 Teflon® beads, (B) 70% amplitude for sonicator 1 and 35% amplitude for sonicator 2 using individual PCR tubes containing 2 Teflon® beads, and (C) 70% amplitude for sonicator 1 and 35% amplitude for sonicator 2 using a PCR microplate containing 40 µL total of PMCA mixture and 1 Teflon® bead. Numbers on top of each lane correspond to the dilution of the HY brain homogenate used to seed amplification reactions.

(Gonzalez-Romero et al., 2008; Moudjou et al., 2013; Saunders et al., 2011a; Saunders et al., 2011b). These changes resulted in slight improvement of sensitivity, particularly for the Misonix S-4000 with detection in PMCAb mixtures seeded with 10^{-6} -diluted HY brain homogenate, yet variations in amplification efficiency occurred between the two sonicators (Figure 3-1b). Recently, a novel method promoting high throughput and consistent amplification was developed that replaced individual tubes with one 96-well PCR microplate (Moudjou et al., 2013). By lowering the total PMCA volume and adding only one Teflon® bead, this new experimental design, designated miniaturized bead-PMCA (mb-PMCA), yielded reproducible detection of PrP^{res} at high sensitivity after one round as reactions seeded with 10^{-7} - and 10^{-8} -diluted brain material were regularly observed (Figure 3-1c, Appendix A-1). From previous attempts, our group saw similar levels of detection after three rounds of PMCAb (Appendix A-2), improving the speed of obtaining results by at least two rounds.

To test if location of the samples within the microplate affects amplification, endpoint titrations were run in triplicate and placed in different locations throughout the plate (Figure 3-2). Slight variations were observed as the samples positioned in the center observed detection in mb-PMCA reactions diluted 10^{-7} -fold while the left side of each sonicator exhibited an approximate 2-log decrease in amplification. One drawback to using tubes for PMCAb is the strong effect of location within the sonicator as the distance from the center of the horn to the tubes is important (Gonzalez-Montalban et al., 2011); the microplate better avoided this limitation and utilized the maximum surface offered by the sonicator (Moudjou et al., 2013). Taken together, these findings suggest microplate-based PMCA is the best choice for amplifying PrP^{TSE}, and plant samples for all subsequent experiments were placed as close to the center of the microplate to promote efficient amplification.

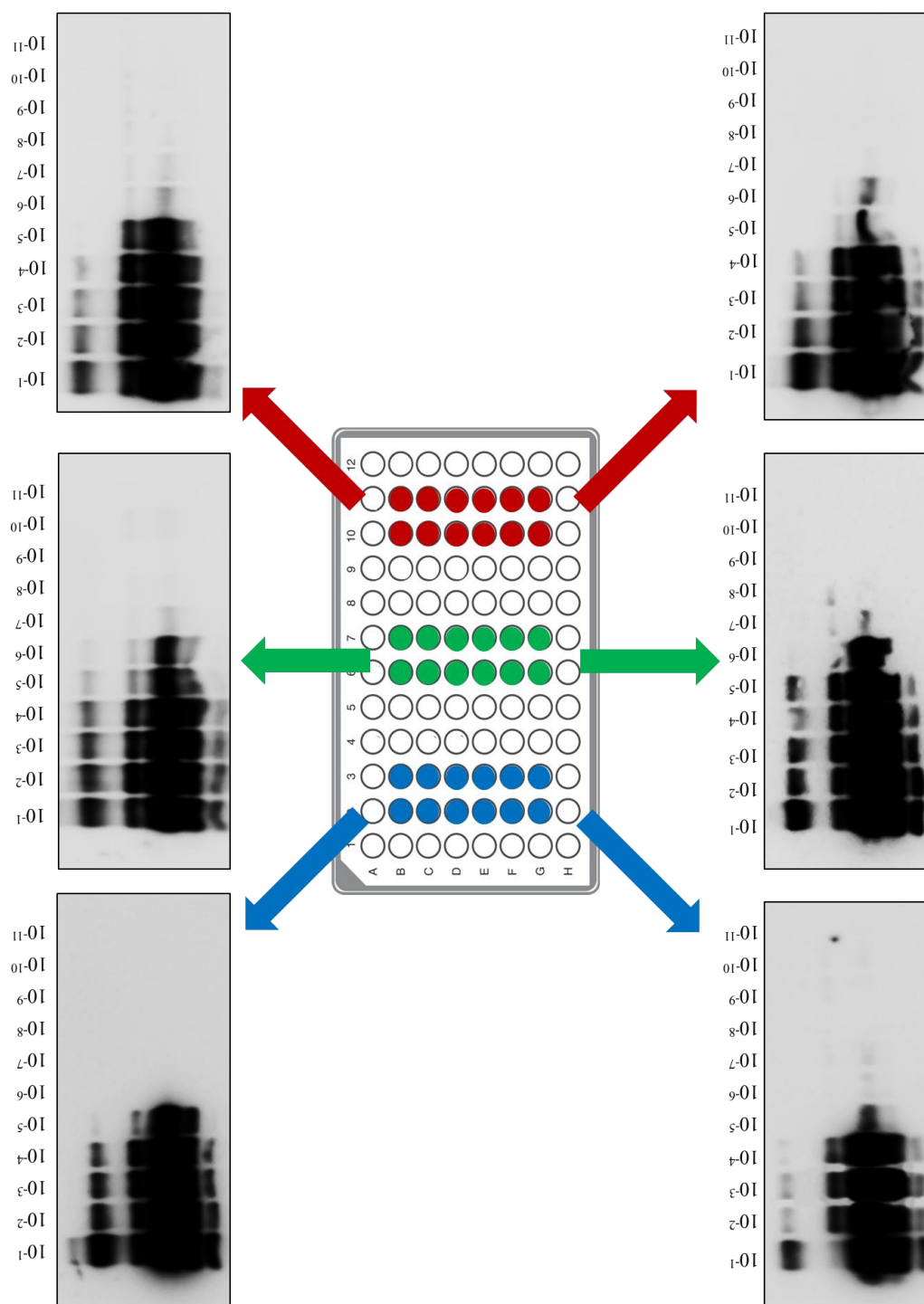


Figure 3-2. Location of samples in PCR microplate affects mb-PMCA amplification. Serial tenfold dilutions of HY-infected BH were prepared in triplicate and placed in one microplate, then subjected to a single round of mb-PMCA. Samples were placed either on the left (blue), center (green), or right (red) of the microplate, and the corresponding results are indicated by the colored arrows. Results displayed above the microplate image are from the Misonix S-4000 while the results below the image are from the Qsonica Q700. Numbers on top of each lane correspond to the dilution of the brain homogenate used to seed amplification reactions.

3.2 Detection of PrP^{TSE} in aerial plant tissues using mb-PMCA

Plants were grown using an adaption of the Ice-Cap culture method developed by Krysan and colleagues (Clark and Krysan, 2007; Krysan, 2004) where roots were directly exposed to prion-containing media and an agar barrier prevented cross-contamination of higher aerial plant tissues (see Materials and Methods). Stem and leaf tissues from plants hydroponically grown in prion-containing media were homogenized to 50% (w/v) and replaced HY-infected brain homogenate as the seed for mb-PMCA reactions. Because PMCA has the ability to detect as little as one particle of oligomeric infectious PrP^{TSE} (Saa et al., 2006b; Silveira et al., 2005), each plant type was subjected to five serial rounds of mb-PMCA to determine potential prion uptake, and the results were verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

The first samples tested were from the plant tissues of *Arabidopsis thaliana*, and mb-PMCA produced detectable levels of PrP^{res} after three 96-cycle rounds of amplification (Figure 3-3). As expected, all six lanes representing PMCA reactions seeded with one HY-grown *A. thaliana* homogenate showed positive signal by the fourth mb-PMCA round. In addition, the three lanes on the far right representing *A. thaliana* grown in non-prion containing media (P1 and P2) and unseeded PMCA substrate (NBH) had no detection, confirming that serial mb-PMCA is able to detect prion protein from plant tissue and is not prone to false-positives, a major drawback to use of earlier PMCA approaches that did not employ the microplate format. Similarly, crop plants corn (*Zea mays*), alfalfa (*Medicago sativa*), and barley (*Hordeum vulgare*) were also hydroponically grown on prion-containing media and all three plant species detected PrP^{TSE} after five serial mb-PMCA rounds (Figure 3-4). We next assessed prion uptake by plants in various soil types incubated with HY-infected brain homogenate enriched for PrP^{res} to observe

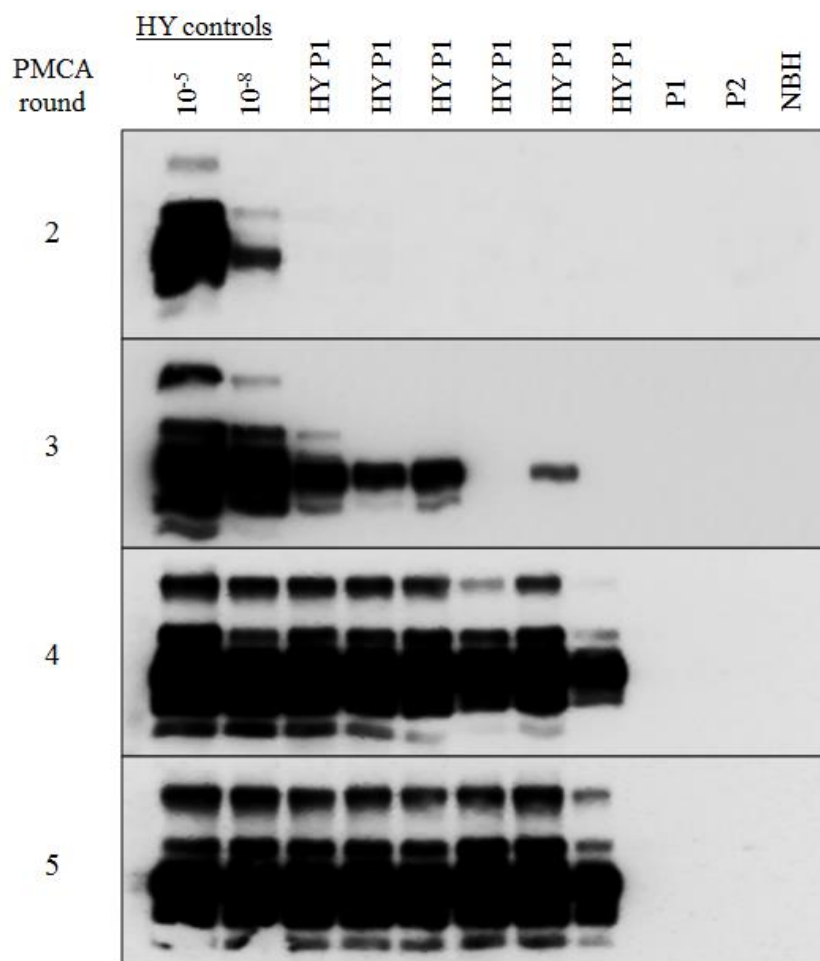


Figure 3-3. Serial mb-PMCA amplification of PrP^{res} in prion-contaminated *A. thaliana*. Plants were grown for 4 days on 2.0% HY-containing hydroponic media using the Ice-Cap method (Clark and Krysan, 2007) and aerial tissues were harvested and homogenized. Four μ L seeds of 50% (w/v) plant homogenates were used to seed 36 μ L hamster NBH and subjected to serial rounds of mb-PMCA, as indicated to left of images, using standard conditions (10 second sonication followed by 29:50 minute incubation at 37°C, 96 cycles per round). Prion-contaminated plants are denoted as “HY PX”, where each unique number assigned to “X” refers to an individual plant homogenate. Controls include 1) tenfold dilutions of HY-positive 10% brain homogenate (dilution factors listed above each figure) were used in the same 4 μ L seed:36 μ L substrate ratio as the plant reactions (first two lanes), and 2) 40 μ L hamster NBH to control for *de novo* prion synthesis during mb-PMCA (labeled “NBH” above each image) that were processed simultaneously with plant reactions. No PrP^{res} was detected in any samples assayed in which plants grown on non-prion containing media were used to seed mb-PMCA reactions (non-prion contaminated plants denoted as “PX” where each unique number assigned to “X” refers to a specific plant).

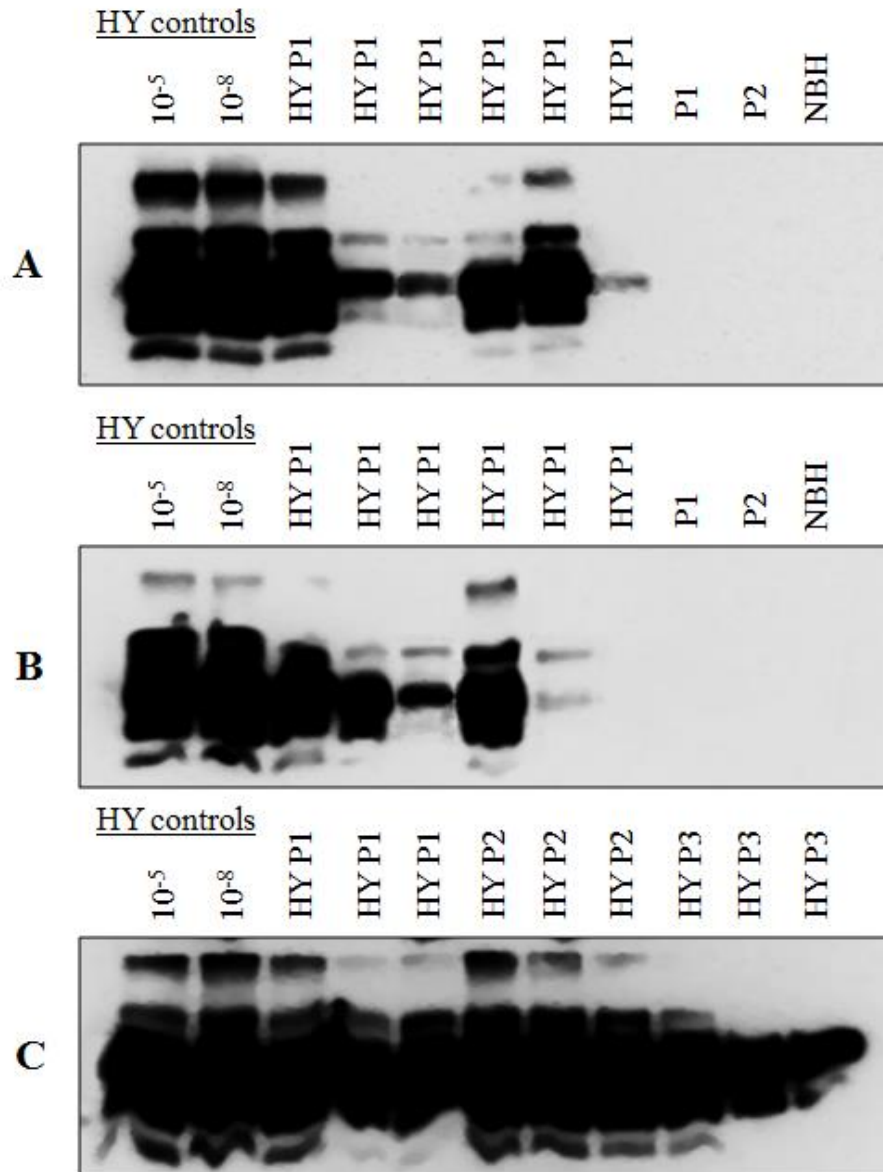


Figure 3-4. Detection of PrP^{res} in various prion-contaminated plant tissues. Plants were grown for 25-50 days on 0.1-2.0% HY-containing hydroponic media using the Ice-Cap method (Clark and Krysan, 2007) and aerial tissues were harvested and homogenized. Products from the fifth round of mb-PMCA amplification are shown. The same procedure and denominations detailed in Figure 3-3 were used to detect and denote PrP^{res} -positive bands in (A) corn, (B) alfalfa, and (C) barley.

potential effects a soil matrix has on uptake. Because *A. thaliana* displayed the highest signal intensity throughout all plant species tested (Figure 3-3) and has a shorter life cycle than the other plant species, it was selected for prion uptake experiments using four different soils – two artificial, quartz and potting mix, and two obtained from natural environments, bluestem clay and Plainfield soil – with PrP^{TSE} as the likely organic nitrogen source. After three weeks of growth, stem and leaf tissues were harvested and separately homogenized and used to seed reactions that were subjected to five serial rounds of mb-PMCA. Evidence of uptake by the roots and translocation into the stem and leaves were observed in every soil type with varying degrees of PrP^{TSE} detection (Figure 3-5). All replicates in artificial soils detected PrP^{TSE} with the exception of the third *A. thaliana* replicate grown in potting mix, most likely resulting from poor germination or lack of access to the soil (Appendix B-1). Interestingly, bluestem clay had the greatest level of detection as determined by signal intensity compared to the other soils while Plainfield did not observe any positive signal even though *A. thaliana* reached the four-leaf stage in this soil (Appendix B-1).

With high mb-PMCA sensitivity, concerns arose that PrP^{TSE} might be generated spontaneously and produce false-positive results as previously reported for other PMCA methods (Cosseddu et al., 2011; Saa et al., 2006b). To date, we have detected no false-positive amplifications in control reactions of only unseeded NBH substrate (Figure 3-3, Figure 3-4a and b, Figure 3-5c). Furthermore, in order to ensure newly-formed PrP^{TSE} was converted from hamster PrP^C and not from *de novo* formation (Deleault et al., 2007), five homogenates of each plant type (50% w/v) hydroponically grown in non-prion containing media and five homogenates of soil-grown *A. thaliana* (50% w/v) exposed to uninfected, enriched brain homogenate were used to seed hamster NBH and subjected to serial rounds of mb-PMCA. No *de novo* formation of

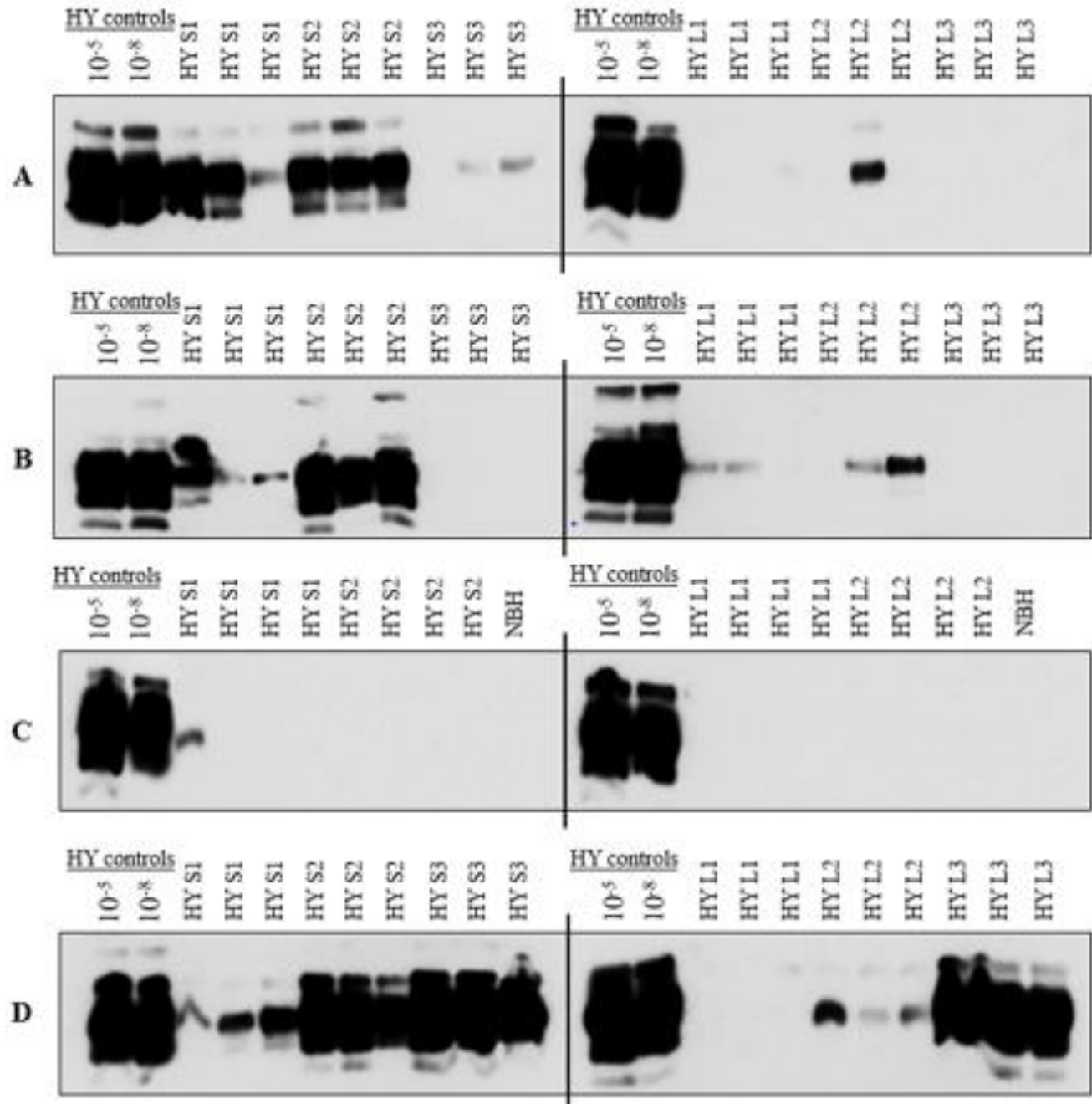


Figure 3-5. Detection of PrP^{res} in soil-grown *A. thaliana*. Two artificial soils, (A) quartz and (B) potting mix, and two naturally-occurring soils, (C) Plainfield and (D) bluestem clay, were subjected to five rounds of mb-PMCA under standard conditions previously described. Results from the fifth round only are shown. Prion-contaminated plants are denoted as “HY SX” or “HY LX”, where “S” represents the stem, “L” represents the leaves, and each unique number assigned to “X” refers to a specific plant homogenate taken from the soil culture (refer to Appendix B-1). Controls include tenfold dilutions of HY-positive 10% (w/v) brain homogenate (dilution factors listed above the figure) that were used to seed hamster NBH in the same 4:36 μ L seed:substrate ratio used for plant reactions.

PrP^{TSE} was detected in any sample by western blotting after five mb-PMCA rounds while one or two positive controls were carried through to verify the amplification process was properly conducted (Figure 3-6). Overall, these findings indicate that not only is mb-PMCA a highly effective tool for amplification of minute amounts of PrP^{TSE} from plant tissues, but can also avoid spontaneous *de novo* generation and false-positive results.

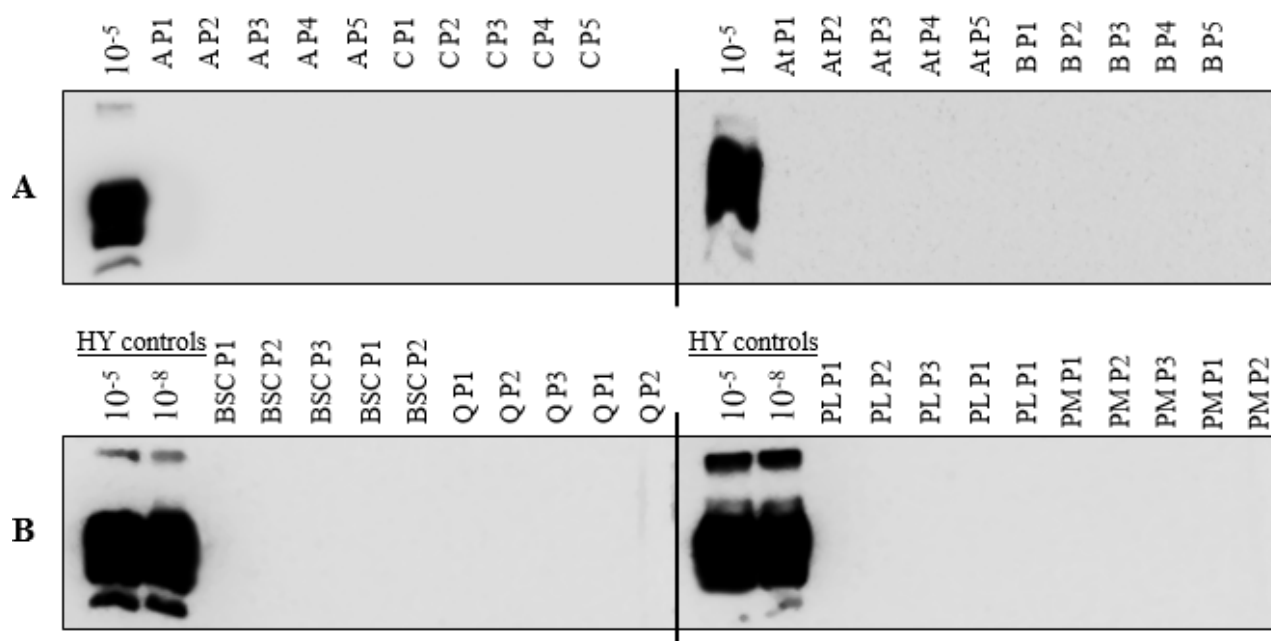


Figure 3-6. High specificity in serial mb-PMCA leads to reliable results. Plants grown in hydroponic and soil cultures were exposed to non-prion containing media and aerial tissues were harvested and homogenized. (A) Twenty 4 μ L HY-negative plant homogenates (five for each plant type) were added to 36 μ L NBH and subjected to five serial rounds of 96-cycle mb-PMCA using standard conditions previously described. Designations include “A” for alfalfa, “At” for *A. thaliana*, “B” for barley, “C” for corn, and “PX” where each unique number assigned to “X” refers to a specific plant homogenate. (B) Twenty 4 μ L HY-negative *A. thaliana* plant homogenates (five from each soil type) were added to 36 μ L NBH and subjected to five serial rounds of 96-cycle mb-PMCA using similar conditions and PK digestion. Designations include “BSC” for bluestem clay, “Q” for quartz, “PL” for Plainfield, “PM” for potting mix, and “PX” where each unique number assigned to “X” refers to a specific *A. thaliana* homogenate. All results from serial mb-PMCA of uninfected samples were negative, confirming high specificity. One or two positive controls representing dilutions of HY-positive 10% (w/v) brain homogenate were also included.

3.3 Quantification of PrP^{TSE} concentrations in plant tissues

While serial PMCA has been established as an ultrasensitive prion detection method, efforts to quantify prion titers in infected tissues have been sparse. Recently, one technical advancement developed by Chen and colleagues demonstrated that estimations of prion concentrations are biochemically feasible *in vitro* with high sensitivity and accuracy from PMCA data (Chen et al., 2010). To quantify prion concentrations in plant tissues, known quantities of PrP^{TSE} were spiked into NBH substrate, similar to the endpoint titrations used for optimizing PMCA settings, and subjected to serial rounds of mb-PMCA. A direct relationship was observed between the amount of PrP^{TSE} in a given sample and the number of PMCA rounds necessary for detection (Chen et al., 2010), and comparing this correlation with the number of PMCA rounds needed to detect PrP^{TSE} in plant tissues provides a rough estimation of PrP^{TSE} initially present in the sample (Morales et al., 2012). Figure 3-7 shows a full endpoint titration series where we determined how many rounds were required to produce a PrP^{TSE} signal detectable by western blotting. Additional dilutions ranging from 10⁻⁶- to 10⁻¹⁵-fold of 10% (w/v) HY-infected BH were spiked into NBH substrate (Figure 3-8a, Appendix C-1), and the densitometric information from each dilution in the five replicated endpoint titrations were averaged and used for quantifying PrP^{TSE} concentrations in plant tissues.

Further analysis was conducted on the titration series by comparing the sensitivity of mb-PMCA to typical animal bioassays. Marsh and Kimberlin previously demonstrated that the infectious dose titer for 50% (ID₅₀) of healthy hamsters to become infected with HY prions via i.c. inoculation was 10^{10.1} ID₅₀ units per gram of brain tissue (Marsh and Kimberlin, 1975). Conversely, the fraction of mb-PMCA products with positive signal from the five endpoint titrations was plotted against the logarithm of 10% (w/v) HY-infected BH dilutions, and the

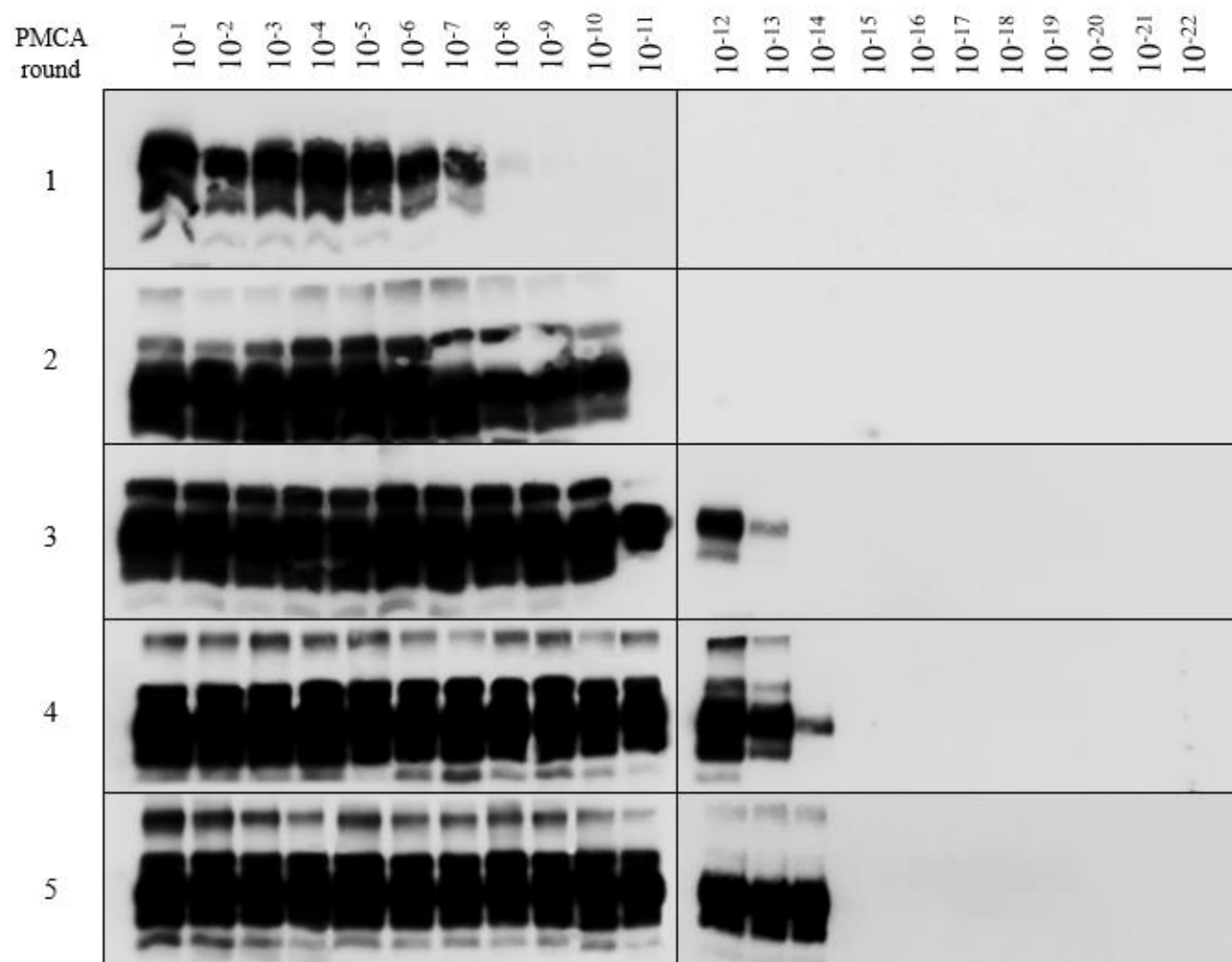


Figure 3-7. Endpoint titration to determine relationship between HY concentration and PMCA rounds required for detection. Four μL aliquots from 10% HY-infected brain homogenate were diluted in 36 μL hamster NBH and subjected to five serial rounds of mb-PMCA. PrP^{TSE} was detected by western blotting using the 3F4 antibody after PK digestion. Numbers on top of each lane correspond to the dilution of the brain homogenate used to seed amplification reactions.

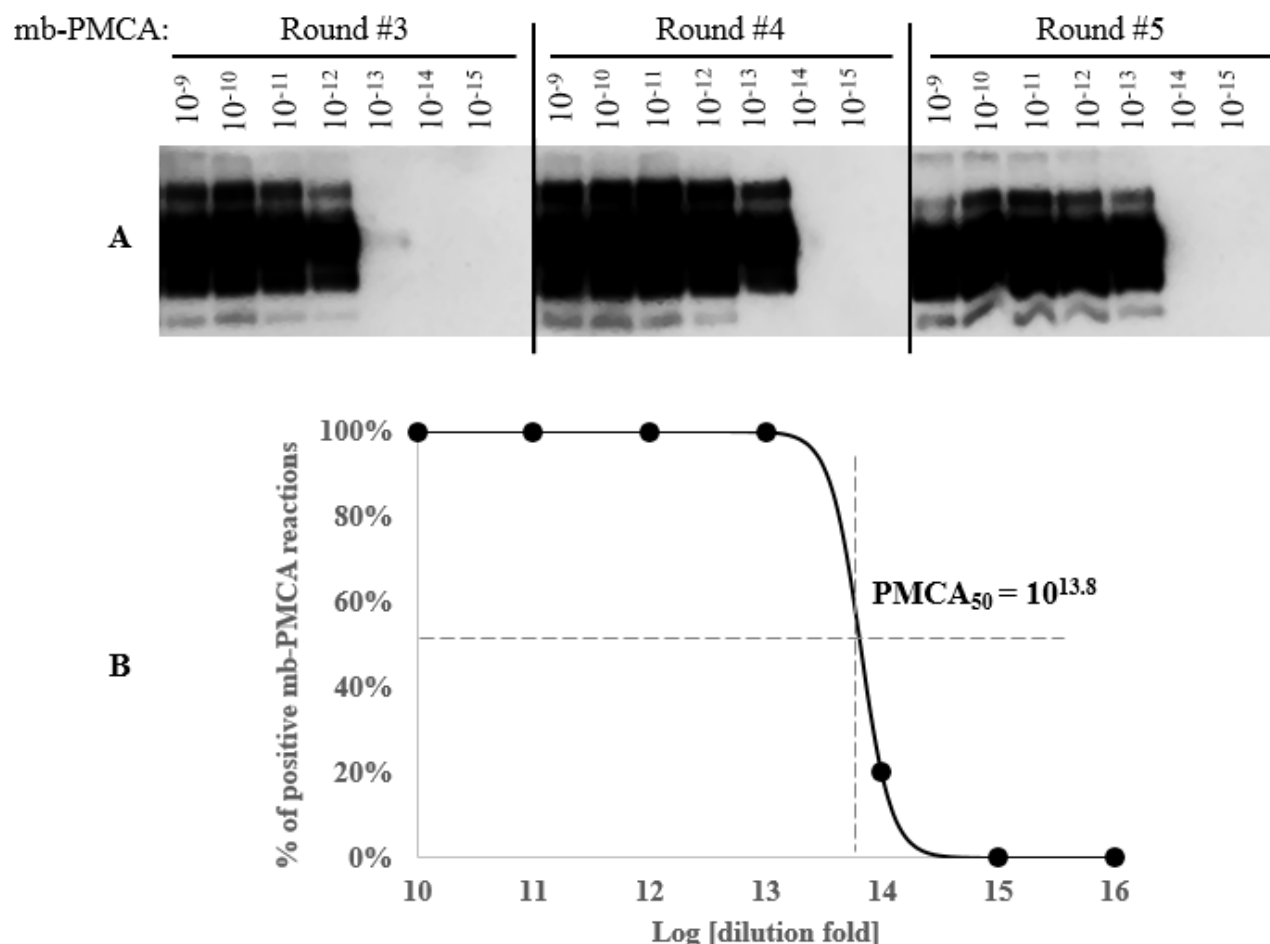


Figure 3-8. Analysis of serial mb-PMCA endpoint titration. 10% brain material from HY-infected hamsters was serially diluted up to 10⁻¹⁵-fold, and aliquots from each dilution were seeded with hamster NBH for mb-PMCA reactions. (A) Example results are shown on Western blots stained with 3F4 antibody where mb-PMCA products were amplified for three, four, or five serial rounds. (B) Analysis of the data using a sigmoidal function occurred through plotting each dilution of brain material with a positive signal during serial mb-PMCA against the logarithm of dilution (•). The solid curve represents the best fit of a non-linear least-squares regression used to calculate PMCA₅₀ titer.

experimental data was fitted using a sigmoidal function and non-linear regression analysis to calculate a PMCA₅₀ titer of $10^{13.8}$ (Figure 3-8b). Similar to the animal bioassay, PMCA₅₀ represents the dilution level at which only 50% of the mb-PMCA reactions are capable of initiating amplification (Makarava et al., 2011; Makarava et al., 2012). The minuscule amounts of PrP^{TSE} that are detected demonstrate the usefulness of the mb-PMCA technique as the sensitivity of mb-PMCA in five serial 96-cycle rounds was higher than that of the bioassay by a factor of ~5,000.

Prion concentrations in plants were quantified when the signal from the earliest PMCA round was at least five times greater than the densitometric signal of the background (Chen et al., 2010). Analysis of hydroponically-grown plant species was first conducted (Table 3-1). *A. thaliana* contained the greatest amount of PrP^{TSE} because it was the only plant type to have detection after three mb-PMCA rounds. The average calculated prion concentration for *A. thaliana* was 88 fg PrP^{TSE}/g wet plant while concentrations of PrP^{TSE} based on the wet weight of barley, corn and alfalfa were also calculated and determined to be 59, 40 and 32 fg PrP^{TSE}/g wet plant, respectively. Conversions from the wet weight of each plant to the dry weight were used to further assess the concentrations of PrP^{TSE}, which varied depending on the moisture content of the plant species (Al-Johani et al., 2012; Khosrowchahli et al., 2013; Weise, 2013; Zhang et al., 2012). In addition, ID₅₀ concentrations were determined from the ID₅₀ value of the HY strain in hamsters (Marsh and Kimberlin, 1975) and the PrP^{TSE} concentration found in the brain tissue of hamsters (Chen et al., 2010). Again, *A. thaliana* had the highest values for the dry weight and ID₅₀ concentrations for plants grown hydroponically (Table 3-1).

In the soil cultures, *A. thaliana* stems had higher calculated concentrations of PrP^{TSE} compared to the leaf homogenates (Table 3-2). Because little-to-no detection in the stem and the

Table 3-1. Quantification of PrP^{TSE} concentrations in hydroponically-grown plant tissues¹

Plant Type	Wet Concentration, g PrP^{TSE}/g wet weight	Dry Concentration², g PrP^{TSE}/g dry weight	ID₅₀ Concentration³, ID₅₀ U/g dry weight
Alfalfa	$3.2 \times 10^{-14} \pm 1.3 \times 10^{-14}$	$1.0 \times 10^{-13} \pm 4.1 \times 10^{-14}$	$5.5 \times 10^1 \pm 2.3 \times 10^1$
<i>A. thaliana</i>	$8.8 \times 10^{-14} \pm 1.3 \times 10^{-14}$	$1.4 \times 10^{-12} \pm 2.1 \times 10^{-13}$	$7.5 \times 10^2 \pm 1.1 \times 10^2$
Barley	$5.9 \times 10^{-14} \pm 4.1 \times 10^{-15}$	$7.1 \times 10^{-13} \pm 4.9 \times 10^{-14}$	$3.9 \times 10^2 \pm 2.7 \times 10^1$
Corn	$4.0 \times 10^{-14} \pm 1.2 \times 10^{-14}$	$4.2 \times 10^{-14} \pm 1.3 \times 10^{-14}$	$2.3 \times 10^1 \pm 7.1 \times 10^0$

¹PrP^{TSE} concentrations were estimated by determining the number of PMCA rounds required to detect signal by western blotting and interpolating the signal strength with the endpoint titration series from the same round. Indicated values are means $\pm$ s.e.m.

²The wet weight (WW) to dry weight (DW) conversions utilized for each plant type are as follows: Alfalfa – 3.165 g WW/g DW (Khosrowchahli et al., 2013); *A. thaliana* – 15.625 g WW/g DW (Weise, 2013); Barley – 11.905 g WW/g DW (Al-Johani et al., 2012); Corn – 1.068 g WW/g DW (Zhang et al., 2012).

³ID₅₀ concentrations were calculated assuming the amount of PrP^{TSE} in prion-infected hamster brain is $10^{-4.6}$ g PrP^{TSE}/g brain tissue (Chen et al., 2010) and ID₅₀ of Hyper-infected hamsters is $10^{10.1}$ ID₅₀ U/g brain tissue (Marsh and Kimberlin, 1975).

leaves of the plants grown in Plainfield soil and leaves from the quartz plants was observed, average PrP^{TSE} concentrations were difficult to obtain. Nevertheless, congruent with the western blot results, *A. thaliana* grown in bluestem clay resulted in the highest average concentration of PrP^{TSE} at 43 fg PrP^{TSE}/g wet plant in the stem and 18 fg PrP^{TSE}/g wet plant in the leaves. Taken together, these results demonstrate that *A. thaliana*, alfalfa, barley, and corn contain wet concentrations of PrP^{TSE} within 2-log from hydroponic or soil cultures. Because amplification in the endpoint titrations concludes after the fourth round, PrP^{TSE} concentrations from the fifth mb-PMCA round are similar to ones calculated from the third and fourth rounds. In addition, HY-

Table 3-2. Quantification of PrP^{TSE} concentrations in soil-grown *Arabidopsis thaliana* stem and leaves¹

Soil Type	Stem Wet Concentration, g PrP ^{TSE} /g wet weight	Stem Dry Concentration ² , g PrP ^{TSE} /g dry weight	Stem ID ₅₀ Concentration ³ , ID ₅₀ U/g dry weight
Bluestem Clay	$4.3 \times 10^{-14} \pm 9.2 \times 10^{-15}$	$6.7 \times 10^{-13} \pm 1.4 \times 10^{-13}$	$3.7 \times 10^2 \pm 7.8 \times 10^1$
Plainfield ⁴	$4.1 \times 10^{-15} \pm 0$	$6.4 \times 10^{-14} \pm 0$	$3.5 \times 10^1 \pm 0$
Potting Mix	$1.8 \times 10^{-14} \pm 6.6 \times 10^{-15}$	$2.8 \times 10^{-13} \pm 1.0 \times 10^{-13}$	$1.5 \times 10^2 \pm 5.8 \times 10^1$
Quartz	$2.4 \times 10^{-14} \pm 6.7 \times 10^{-15}$	$3.7 \times 10^{-13} \pm 1.1 \times 10^{-13}$	$2.0 \times 10^2 \pm 5.8 \times 10^1$
Soil Type	Leaf Wet Concentration, g PrP ^{TSE} /g wet weight	Leaf Dry Concentration ² , g PrP ^{TSE} /g dry weight	Leaf ID ₅₀ Concentration ³ , ID ₅₀ U/g dry weight
Bluestem Clay	$1.8 \times 10^{-14} \pm 7.9 \times 10^{-15}$	$2.8 \times 10^{-13} \pm 1.2 \times 10^{-13}$	$1.5 \times 10^2 \pm 6.7 \times 10^1$
Plainfield	N/A	N/A	N/A
Potting Mix	$2.1 \times 10^{-15} \pm 9.3 \times 10^{-16}$	$3.3 \times 10^{-14} \pm 1.5 \times 10^{-14}$	$2.7 \times 10^1 \pm 7.9 \times 10^0$
Quartz ⁴	$6.5 \times 10^{-15} \pm 0$	$1.0 \times 10^{-13} \pm 0$	$5.6 \times 10^1 \pm 0$

¹PrP^{TSE} concentrations were estimated by determining the number of PMCA rounds required to detect signal by western blotting and interpolating the signal strength with the endpoint titration series from the same round. Indicated values are means $\pm$ s.e.m.

²The wet weight (WW) to dry weight (DW) conversions utilized for each plant type are as follows: Alfalfa – 3.165 g WW/g DW (Khosrowchahli, 2013); *A. thaliana* – 15.625 g WW/g DW (Weise, 2013); Barley – 11.905 g WW/g DW (Al-Johani, 2012); Corn – 1.068 g WW/g DW (Zhang, 2012).

³ID₅₀ concentrations were calculated assuming the amount of PrP^{TSE} in prion-infected hamster brain is $10^{-4.6}$ g PrP^{TSE}/g brain tissue (Chen et al, 2010) and ID₅₀ of Hyper-infected hamsters is $10^{10.1}$ ID₅₀ U/g brain tissue (Marsh and Kimberlin, 1975).

⁴Not enough positive signals to calculate a standard error of mean value

infected plant homogenate may amplify differently during mb-PMCA compared to dilutions of 10% HY-infected hamster brain homogenate; therefore, the concentrations of PrP^{TSE} may reflect overestimations of the true PrP^{TSE} content in plant tissues (Carlson, 2013; Gregori et al., 2008a)

3.4 PrP^{TSE} amplification is inhibited by plant material and affects quantification

Unidentified components of blood and plasma have been previously demonstrated to interfere with PrP^{TSE} amplification during PMCA (Barria et al., 2012; Castilla et al., 2006; Properzi and Pocchiari, 2013) as well as samples that include metal cations like copper and zinc (Orem et al., 2006). Therefore, plant homogenates may also exhibit inhibitory effects throughout the amplification process because of the compounds present in the stem and leaf tissues. The effect of plant tissue on HY PMCA amplification was conducted by spiking HY-infected brain dilutions with a 50% (w/v) *A. thaliana* homogenate at a 1:4 plant homogenate:brain homogenate ratio. The plant-spiked dilutions were used to seed NBH substrate, then subjected to serial rounds of mb-PMCA. A separate control series containing HY-infected dilutions spiked with PMCA conversion buffer at the same ratio was also used and run in the same microplate. The addition of stem and leaf tissues resulted in a lack of amplification in reactions seeded with low dilutions of HY brain material (Figure 3-9a). A reduction of amplification capability greater than 50% was observed during the first three rounds of mb-PMCA while amplification increased in later rounds once the plant homogenate diminished in each dilution, providing evidence of the inhibitory effect has plant material on PMCA. This result could explain observations of little-to-no detection in each plant species through the first three rounds of mb-PMCA.

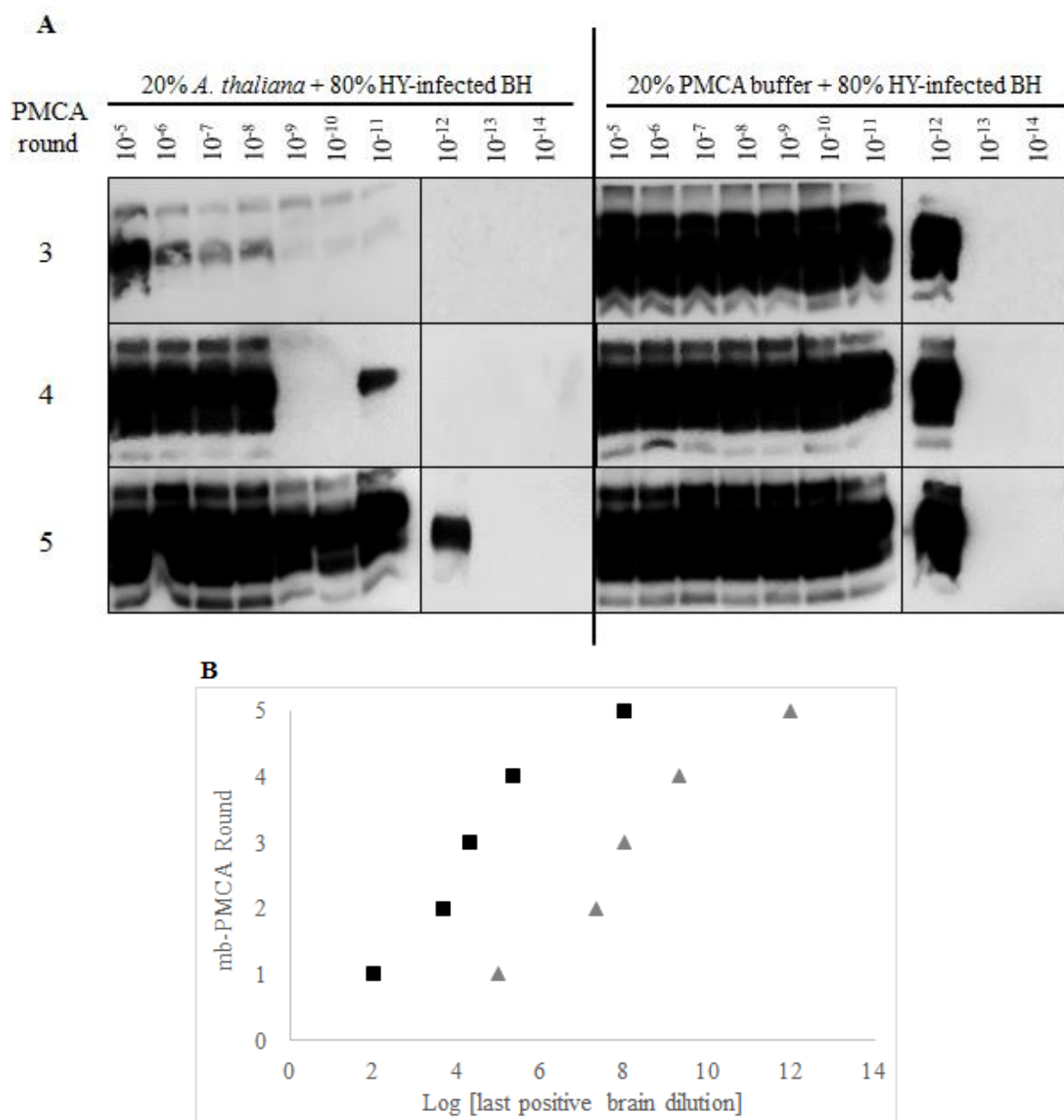


Figure 3-9. Inhibition of plant homogenate on PrP^{TSE} amplification during mb-PMCA. (A) 50% (w/v) homogenate of *A. thaliana* was incubated with ten-fold dilutions of HY-positive 10% brain homogenate for two hours to create seeds for mb-PMCA. A 1:4 ratio of plant homogenate:brain homogenate in the seeds was used to dilute plant homogenate to 10%. A second series was prepared by spiking 10% (w/v) PMCA conversion buffer into dilutions of HY-positive brain homogenate in the same 1:4 ratio and used as a control. The two spiked dilution series were used to seed PMCA reactions (4 μ L) with NBH substrate (36 μ L) and subjected to five serial rounds of mb-PMCA, as indicated. Numbers on top of each lane correspond to the dilution of the brain homogenate used seed amplification reactions. (B) Western blots from three independent experiments [example shown in (A), Appendix D] were analyzed, and the average of the last detectable signal after each mb-PMCA round in the three plant-spiked (squares) and PMCA buffer-spiked (triangles) dilution series was plotted.

We next determined an approximate correction factor to account for inhibition in order to better estimate the true PrP^{TSE} content in aerial plant tissues (Figure 3-9b). For this model, similar to the quantification process, a signal was considered positive only if the optimal density was five times greater than the densitometric signal of the background. The difference between the two dilution series at a particular mb-PMCA round was used as the calculated factor to correct for the inhibitory effects observed when using plant material during PMCA. During round three of mb-PMCA, inhibition was measured at $10^{3.7}$, after converting from the logarithm dilution, while rounds four and five calculated the difference between the last detectable signals of PMCA buffer-spiked and plant-spiked to be $10^{4.0}$. The average PrP^{TSE} concentrations previously calculated were corrected with either one of these factors, dependent on which mb-PMCA round PrP^{TSE} was first detected in a particular plant sample, and the results are listed in Tables 3-3 and 3-4. Hydroponically-grown barley, corn, and alfalfa all had initial positive PrP^{TSE} signal starting in the fourth mb-PMCA round and, because the correction factor is equivalent in rounds four and five, average prion concentrations in each crop species increased proportionally by 4-log (0.3-0.6 ng PrP^{TSE} /g wet plant). *A. thaliana* increased at a slightly lower rate because of detection in the third mb-PMCA round (Figure 3-3); nevertheless, it still had the highest prion concentration from plants grown in the hydroponic culture at 0.9 ng PrP^{TSE} /g wet plant. Similar trends were observed in soil-grown *A. thaliana* where prion concentrations from each soil increased 10,000-fold for both stem and leaf samples while the calculated ID_{50} concentrations ranged from $10^{5.4}$ - $10^{6.9}$ ID_{50} U/g dry plant, similar to the estimated titer of CWD prions in saliva over a 10-month period (Tamguney et al., 2012). These data suggest that prions are taken up by roots of various plants species from hydroponic and soil culture systems and transported to aerial tissues in sufficient quantities to cause prion disease in mammalian hosts.

Table 3-3. PrP^{TSE} concentrations in hydroponically-grown plant tissues considering inhibition from plant material¹

Plant Type	Wet Concentration, g PrP^{TSE}/g wet weight	Dry Concentration², g PrP^{TSE}/g dry weight	ID₅₀ Concentration³, ID₅₀/g dry weight
Alfalfa	$3.2 \times 10^{-10} \pm 1.3 \times 10^{-10}$	$1.0 \times 10^{-9} \pm 4.1 \times 10^{-10}$	$5.5 \times 10^5 \pm 2.3 \times 10^5$
<i>A. thaliana</i>	$8.5 \times 10^{-10} \pm 1.4 \times 10^{-10}$	$1.3 \times 10^{-8} \pm 2.1 \times 10^{-9}$	$7.3 \times 10^6 \pm 1.3 \times 10^6$
Barley	$5.9 \times 10^{-10} \pm 2.1 \times 10^{-10}$	$7.1 \times 10^{-9} \pm 2.5 \times 10^{-9}$	$3.8 \times 10^6 \pm 1.4 \times 10^6$
Corn	$4.0 \times 10^{-10} \pm 1.2 \times 10^{-10}$	$4.2 \times 10^{-10} \pm 1.3 \times 10^{-10}$	$2.3 \times 10^5 \pm 7.1 \times 10^4$

¹PrP^{TSE} concentrations were estimated by determining the number of PMCA rounds required to detect signal by western blotting and interpolating the signal strength with the endpoint titration series from the same round. Indicated values are means $\pm$ s.e.m.

²The wet weight (WW) to dry weight (DW) conversions utilized for each plant type are as follows: Alfalfa – 3.165 g WW/g DW (Khosrowchahli et al., 2013); *A. thaliana* – 15.625 g WW/g DW (Weise, 2013); Barley – 11.905 g WW/g DW (Al-Johani et al., 2012); Corn – 1.068 g WW/g DW (Zhang et al., 2012).

³ID₅₀ concentrations were calculated assuming the amount of PrP^{TSE} in prion-infected hamster brain is $10^{-4.6}$ g PrP^{TSE}/g brain tissue (Chen et al., 2010) and ID₅₀ of Hyper-infected hamsters is $10^{10.1}$ ID₅₀/g brain tissue (Marsh and Kimberlin, 1975).

Table 3-4. PrP^{TSE} concentrations in soil-grown *Arabidopsis thaliana* stem and leaves considering inhibition from plant material¹

Soil Type	Stem Wet Concentration, g PrP ^{TSE} /g wet weight	Stem Dry Concentration ² , g PrP ^{TSE} /g dry weight	Stem ID ₅₀ Concentration ³ , ID ₅₀ /g dry weight
Bluestem Clay	$3.8 \times 10^{-10} \pm 8.1 \times 10^{-11}$	$6.0 \times 10^{-9} \pm 1.3 \times 10^{-9}$	$3.3 \times 10^6 \pm 7.0 \times 10^5$
Plainfield ⁴	$4.1 \times 10^{-11} \pm 0$	$6.4 \times 10^{-10} \pm 0$	$3.5 \times 10^5 \pm 0$
Potting Mix	$1.8 \times 10^{-10} \pm 6.7 \times 10^{-11}$	$2.8 \times 10^{-9} \pm 1.0 \times 10^{-9}$	$1.5 \times 10^6 \pm 5.7 \times 10^5$
Quartz	$2.5 \times 10^{-10} \pm 6.9 \times 10^{-11}$	$3.9 \times 10^{-9} \pm 1.1 \times 10^{-9}$	$2.1 \times 10^6 \pm 5.9 \times 10^5$
Soil Type	Leaf Wet Concentration, g PrP ^{TSE} /g wet weight	Leaf Dry Concentration ² , g PrP ^{TSE} /g dry weight	Leaf ID ₅₀ Concentration ³ , ID ₅₀ /g dry weight
Bluestem Clay	$1.8 \times 10^{-10} \pm 2.2 \times 10^{-10}$	$2.8 \times 10^{-9} \pm 3.5 \times 10^{-9}$	$1.5 \times 10^6 \pm 1.9 \times 10^6$
Plainfield	N/A	N/A	N/A
Potting Mix	$2.1 \times 10^{-11} \pm 2.7 \times 10^{-11}$	$3.3 \times 10^{-10} \pm 4.1 \times 10^{-10}$	$1.8 \times 10^5 \pm 2.2 \times 10^5$
Quartz ⁴	$6.5 \times 10^{-11} \pm 0$	$1.0 \times 10^{-9} \pm 0$	$5.6 \times 10^5 \pm 0$

¹PrP^{TSE} concentrations were estimated by determining the number of PMCA rounds required to detect signal by western blotting and interpolating the signal strength with the endpoint titration series from the same round. Indicated values are means $\pm$ s.e.m.

²The wet weight (WW) to dry weight (DW) conversions utilized for each plant type are as follows: Alfalfa – 3.165 g WW/g DW (Khosrowchahli, 2013); *A. thaliana* – 15.625 g WW/g DW (Weise, 2013); Barley – 11.905 g WW/g DW (Al-Johani, 2012); Corn – 1.068 g WW/g DW (Zhang, 2012).

³ID₅₀ concentrations were calculated assuming the amount of PrP^{TSE} in prion-infected hamster brain is $10^{-4.6}$ g PrP^{TSE}/g brain tissue (Chen et al, 2010) and ID₅₀ of Hyper-infected hamsters is $10^{10.1}$ ID₅₀/g brain tissue (Marsh and Kimberlin, 1975).

⁴Not enough positive signals to calculate a standard error of mean value

CHAPTER FOUR:

Discussion

Collectively, these results reveal that infectious prions are taken up by plants through root hairs and are transported to the aerial plant tissues where they are readily detectable by serial mb-PCMA. The additional confirmation that various crop species, including alfalfa, barley, and corn, can internalize prions highlights a previously unrecognized route of horizontal transmission in CWD-contaminated environments along with risks of potential prion exposure to domestic animals and humans that consume these crops.

The PMCA assay has emerged in the last decade as a very efficient procedure to amplify prions *in vitro* that mimics the replication thought to occur *in vivo* during disease progression (Saborio et al., 2001). Optimization of the PMCA technique lead to the use of 96-well microplates supplemented with one Teflon® bead, which increased the sensitivity of HY prion amplification by at least 1,000-fold compared to PMCAb after one round (Figure 3-1). The use of microplates also reduces variations in sonication conditions that seemed to affect amplification in individual tubes for PMCAb. Distribution of sonic energy could vary throughout the sonicator horn, and our data, in conjunction with previous reports, have demonstrated amplification efficiency depends on the position of the tubes within the sonicator and their distance from the center of the horn (Castilla et al., 2006; Gonzalez-Montalban et al., 2011; Morales et al., 2012). One potential effort to eliminate position bias in the sonicator horn is to change sample location each time serial PMCA rounds were utilized (Johnson et al., 2012). Moudjou and colleagues claimed position of the samples within the microplate does not affect amplification (Moudjou et al., 2013); however, his group only tested prion strains run in two adjacent rows of one microplate. While they did observe consistent results for each strain, the samples were not relocated to difference positions throughout the microplate to test the reproducibility of amplification. Even though mb-PMCA allowed the use of up to 96 samples to

be run at the same time, decreases in amplification can arise depending on the placement of samples within the microplate. Based on the results displayed in Figure 3-2, variations in amplification efficiency can occur with mb-PMCA; to combat this issue, samples were kept as close to the center of the microplate as possible for optimal results. Using the mb-PMCA protocol also significantly reduced the total time needed to evaluate all samples for PrP^{TSE} detection. Up to three sample sets were positioned near the center of one microplate at a time, and little-to-no variation was observed. For PMCAb, recommended spacing of each individual tube limited the number of samples that could be run at the same time, and, when high levels of amplification were necessary, the *in vitro* results were inconsistent and lanes were skipped in serial dilutions (Appendix A-2). Conversely, no reruns were required, fewer serial rounds achieved maximum amplification, and more sample sets were exposed to sonication at one time with the enhanced strain-specific mb-PMCA, allowing much faster PrP^{TSE} detection to occur.

To test the effectiveness of mb-PMCA in amplifying minute amounts of PrP^{TSE}, serial dilutions of HY-infected BH were used to seed PMCA reactions where 10¹³-diluted HY brains showed a positive signal in 100% of reactions while 10¹⁴-diluted was detected in only one out of five reactions after five rounds of amplification. Dilutions of 10⁻¹⁵ and higher were all negative, suggesting HY-infected BH that was diluted 10⁻¹⁴ has very few active PrP^{TSE} particles per 40 μ L of total PMCA reaction volume able to be detected after serial mb-PMCA rounds. Comparison of the data obtained from our endpoint titrations using mb-PMCA and the HY bioassay establishes a quantitative relationship between PMCA₅₀ and ID₅₀. The sensitivity reached by the mb-PMCA assay exceeded that of the most sensitive hamster bioassay by approximately 5,000-fold (Figure 3-8), which is consistent with other studies that previously determined this ratio (Makarava et al., 2012; Moudjou et al., 2013; Saa et al., 2006b). PMCA₅₀ values are consistently

observed to be higher than ID₅₀ values, which could be attributed to the ability of seeded and generated PrP^{TSE} from PMCA to degrade *in vivo* during animal bioassays (Safar et al., 2005), or a portion of PrP^{TSE} amplifiable during mb-PMCA having little-to-no infectivity (Klingeborn et al., 2011; Makarava et al., 2012).

Because only one of five endpoint titrations saw detection in the 10¹⁴-fold dilution, a potential cross-contamination event cannot be ruled out. Spontaneous *de novo* generation of PrP^{TSE} was not observed using mb-PMCA as the specificity of the plant species grown in non-prion containing media for both hydroponic and soil cultures was 100% (Figure 3-6). In addition, decreasing the total reaction volume from 100 µL for PMCAb to 40 µL for mb-PMCA and fastening each microplate with a tight-fitting sealing plate that covered each individual well prevented any inadvertent sample penetration through microscopic cavities between the top of the wells and the sealing plate during sonication (Barria et al., 2009; Cosseddu et al., 2011). The most likely factor influencing potential cross-contamination is the simultaneous manipulation of seeded and unseeded samples in the same microplate (Cosseddu et al., 2011). The 10¹³-fold and 10¹⁴-fold HY brain dilutions were positioned in adjacent wells, suggesting that the handling of these products could have led to accidental cross-contamination during the preparation of serial mb-PMCA rounds.

To date, two unpublished studies have evaluated prion interactions with plants, and both investigated the effectiveness of prion protein binding to outer root and leaf surfaces (Pritzkow et al., 2014; Rasmussen et al., 2014). Our group went further in-depth and found direct evidence of prion uptake through root hairs, and we were able to estimate the prion concentrations present in the aerial tissues. Even though the mechanism by which plants take up whole proteins remains to be determined, it has been hypothesized that protein enters root hairs through endocytotic

processes (Nasholm et al., 2009; Paungfoo-Lonhienne et al., 2008; Samaj et al., 2005). If this is the preferred mechanism of uptake, plants may selectively internalize proteins as endocytotic absorption limits the size of proteins to up to 200 nm (Zuhorn et al., 2002). However, because internalization of particles with diameters of less than 100 nm occurred rapidly with little stress on cells (Rejman et al., 2004) and prion protein particles associated with the highest infectivity were measured at 17-27 nm (Silveira et al., 2005), plants could unintentionally take up the most infectious PrP^{TSE} oligomers. Our results demonstrating PrP^{TSE} absorption primarily by root hairs is consistent with previous studies that observed uptake of proteins in plants and hypothesized an endocytotic route of uptake (Nishizawa and Mori, 1977; Nishizawa and Mori, 1980; Paungfoo-Lonhienne et al., 2008)

Once the magnitude of inhibition by plant material on mb-PMCA was determined, PrP^{TSE} concentrations in *A. thaliana*, alfalfa, barley, and corn grown in the hydroponic culture were calculated and ranged from 0.42-13 ng PrP^{TSE}/g dry plant. Similarly, infectivity estimates based on previously published results (Chen et al., 2010; Marsh and Kimberlin, 1975) varied from $10^{5.4}$ - $10^{6.9}$ ID₅₀ U/g dry weight. Concentrations of PrP^{TSE} were lower in soil-grown *A. thaliana* compared to hydroponic plants when the same growth conditions were implemented (room temperature, light intensity, access to media in humidity chamber) as the concentrations and infectivity ranged from 0.64-8.8 ng PrP^{TSE}/g dry plant and $10^{5.5}$ - $10^{6.7}$ ID₅₀ U/g of dry weight of *A. thaliana*, respectively, when stem and leaf concentrations are combined. These quantities likely represent a maximum prion concentration able to be taken up by plants as we “overloaded” both culture systems with an initial 2.0% prion-containing media, likely a concentration that would not be found in naturally prion-contaminated environments. Obtaining accurate estimates was difficult as endpoint titrations and ID₅₀ values varied throughout this

study and others. Not only could there have been a contamination issue in one endpoint titration as the highest reported detection using PMCA was from a 10^{13} -fold dilution of original brain homogenate (Saa et al., 2006b), but ID_{50} concentrations have been recently measured at slightly lower values, ranging from $10^{9.2}$ - $10^{9.5}$ ID_{50}/g of brain tissue, than the $10^{10.1}$ concentrations used for our calculations (Ayers et al., 2009; Bartz et al., 2007; Kincaid and Bartz, 2007). Furthermore, the concentration of PrP^{TSE} quantified from brain tissue was determined using a different hamster-passaged prion strain (263K) and may not be equivalent to HY PrP^{TSE} concentrations (Chen et al., 2010). These variations could significantly alter the calculated infectivity and lead to discrepancies between estimated and observed infectivity.

Nevertheless, our calculated ID_{50} values indicate prion interaction with plant tissues can reduce TSE infectivity when compared to a typical HY bioassay, which was confirmed by the observance of lower rates of infection when prion-contaminated *A. thaliana* samples were orally fed to mice (Carlson, 2013; Johnson et al., 2014). One explanation for the reduced oral transmissibility is that plants may contain proteases capable of degrading PrP^{TSE} . Previous studies have determined *A. thaliana* plants encode 828 proteases that assist with nutrient metabolism and protein breakdown in the rhizosphere (Godlewski and Adamczyk, 2007; Hamilton et al., 2003; Paungfoo-Lonhienne et al., 2008; Rawlings et al., 2006; Tornero et al., 1996). One or a combination of these plant proteases could degrade PrP^{TSE} , leading to the decrease in infectivity. Additionally, once contaminated plants are consumed, prions may bind to plant tissue components that do not break down in monogastric animal guts. While enzymatic degradation of PrP^{TSE} can occur at low levels in the gastrointestinal tract (Kruger et al., 2009; Scherbel et al., 2006), plant-bound prions may promote further clearance in the gut and reduce PrP^{TSE} translocation throughout the body.

There are a number of ways to explain the discrepancy in prion concentrations between plants grown hydroponically and *A. thaliana* grown in various soils. First, the hydroponic system represents a simplified culture where the plant growth media could be manipulated by removing inorganic nitrogen from the composition to force plants to take up prions as a nitrogen source. Conversely, soil is a competitive matrix and was not altered for our studies; as a result, prions had to compete with other components for uptake into plants. Soils from natural ecosystems contain microorganisms, organic matter, roots from surrounding plants, and other components that can reduce the amount of prions available for plant uptake. Therefore, the observed concentrations of PrP^{TSE} could be higher in hydroponically-grown plants than that of plants grown in simulated natural environments. Second, prions can bind to a range of soils and soil minerals (Johnson et al., 2006; Leita et al., 2006; Ma et al., 2007; Maddison et al., 2010a; Smith et al., 2011). Desorption of prions from soil has not been observed under mild, environmentally-relevant conditions (Cooke et al., 2007; Johnson et al., 2006; Seidel et al., 2007), suggesting they become fixed to the mineral structure and unavailable for plant uptake once in this bound state. In order for the soil-grown plants to take up infectious prions, they most likely have to acquire unbound PrP^{TSE}; however, in our study, because of extended contact time prions experienced with soil minerals before roots reached the soil, some aqueous PrP^{TSE} became soil bound and, potentially not accessible to plants. Third, the preparation of HY-infected BH differed between the two growth cultures as the prion-containing media used for the soil system was enriched by enzymatic degradation (see Materials and Methods) while the hydroponic system did not undergo the prion enrichment method. It is possible that different interactions could occur when infected brain homogenate is exposed to soil as oppose to enriched PrP^{TSE}. Brain homogenate is complex and PrP^{TSE} will compete for sorption sites with many other constituents once incubated

with soil, potentially leading to less PrP^{TSE} binding to soil minerals (Saunders et al., 2009) and more available for plant uptake. Additionally, non-enriched brain material could contain compounds that assist in the degradation of cell wall structures that allows PrP^{TSE} direct penetration into root hairs. Initial treatment of brain homogenate (i.e., enriched or non-enriched) can be important in studying the environmental fate of prions, particularly as prion interactions with soil and plants are currently still being investigated. Fourth, hydroponically-grown plants had better access to prion-containing media than the soil-grown *A. thaliana*. Once root hairs extended past the agar media from the hydroponic system, each plant species could access an inverted box acting as a large reservoir that contained the prion media. Daily shaking also ensured proper mixing of the media to avoid any sequestering of prions in one area of the reservoir. On the other hand, the soil culture had one small opening roots could grow down into while no shaking could be performed because the glass vials were packed tightly. Attempts were made to confirm prion media was evenly dispersed throughout each individual soil culture system by adding one-third of the soil, then one-third of the prion media, and repeating this process two more times, but it was difficult to distinguish the effectiveness of this approach.

For plants grown hydroponically via the Ice-Cap method, PrP^{TSE} concentrations were within two-logs after converting to the dry weight concentrations. *A. thaliana* and barley had greater PrP^{TSE} uptake compared to alfalfa and corn, which likely resulted from a couple factors. The amount of plant samples tested for *A. thaliana* (8 total plant homogenates) and barley (9 plant homogenates) was representative of an average concentration while alfalfa and corn each had only one homogenate subjected to mb-PMCA amplification. The calculated PrP^{TSE} concentrations from these two crop species may be lower than the actual concentrations present in the aerial tissues. The root structures of each plant also play an important part in nutrient

uptake. Roots from *A. thaliana* are smaller and simpler as the branching pattern consists of one main primary root with less lateral root development (Armengaud et al., 2009; Coudert et al., 2010; Esau, 1965). On the other hand, crop species have large and complex root systems that form many lateral roots branching from the primary root as well as seminal roots that assist in nutrient uptake during germination and plant development (Hochholdinger and Zimmermann, 2008; Smith and De Smet, 2012). While *A. thaliana* is dependent on the main parent root for nutrient uptake, the more complex roots in crop species extend farther from the stem and, when grown in close proximity, could create competition for uptake between plants. Being contained in one reservoir could have limited PrP^{TSE} uptake in barley and alfalfa. Furthermore, while individual corn stalks were grown separately (see Materials and Methods), they were exposed to a hydroponic media containing a 0.1% prion concentration whereas the other plants were grown in 2.0% prion-containing media. To compensate for this difference, the length of PrP^{TSE} exposure for corn was increased to 50 days instead of the 25-day durations for alfalfa and barley. Nevertheless, because of the poor understanding of the kinetics in crop species compared to *A. thaliana* and greater translocation of prions over a larger overall area in corn stalks that could result in a dilution effect throughout the stalk, the true PrP^{TSE} content in plant species may vary. Further investigations that alter growth conditions may elucidate these factors.

Minimal prion uptake occurred from the Plainfield soil even though *A. thaliana* appeared to grow the best in this culture (Appendix B-1), indicating that the soil could have contained enough nutrients already present that no exogenous protein was needed to stimulate plant growth. Similarly, the potting mix was enriched with natural organic materials and mineral supplements, but, even with the known additives, still detected PrP^{TSE} in the two replicates that experienced no bacterial growth. The use of coarse quartz (mesh 20-30), while not ideal for plant

development or typically found in natural environments, was expected to encourage prion uptake because of its larger porosity and limited binding capacity of prions (Johnson et al., 2006) that made accessing the prion-containing media easier for root hairs. All replicates from the quartz culture showed positive PrP^{TSE} signal at levels slightly higher than those from the potting mix. Interestingly, the greatest prion concentrations calculated from soil-grown *A. thaliana* were grown in bluestem clay and were comparable to the estimated prion concentration in hydroponically-grown *A. thaliana* (Figure 3-5). PrP^{TSE} appears to have a particularly strong affinity for clay minerals (Cooke et al., 2007; Johnson et al., 2006; Ma et al., 2007; Rigou et al., 2006), which could make accessing prions difficult for plants. One possible explanation for the higher observance of uptake is that clay particles, because of their extremely small sizes, have micropores small enough that the adhesive forces holding water and other aqueous solutions to the pore wall are stronger than the gravitational forces trying to drain the soil (Idaho Department of Environmental Quality, 2014). Because we overloaded the system with a high initial prion concentration and because of the limited mobility of PrP^{TSE} in soils, the prion-containing media may have stayed more readily accessible in this soil type than the others. Another possibility includes pathogen entry through natural openings or wounds caused by lateral root formation or breakage of root hairs (Jiang et al., 2014; Melotto et al., 2008). Soil minerals produce wounds in the hairs as the root systems expand during plant growth, potentially generating a greater number of entry points for PrP^{TSE} not present in hydroponically-grown plants. The quantities of PrP^{TSE} found in aerial tissues likely vary in natural environments depending on the degree of CWD deposition, soil clay content, and length of time plants have been growing in a particular region.

While detection was observed as early as the third mb-PMCA round in hydroponically-grown *A. thaliana*, the endpoint titrations spiked with plant homogenate exhibited significant

inhibition in the same round. Comparing PMCA reactions seeded with PMCA buffer-spiked and plant-spiked PrP^{TSE} prepared from the same HY BH further suggests the presence of amplification inhibitor(s) in plant tissues (Figure 3-9). Supplementing each PMCA reaction with one Teflon® bead every round might have assisted in the breakage of cellular debris that might contain inhibitory compounds and increased the accessibility of PrP^{C} and other cofactors essential for conversion (Gonzalez-Montalban et al., 2011). In addition, as serial mb-PMCA rounds were conducted, the more dilute the original inoculum becomes and less inhibitory plant material is present in the reaction. This could explain why we observe low PrP^{TSE} detection in some hydroponically- and soil-grown plant samples after five rounds of amplification as the seeds in these PMCA reactions are composed entirely of homogenized plant material. The plant-spiked PrP^{TSE} seeds contained only 20% plant material, leading to efficient amplification in later round of mb-PMCA. Interestingly, discrepancies in amplification occurred between the two spiked endpoint titrations run in the same microplate (Appendix D) and the third run independently in its own microplate (Figure 3-9). Because aliquots of each plant-spiked and PMCA buffer-spiked dilution were taken from an original stock solution and used to seed PMCA reactions, these differences in amplification suggest that perhaps variability in the levels of PrP^{C} and other conversion factors from individual NBH substrate may lead to changes on the extent of *in vitro* prion conversion (Saa et al., 2006b). Another potential issue could have involved the temperature that was maintained in each incubator as deviations from the optimal 37°C were measured occasionally, which could also result in poor amplification (Barria et al., 2012; Morales et al., 2012). Plant inhibition was modeled only for *A. thaliana* homogenate for this study; therefore, further experiments are required to elucidate the effect alfalfa, barley, and corn

homogenates have on amplification to determine a more representative prion concentration in the crop species.

In closing, our data demonstrates that PrP^{TSE} is taken up by root hairs, likely as a nitrogen source, and transported to the aerial tissues, supporting the hypothesis that plants serve as a currently unrecognized route of environmental transmission for prion disease. These findings also suggest PrP^{TSE} is present at concentrations detectable by western blotting after mb-PMCA amplification and in sufficient quantities to cause TSE infectivity. *Arabidopsis thaliana* was used as a model plant to investigate PrP^{TSE} uptake as well as alfalfa, barley, and corn to represent crop species. While infectivity estimates indicate that PrP^{TSE} can remain infectious after internalization into root hairs, the mechanism by which prions enter the plants and kinetics of prion persistence within plant tissues are considerations to examine in future investigations. This study established the potential for CWD prion transmission via plant uptake and highlighted the application of sensitive detection methods like serial mb-PMCA in recognizing CWD infection and prevalence.

CHAPTER FIVE:
Conclusions and Future Directions

5.1 Conclusions

The overall goal of this project was to assess uptake of prion protein in plants in order to further comprehend potential environmental routes of horizontal transmission for natural animal prion diseases. This thesis work demonstrated that prions are found in aerial plant tissues of the model plant *Arabidopsis thaliana* and several crop species in quantities estimated to cause prion infectivity in mammalian hosts. Previous research alluded to a potential interaction between prions and root systems because of the survival of the TSE agent in natural environments and its limited mobility from the soil surface (Brown and Gajdusek, 1991; Cooke and Shaw, 2007; Georgsson et al., 2006; Jacobson et al., 2009; Jacobson et al., 2010; Seidel et al., 2007), soil serving as an environmental reservoir for prions (Johnson et al., 2007; Johnson et al., 2006; Leita et al., 2006; Schramm et al., 2006), and the observance of whole protein uptake into plants (Drew et al., 1970; McLaren et al., 1960; Paungfoo-Lonhienne et al., 2008; Seear et al., 1968; Sung, 1974; Ulrich et al., 1964). To confirm whether prion protein remained in the root tissues or was translocated within the plant to aerial tissues, amplification of prions using mb-PMCA was performed on *A. thaliana*, alfalfa, barley, and corn grown hydroponically on prion-containing media as well as *A. thaliana* grown in cultures with prion-containing media incubated with various soil types. Of the four species tested using this sensitive detection method, all plants from both cultures accumulated prions via root uptake and transported them to stem and leaf tissues. Estimated prion concentrations within the tissues were also calculated using serial mb-PMCA and, after taking inhibition of plant material into consideration, were determined to be present in sufficient amounts that can serve as an additional environmental source of TSE infectivity. These data support the concept that plants may be capable of absorbing prions shed

from infected animals and that internalized prions represent a previously unrecognized route of prion transmission on the landscape.

5.2 Future Directions

While investigations have already been conducted on uptake of bacterial and viral pathogens by plants (Franz et al., 2007; Hirneisen and Kniel, 2013; Jablasone et al., 2005; Solomon and Matthews, 2005; Urbanucci et al., 2009), this is one of the first reports demonstrating the internalization of infectious prions. Further research is needed to better comprehend the risk of plants as reservoirs for CWD transmission. In particular, understanding the fate of prions once taken up by plants is a next logical step. Because the rate of prion accumulation inside plants is unknown, a detailed kinetics study examining how quickly plants become contaminated with prions after initial exposure and how long prions remain infectious once absorbed would help clarify the risk prolonged exposure to prions has on plants grown in CWD-contaminated environments. Moreover, time-course studies can establish conditions permitting maximal levels of prion uptake in different plant species during various growth stages. Should plants show a propensity to acquire prions and effectively degrade or inactivate them, those species could be grown in known contaminated areas and harvested after some time to potentially remove prions from the soil. From the work presented here, *Arabidopsis thaliana* demonstrated uptake in four separate soil types; however, the aerial tissues remain infectious once orally fed to mice (Carlson, 2013; Johnson et al., 2014). Other species not tested yet may have the ability to break down environmentally shed prion protein.

Determining the mechanism of prion uptake into plants is another key to designing practices to minimize plant contamination. Our studies revealed roots have the capability of

absorbing prions, and several mechanisms are known to be used by pathogens to gain entry into the root hairs. Uptake through endocytotic processes have been hypothesized as a main route by which plants are able to internalize whole proteins (Adlassnig et al., 2012; Nishizawa and Mori, 1977; Nishizawa and Mori, 1980; Paungfoo-Lonhienne et al., 2008); other proposed mechanisms include direct penetration via enzymatic degradation of plant cell wall structures and entry through natural openings or wounds (Dou and Zhou, 2012; Guest and Brown, 1997; Jiang et al., 2014). Using confocal microscopy, exposure of *Arabidopsis*-derived plant cells to metabolic inhibitors that prevent plant endocytotic processes while incubating the cells with PrP^{TSE} would provide additional knowledge to the basic mechanism by which plants gain access to prions. Of course, uptake mechanisms may vary between plant types and could indicate fundamental differences in nutrient metabolism across each species. Similar confocal microscopy studies can be conducted to test for uptake mechanism of multiple species of plants. Though our prion uptake experiments do not perfectly mimic prion uptake by plants in complex natural environments, the results are critical toward understanding the threat of prion infectivity in crop food sources and can assist in devising measures that interrupt disease transmission.

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CHAPTER SIX:
Appendix

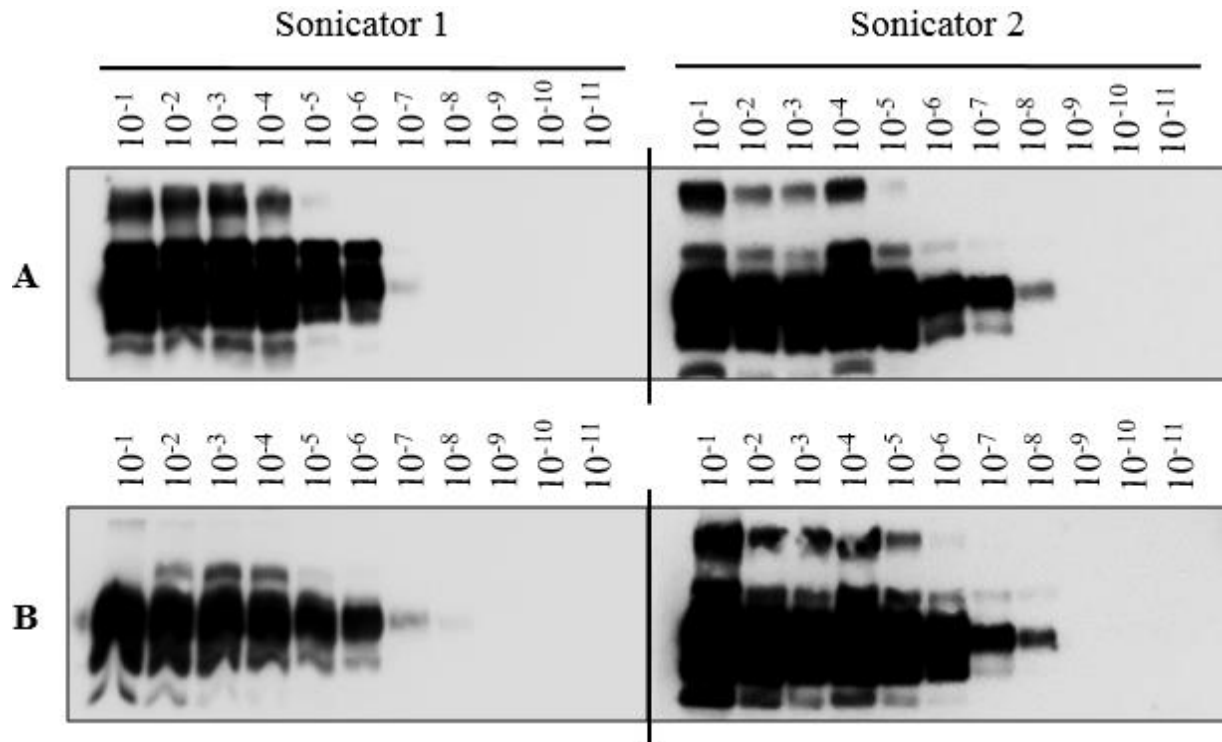


Figure A-1. Verification of consistent amplification after one round of mb-PMCA. Four independent endpoint titrations of 10% HY-infected brain homogenates were positioned in the center two lanes of each microplate and subjected to a single round of mb-PMCA. Samples from (A) and (B) were amplified at the same time, but exposed to 96-cycle PMCA rounds from different sonicators as Misonix S-4000 represents sonicator 1 and Qsonica Q700 represents sonicator 2.

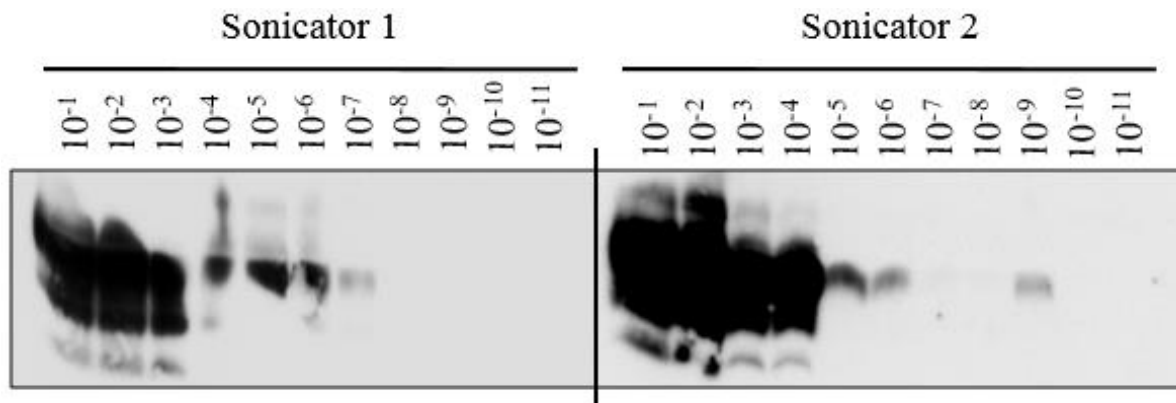


Figure A-2. Low sensitivity and inconsistent amplification using PCR tubes for PMCAb. 10% HY-infected brain material was serially diluted into hamster NBH substrate and subjected to three rounds of PMCA using individual PCR tubes. Amplification from two independent experiments, one using the Misonix S-4000 (sonicator 1) and the other using the Qsonica Q700 (sonicator 2) were conducted, and the third round results are shown.

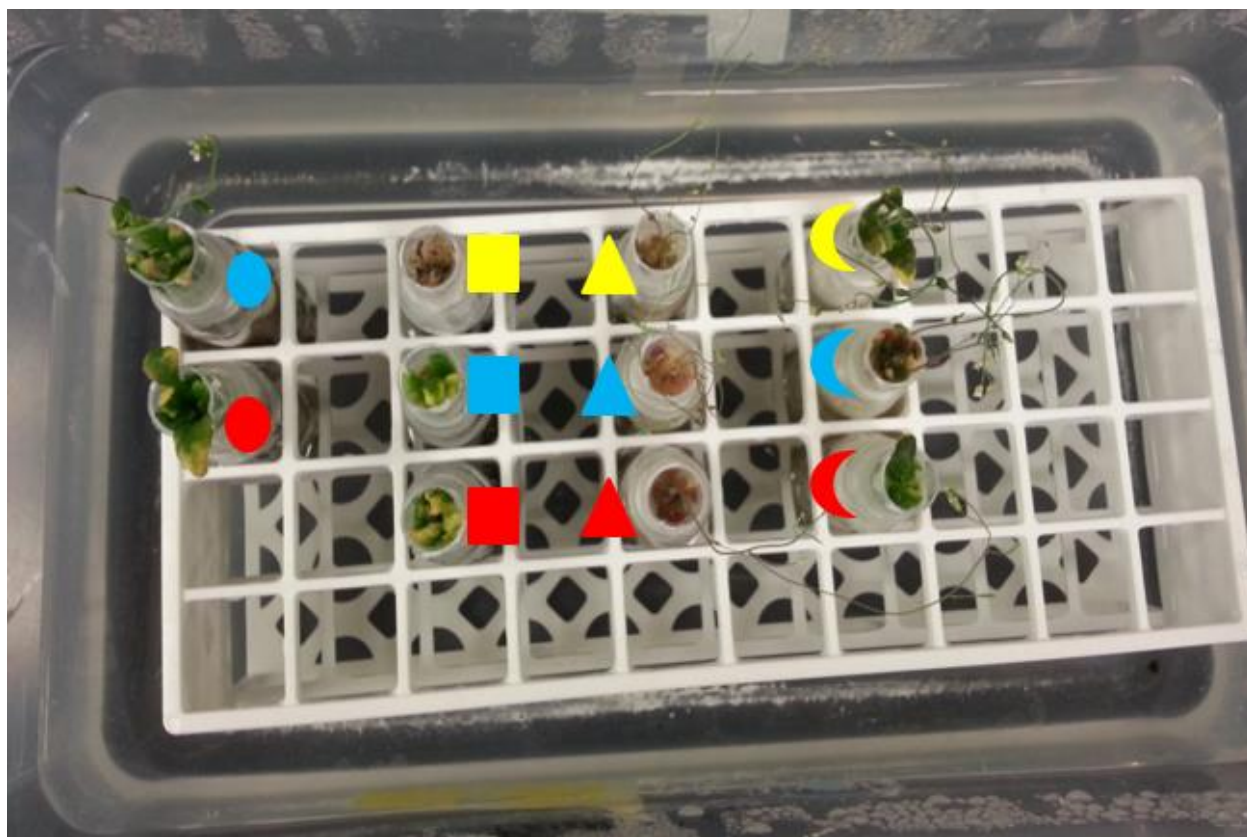


Figure B-1. Image of soil-grown culture for *A. thaliana*. Enriched HY-containing or non-infected brain homogenate was dialyzed against a 1:1 mixture of tap and distilled water and added to a final concentration of 2% (w/v) to 15 mL of the following soils in glass vials: Plainfield A soil (circles), Miracle-Gro® potting mix (squares), Ottawa sand (triangles), and Bluestem clay (crescents). Seeded *A. thaliana* on agar tips were placed onto the tops of the vials containing soil-BH mixtures such that only plant roots would germinate downward into the soil medium below. After three weeks of growth in a humidity-controlled culture, plants were separated from the roots and divided into stem and leaf homogenates, respectively. Each soil culture was run in triplicate, except for Plainfield A soil, where each colored dot represents a specific homogenate where red is plant #1 (P1), blue is plant #2 (P2), and yellow is plant #3 (P3).

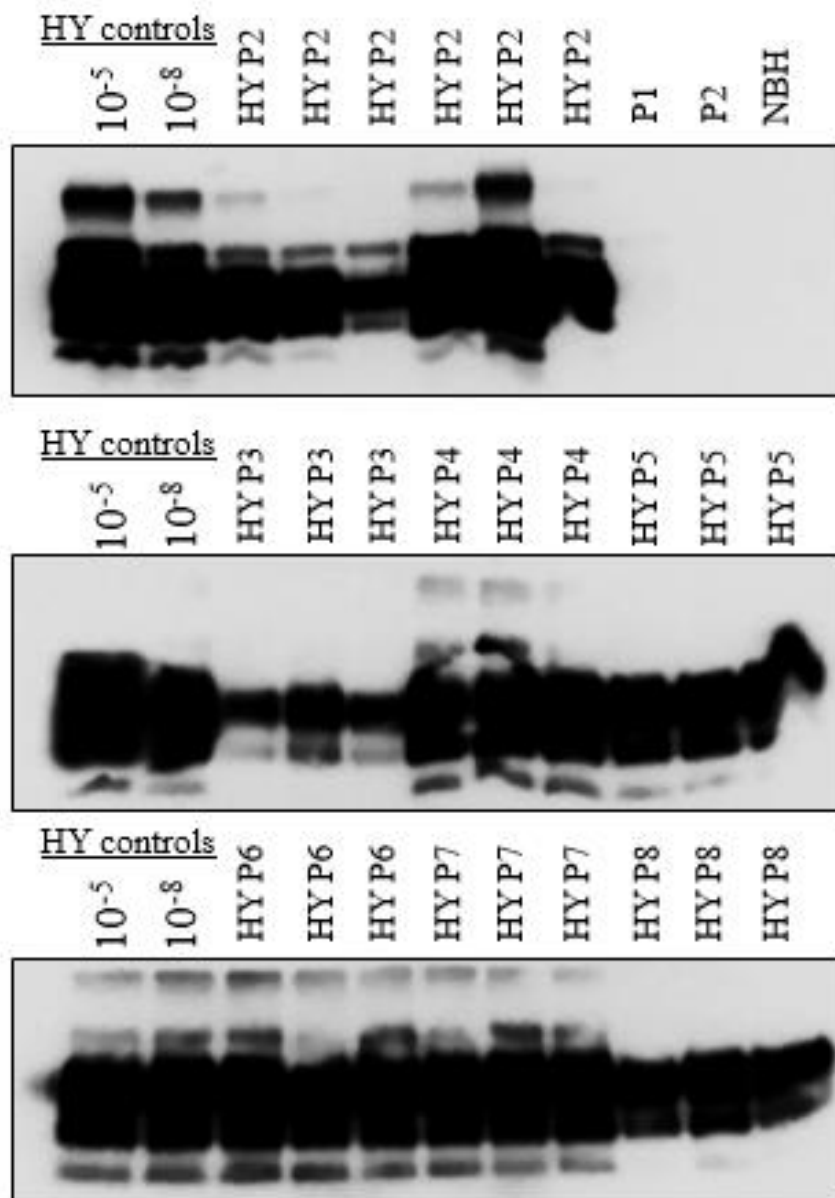


Figure B-2. Additional mb-PMCA results from hydroponically-grown *A. thaliana*. Four μL seeds of 50% (w/v) *A. thaliana* plant homogenates were seeded in 36 μL hamster NBH and subjected to serial rounds of mb-PMCA using standard conditions (10 second sonication followed by 29:50 minute incubation at 37°C, 96 cycles per round). Prion-contaminated plants are denoted as “HY PX”, where each unique number assigned to “X” refers to a specific plant homogenate. Controls include 1) tenfold dilutions of HY-positive 10% brain homogenate (dilution factors listed above each figure) were used in the same 4 μL seed:36 μL substrate ratio as the plant reactions, and 2) 40 μL hamster NBH to control for *de novo* prion synthesis during mb-PMCA (labeled “NBH” above each image) that were subjected simultaneously with plant reactions. No PrP^{res} was detected in any samples assayed in which plants grown on non-prion containing media were used to seed mb-PMCA reactions (non-prion contaminated plants denoted as “PX” where each unique number assigned to “X” refers to a specific plant). Results from the fifth round are shown here.

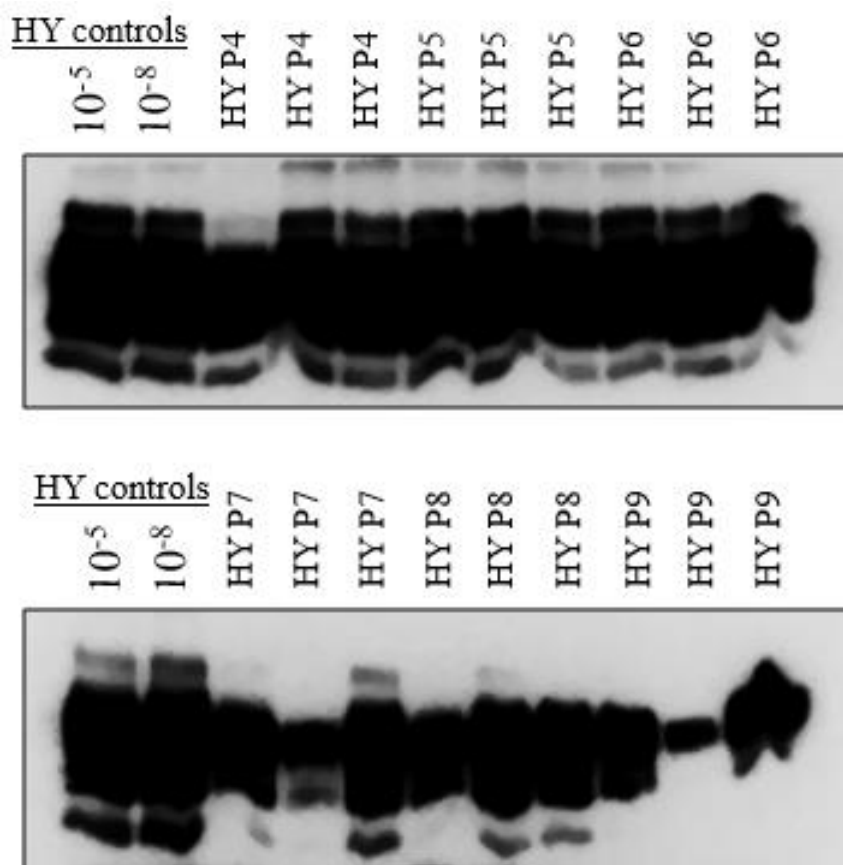


Figure B-3. Additional mb-PMCA results from hydroponically-grown barley. After growth for 25 days on 2% HY-containing hydroponic media, four μ L seeds of 50% (w/v) barley plant homogenates were seeded in 36 μ L hamster NBH and subjected to serial rounds of mb-PMCA using standard conditions (10 second sonication followed by 29:50 minute incubation at 37°C, 96 cycles per round). The same denominations detailed in Figure B-2 are utilized here, and the products from the fifth round of mb-PMCA amplification are shown.

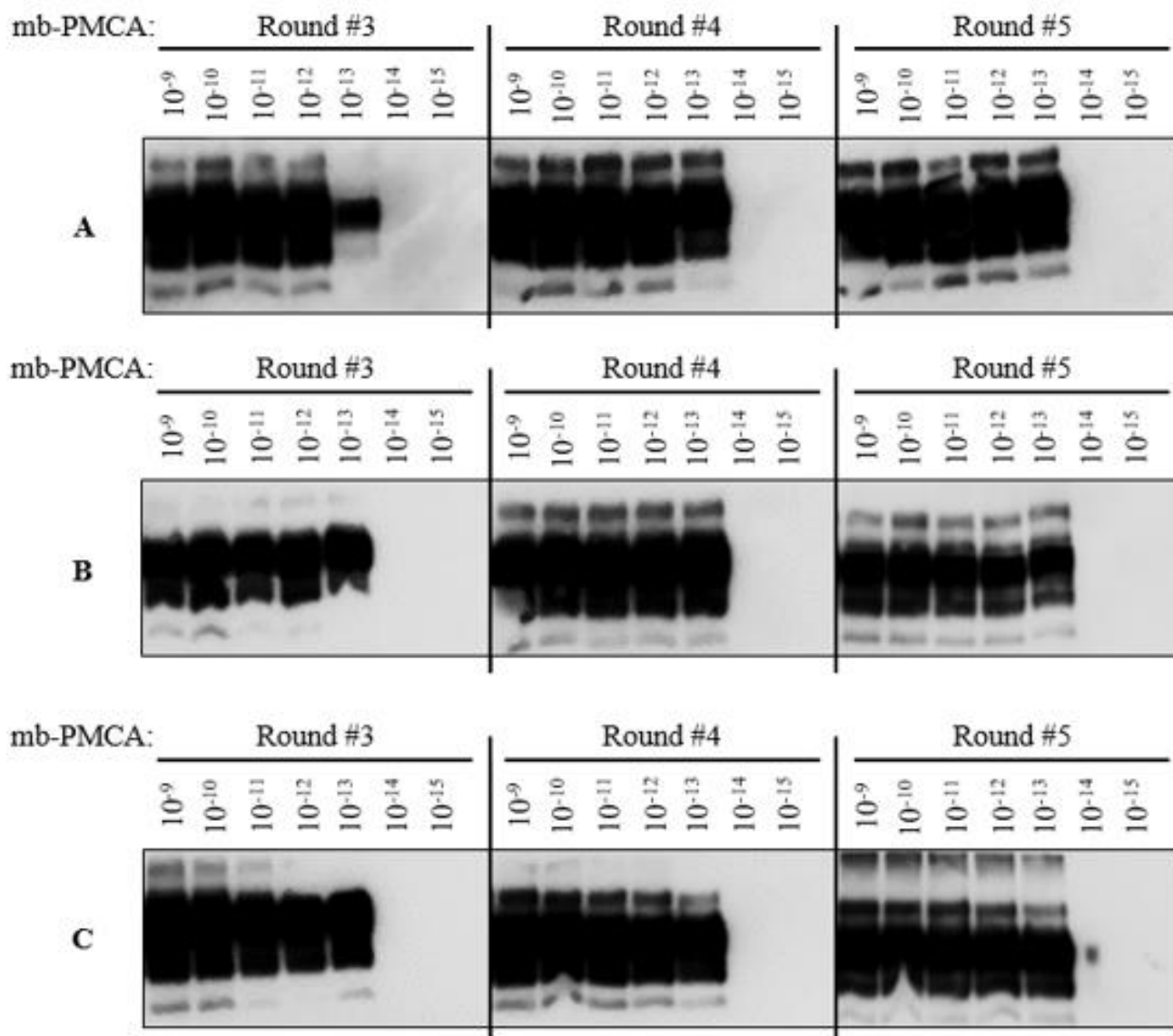


Figure C-1. Additional endpoint titrations utilized for quantifying PrP^{TSE} concentrations. Three independent endpoint titrations of 10% HY-infected brain homogenate were positioned either (A) on the left or (B and C) on the right sides of the microplate similar to previous runs (Figure 3-2) and subjected to serial 96-cycle rounds of mb-PMCA using the Misonix S-4000 sonicator. Products of mb-PMCA from rounds three, four, and five are indicated above each image. Including the two previously mentioned endpoint titrations (Figure 3-7, Figure 3-8a), the densitometric information was determined from all five series, and an overall average signal strength for each brain dilution could be used for the quantification process.

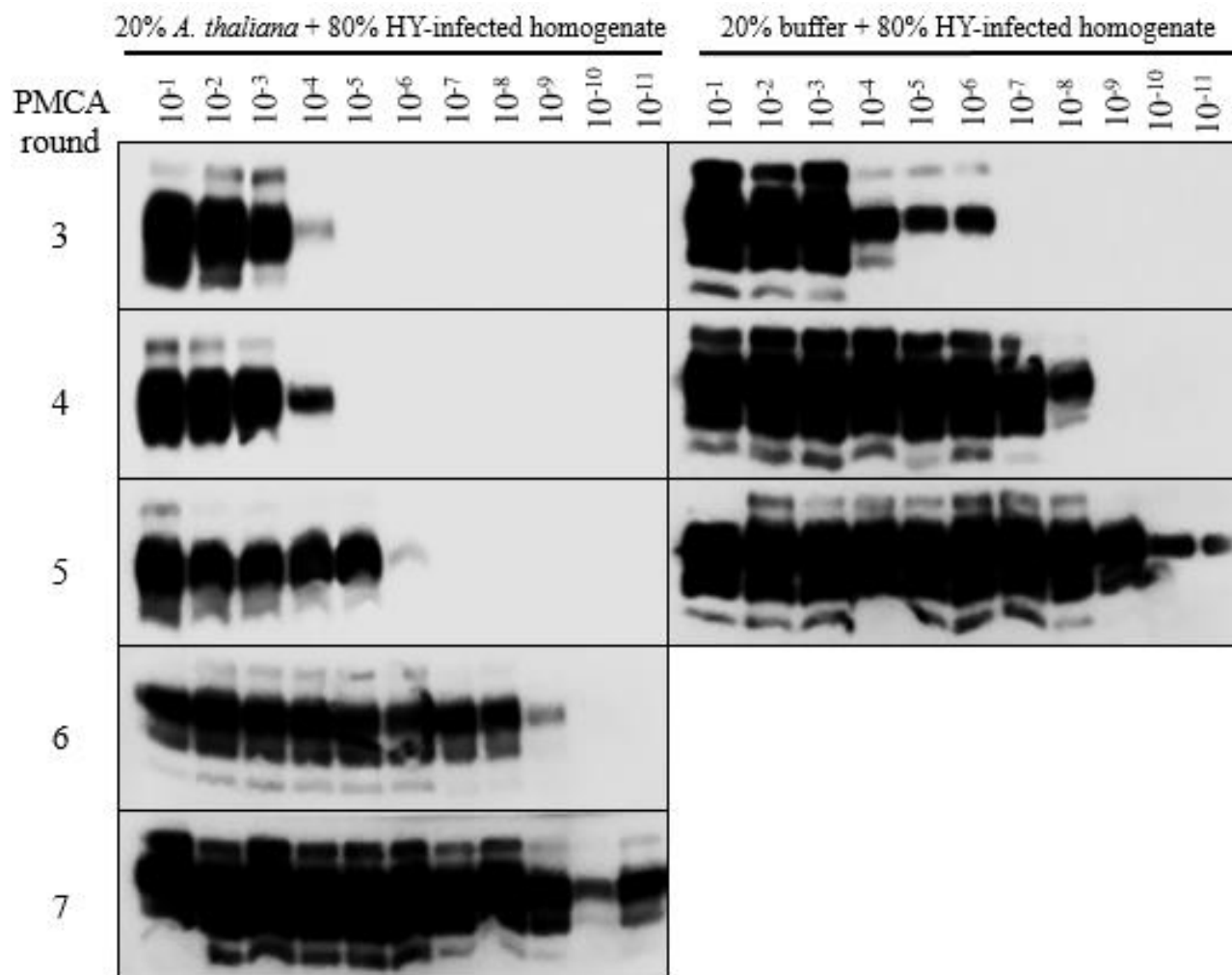


Figure D-1. Additional spiked endpoint titration utilized for observance of inhibitory effects during mb-PMCA. 50% (w/v) *A. thaliana* homogenate was incubated for two hours with serial tenfold dilutions of 10% HY-infected brain homogenate at a 1:4 ratio to create seeds, which were combined with hamster NBH substrate and subjected to serial rounds of mb-PMCA (96 cycles each), as indicated. 10% PMCA conversion buffer was also spiked into dilutions of HY-positive brain homogenate in the same 1:4 ratio and utilized as a control to determine inhibition per PMCA round. Each endpoint titration was run using the Misonix S-4000 sonicator. Numbers on top of each lane correspond to the dilution of the brain homogenate used to seed amplification reactions.

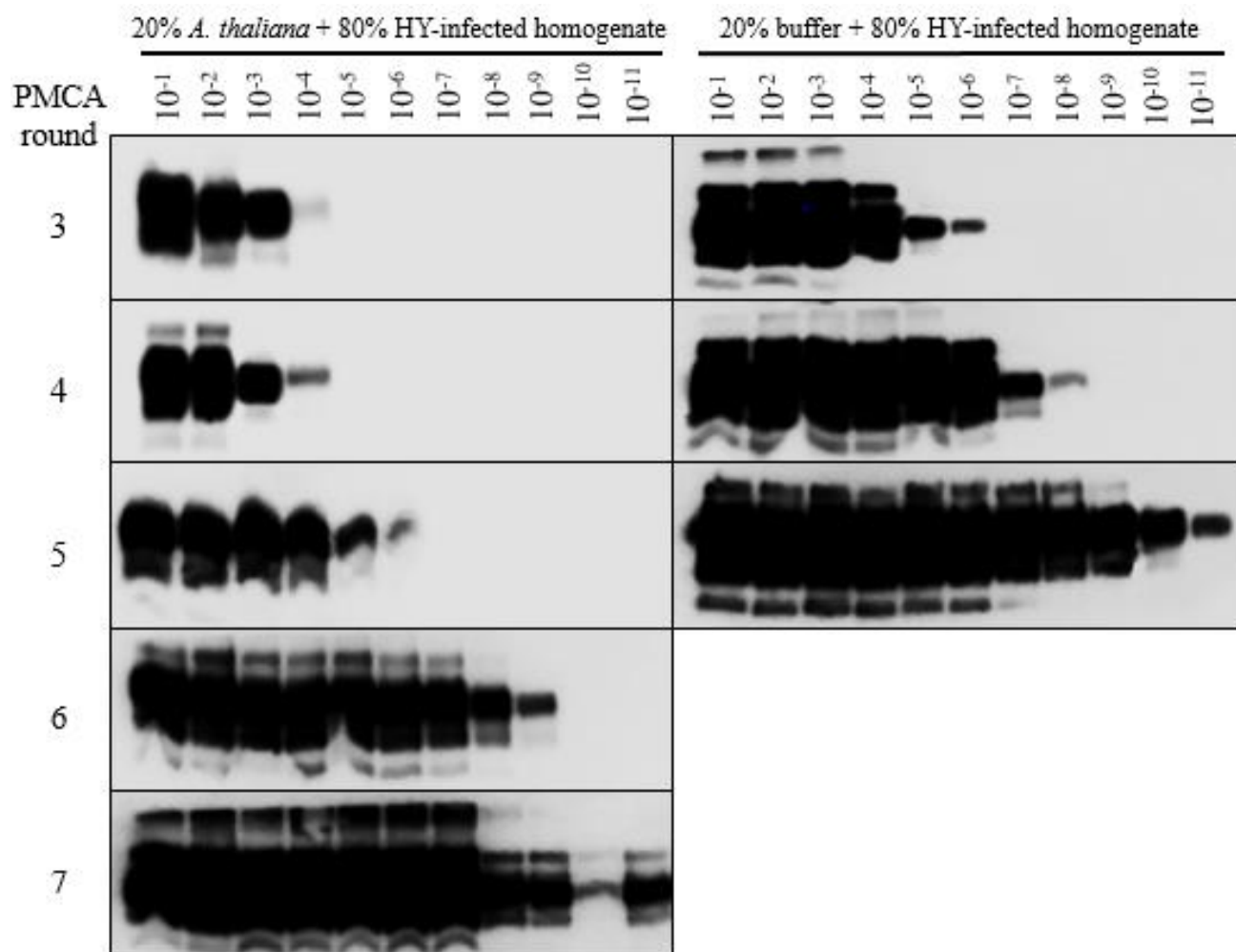


Figure D-2. Final spiked endpoint titration utilized for observance of inhibitory effects during mb-PMCA. 50% (w/v) *A. thaliana* homogenate and 10% PMCA conversion buffer was incubated for two hours with serial tenfold dilutions of 10% HY-infected brain homogenate at a 1:4 ratio to create seeds, which were combined with hamster NBH substrate and subjected to serial rounds of mb-PMCA (96 cycles each), as indicated. Plant-spiked and buffer-spiked endpoint titrations were run in the Qsonica Q700 and positioned as close to the center of the microplate as possible. Numbers on top of each lane correspond to the dilution of the brain homogenate used to seed amplification reactions.