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Investigation on the uptake of functional proteins and infectious prions into wheat plants through the root system

by

Jay Douglas Rasmussen

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ABSTRACT

Prions are the proteinaceous particle responsible for infections in a class of neurodegenerative diseases. These diseases affect a number of mammals including cervids where it is termed Chronic Wasting Disease (CWD). Prions enter the environment and persist for years. Plants have the ability to take up large organic molecules like proteins and bacteria as a potential nitrogen source. This project used wheat (*Triticum aestivum* L.) to determine the reason for protein uptake in plants and if prions are also taken up by plants. We found that bovine serum albumin was not a suitable nitrogen source for plants but uptake of ovalbumin into the stem was possible when minor root damage was present. Conversely, CWD prions bound to the outside of wheat roots and were not taken up into the stem. This work suggests that plants do not act as a vector in the transmission of prion diseases such as CWD.

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AMF	arbuscular mycorrhizal fungi
AR	attack rate
BH	brain homogenate
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
C	combination treatment
CJD	Creutzfeldt-Jakob disease
CPP	cell-penetrating peptide
CPW	cell and protoplast wash solution
CWD	chronic wasting disease
FDC	follicular dendritic cells
GFP	green fluorescent protein
H	high treatment
HATS	high-affinity transport system
IN	inorganic nitrogen
IP	incubation period
L	low treatment
LATS	low-affinity transport system
M	medium treatment
mAb	monoclonal antibody
MS	Murashige and Skoog media
Mte	montmorillonite
N	nitrogen
OV	ovalbumin
OVAF488	Alexa Fluor 488 conjugated ovalbumin
OVAF555	Alexa Fluor 555 conjugated ovalbumin
OVAF647	Alexa Fluor 647 conjugated ovalbumin
P	protein nitrogen
PBS	phosphate-buffered saline
PK	proteinase K

PMCA	protein misfolding cyclic amplification
PrP^{C}	cellular prion protein
PrP^{TSE}	infectious prion protein
SDS	sodium dodecyl sulphate
TME	transmissible mink encephalopathy
TME HY	hyper strain transmissible mink encephalopathy
TSE	transmissible spongiform encephalopathy
vCJD	variant Creutzfeldt-Jakob disease
WB	Western Blotting

Chapter 1: Introduction

The growing human population is putting pressure on the agriculture industry to produce more food with less land. Food crops can be used as an option for feeding developing countries based on their relatively lower cost as compared to livestock products. All food crop plants require essential macronutrients that include nitrogen (N), phosphorous and potassium. Nitrogen is commonly a growth limiting factor for plants because of its involvement as a building block in DNA and amino acids. Uptake of N by plants from the surrounding environment is important to acquire enough N for these essential plant molecules. The ability of crop plants to acquire enough N is crucial for maximizing growth.

PLANTS & NITROGEN

In the environment

There are multiple forms of N in the soil environment that are a part of the larger N cycle. Some of the smallest N particles are inorganic molecules such as nitrate and ammonium. These inorganic molecules can be created by various conversion processes. One of these processes is N-fixing where specialized microorganisms convert nutritionally unusable atmospheric N_2 into inorganic N. Nitrogen fixing can be achieved either by free-living soil microorganisms or nodule-inhabiting bacteria that form a symbiosis with certain plants like legumes (Oldroyd and Downie 2008). Nitrogen fixation results in the production of ammonium which can then be converted to nitrate by nitrification (Oldroyd and Downie 2008). This

conversion of atmospheric N is an important step in the cycle that returns usable N to the soil for use by plants and other organisms. A substantial portion of N contained in soil is associated with organic sources (Jan et al. 2009). Organic material is constantly being added to the soil environment through plant and animal decay. The breakdown of organic N sources is facilitated by the release of proteases from soil microorganisms as well as from the roots of some plants (Adamczyk and Godlewski 2007; Adamczyk et al. 2009; Jan et al. 2009). Large proteins in soil that arise from decay are hydrolyzed into smaller peptides and amino acids (Jan et al. 2009).

Application of fertilizer to arable land also represents a large contributor to nutrients and particularly N in the soil environment. The use of inorganic fertilizers is a common practice in agriculture and is a reason for the dramatic increase in crop yields that have been observed in the past ~60 years (Robertson and Vitousek 2009). Application of organic fertilizers such as manure and compost to land is also a strategy on many farms to increase the availability of nutrients for crops (Robertson and Vitousek 2009). Anthropogenic fertilizing results in an increase of N in the locally applied area as well as increasing the risk of it spreading to the broader environment through leaching or flow with surface water (Robertson and Vitousek 2009). The sum of different inorganic and organic N molecules contributes to the total available N within soil.

Root Architecture

The sessile nature of plants represents a limitation to acquiring nutrients from the surrounding soil environment. The root system is the primary organ responsible

for N uptake and the ability to change its architecture allows plants to respond to the environment. One of the most important N molecules based on plant health is nitrate and it is also one of the main factors in root patterning. Nitrate induces local lateral root (LR) proliferation in barley as the plant makes an effort to acquire sufficient N (Drew and Saker 1975; Drew et al. 1973). Not only does nitrate induce LR proliferation it also has an inhibitory effect when supplied in excess by reducing LR proliferation (Zhang and Forde 2000). This nitrate-dependent regulation of LR is linked to the plant hormone, abscisic acid (ABA), based on studies showing that ABA insensitive mutants do not respond to nitrate (Signora et al. 2001). Another plant hormone, auxin, is also involved in patterning of root growth through LR emergence (Casimiro et al. 2001; Malamy and Ryan 2001). Recent work has further identified the involvement of microRNA and auxin response factors in the regulation of LR (Gifford et al. 2008; Vidal et al. 2010). Auxin is localized at the LR points, a phenomenon that is facilitated by the nitrate transporter *NRT1.1,* further establishing a linkage between nitrate and lateral root development (Krouk et al. 2010). When applied externally to roots, the synthetic auxin 2,4-D induces a unique response termed *para*-nodules where more bulbous LR proliferate (Francisco and Akao 1993). Extensive root restructuring in the formation of nodules for symbiosis with N-fixing microorganisms like *Rhizobia spp.* is well known (Oldroyd and Downie 2008). Root growth and response to the local environment bring the root system into contact with nutrients but plants have other strategies to internalize nutrient molecules.

Uptake mechanisms

Plants acquire nutrients for growth, metabolism and reproduction from the surrounding soil environment. There are a diverse number of strategies and mechanisms plants utilize to acquire different N sources (Fig. 1-1). This acquisition is accomplished by specific active transporters for each N source. Some of the most studied transporters are for inorganic N molecules such as nitrate and ammonium (Ludewig et al. 2002; Tsay et al. 2007). Uptake mechanisms are typically divided into two categories designated high and low affinity transport systems (HATS and LATS, respectively) (Gutierrez et al. 2011). High affinity transport systems are active under lower concentrations of a specific nutrient (μM) while LATS work at higher concentrations (mM) (Gutierrez et al. 2011). Uptake of nitrate and ammonium molecules occurs primarily through nitrate transporters (NRTx) and ammonium transporters (AMTx), respectively (Girin et al. 2010; Ludewig et al. 2002).

The ability of plants to access different organic N sources is crucial to satisfy N requirements. Amino acids are absorbed into roots by a number of different transporters. Amino acid permeases (AAPx) transport a number of different neutral amino acids and glutamic acid into roots (Lee et al. 2007). Lysine/histidine transporters (LHTx) are another family of transporters that act on neutral and acidic amino acids as well as lysine and histidine (Chen and Bush 1997; Hirner et al. 2006; Svennerstam et al. 2007). There are also other families of transporters that facilitate the uptake of amino acids into the plant roots. Both di- and tri-peptides can also be directly acquired by the plant roots through the peptide transporter (PTRx) family of transporters (Dietrich et al. 2004; Komarova

et al. 2008). These plant transporters are one of two mechanisms to acquire N with the other being interactions with other organisms that increase N uptake.

Plants form symbiotic relationships with soil microorganisms to enhance their ability to access nutrients within the soil environment. Mycorrhizal fungi associate with plant roots and enhance the ability of plants to acquire nutrients from soil (Parniske 2008). This symbiotic relationship was previously thought to be specific to the acquisition of phosphorous by plants, but the importance of these relationships for N acquisition is becoming increasingly apparent (Harrison and van Buuren 1995). In this symbiosis, fungi acquire N from outside the root zone for the plant and, in return, the plant provides the fungi with carbon in the form of hexose (Parniske 2008). Mycorrhizal fungi are usually classified as either endomycorrhiza or ectomycorrhiza as distinguished by the ability of hyphae to penetrate root cells or not. Mycorrhizae have the ability to uptake different forms of N but the efficiency of transferring these N molecules to the host varies with the form of N. Studies have shown that arbuscular mycorrhizal fungi (AMF) are more efficient at transferring N in the form of ammonium to the host plant than nitrate even though fungi can take up both N sources (Govindarajulu et al. 2005; Jin et al. 2005; Tanaka and Yano 2005). Recent work has also shown that AMF can access organic N directly from the environment (Chapin et al. 1993; Hodge and Fitter 2010; Leigh et al. 2009). Large amounts of organic N are utilized by fungi but only a fraction appears to be transferred to the host plant (Hodge and Fitter 2010). These associations with mycorrhizae have a significant contribution

to N acquisition (up to75%) by most plants (>70%) (Parniske 2008; Tanaka and Yano 2005).

Legumes form symbiotic relationships with N fixing bacteria in order to increase N acquisition. *Rhizobia spp.* are bacteria that colonize roots by communicating directly with the plant through release of signalling molecules called nod factors (Oldroyd and Downie 2008). The roots engulf these bacteria and form specialized organs in the root called nodules (Oldroyd and Downie 2008). The bacteria then convert N_2 into ammonia that can be used by host plant (Oldroyd and Downie 2008).

These different mechanisms of uptake including plant transporters and symbiosis with other organisms allow plants to acquire N from the surrounding environment. Although these strategies are diverse, N deficiency still occurs in natural settings where anthropogenic fertilization does not occur. Accessing other N sources is necessary to reach nutrient requirements in these conditions.

Large organic molecules

In the soil environment there is competition for resources among different organisms. Most soil microbes utilize the same simple inorganic N molecules such as ammonium and nitrate as plants. There is also recent evidence to suggest that plants and microbes compete for organic amino acids and peptides with the same chirality (Hill et al. 2012). Consequently, plants and microbes often have to compete for a limited N pool in soil. The ability of plants to directly utilize larger organic N molecules such as protein could confer a competitive advantage to plants over microbial competitors for N. Initial studies used different methods to

determine uptake of protein into roots excised from barley plants. Radioactive labelling of lysozyme and hemoglobin was used to track the movement of these proteins into barley roots (McLaren et al. 1960). Both proteins were found primarily in the intercellular spaces of the roots (McLaren et al. 1960). Other early work supported these findings with uptake of lysozyme into excised roots being supported by decline in the protein concentration of the surrounding solution (Bradfute and McLaren 1964). Uptake of lysozyme was also confirmed in a number of plant types including maize, vetch, and tomato using fluorescent labelling (Seear et al. 1968). These studies outline that uptake of protein into the root system can occur in some plants and is not specific to one protein. Whole tomato plants were used to document the uptake of radioactively labelled hemoglobin and lactoglobin and the fact that radioactive metabolites from these proteins were detected in the leaves suggests that these proteins were metabolized (Ulrich and McLaren 1965). No radioactive metabolites of lysozyme and ovalbumin were detected, with the radioactivity in leaves appearing to occur as a result of these proteins entering the plant through areas of mechanical damage in the roots (Ulrich and McLaren 1965). If protein enters the roots through damaged sites and reaches the vasculature, it can be transported to the stem and leaves via the xylem (Neumann et al. 2010). More recent work has shown that green fluorescent protein (GFP) has the ability to enter the roots of *Arabidopsis thaliana* and *Hakea actites* (Paungfoo-Lonhienne et al. 2008). These authors suggest that protein moves through the apoplast once taken up by roots (Paungfoo-Lonhienne et al. 2008).

Protoplasts are used in primary cell culture studies and in particular have proven useful for investigating the passive entry of cell penetrating peptides (CPP) rich in positively charged amino acids into plant cells (Eudes and Chugh 2008). Studies have shown that GFP is unable to enter protoplasts without the assistance of a CPP (Qi et al. 2011). Considering that proteins may not be able to directly enter individual cells, apoplastic movement may be the most common mechanism of protein uptake in plants.

The above studies outline the ability of plants to take up larger organic molecules in the form of protein. However, the nutritional value of proteins as a N source for plants remains unclear. Initial studies investigated beta-lactoglobin and lysozyme as sources of N for tomato plants. Beta-lactoglobin had negligible nutritional value in both sterilized sand and soil growth medium (Ulrich et al. 1964). Lysozyme had similar nutritional value to a low nitrate control treatment in tomato plants but high nitrate controls produced significantly more biomass than those grown in nutrient solutions containing lysozyme (Ulrich et al. 1964). Considering that lysozyme can be absorbed by many different types of plant and that it confers some nutritional value, direct utilization of protein N by some plant species may be possible. Other work grew wheat in liquid growth solutions to assess the value of casein as a N source (Adamczyk et al. 2008). Plants grown with casein as the sole N source produced more biomass than plants that were not supplied with N (Adamczyk et al. 2008). Furthermore, proteolytic activity within the growth solution increased as the amount of casein supplied was increased (Adamczyk et al. 2008). This suggests that wheat plants were capable of utilizing

casein protein as a N source after it had been degraded by proteases released from roots (Adamczyk et al. 2008). The two non-mycorrhizal plants, *Hakea actites* and *Arabidopsis thaliana*, have also been examined for their ability to utilize bovine serum albumin (BSA) as a N source (Paungfoo-Lonhienne et al. 2008). *H. actites* exhibited an increase in root biomass, but no significant increase in shoot biomass when grown on BSA as compared to when it was grown with no N (Paungfoo-Lonhienne et al. 2008). In contrast, *A. thaliana* biomass was higher in plants grown with BSA as compared to no N, suggesting that this protein was utilized as a N source (Paungfoo-Lonhienne et al. 2008). These studies outline how the nutritional value of protein for plants is not clearly elucidated, with response depending on plant species, protein source and the nature of the model growth system employed in experiments.

It is somewhat surprising that there is a scarcity of data on the ability of plants to uptake simple proteins, but a rapidly growing body of evidence that pathogenic bacteria and viruses are taken up by plants exists. A number of groups have examined the ability of fresh produce to serve as a vector for transmission of pathogenic bacteria and viruses. Lettuce (*Lactuca sativa*) has been frequently used as the model plant to study the potential uptake of viruses. Canine *calicivirus* has been detected in leaves of lettuce that lacked and exhibited root damage after being grown hydroponically or in soil (Urbanucci et al. 2009). In contrast, human *norovirus G2* was not detected in the leaves of lettuce grown under similar conditions (Urbanucci et al. 2009). Others have documented the internalization of norovirus in lettuce leaves (Dicaprio et al. 2012; Wei et al. 2011). Uptake of

norovirus and transport to leaves was more commonly documented when lettuce was grown hydroponically with a one-time high dose as opposed to a sustained low-dose of virus medium (Wei et al. 2011). Another member of *Caliciviridae*, porcine sapovirus, has also been shown to enter lettuce roots and be transported to leaf tissues (Esseili et al. 2012).

More recently, researchers have focused on documenting the extent to which internalized bacteria in fruits and vegetables may be responsible for the spread of disease. *Escherichia coli* and *Salmonella enterica* remain viable on the surface of a variety of plants for a considerable period of time enabling plants to act as a source of disease transmission (Roy et al. 2013; Zheng et al. 2013). Interestingly, *A. thaliana* and lettuce have defenses such as stomatal closure and pattern recognizing innate immune responses to pathogenic bacteria. These plant defenses appear to depend on the nature of the bacteria, with *E. coli* O157:H7 inducing a strong immune response and *S. enterica* Typhimurium SL1344 causing a negligible response (Roy et al. 2013). The ability of different plants to internalize bacteria through roots has also been studied. Corn was shown to be able to uptake *E. coli* strain TG1 through roots and transport it to the stem (Bernstein et al. 2007). If roots were damaged, uptake and movement of *E. coli* into the stem of corn was accentuated (Bernstein et al. 2007). Similarly, *E. coli* O157:H7 has been shown to enter the roots of both lettuce and spinach in a study where root damage was not manipulated (Wright et al. 2013). Not only does the uptake of bacteria into plants appear to depend on the species of bacteria, it even appears to vary within a species as *E. coli* O157:H7 was readily internalized in

spinach but *E. coli* K-12 failed to enter spinach roots (Wright et al. 2013). *S. enterica* is also taken up via roots and transported to the stem and leaves in sweet basil plants, but the pathogen only survived for ~24 h as compared to more than a week on the plant surface (Gorbatsevich et al. 2013). Tomato plants have been shown to internalize *S. enterica* through the roots, but transport of it to other areas of the plants occurred at low frequency. Movement to the stem was increased if *S. enterica* was inoculated into a sandy loam soil immediately after tomatoes were transplanted (Zheng et al. 2013). In a comparative study with leeks (*Allium porrum*), *S. enterica* was internalized through roots and transported to the shoots at higher numbers than *E. coli* O157:H7 (Gurtler et al. 2013). This study also found that arbuscular mycorrhizal fungi increased the frequency of internalization and transport of both *S. enterica* and *E. coli* O157:H7 to shoots of leeks (Gurtler et al. 2013). Exposure of both tomato plants and *A. thaliana* to *E. coli* strain Bl21 showed uptake of this bacterium into the roots and there was evidence that it was used as a N source by the plant (Paungfoo-Lonhienne et al. 2010). These studies outline that uptake of different pathogenic and non-pathogenic bacteria is possible in several plant types but frequently requires root damage and is dependent on growth conditions.

These studies show that bacterial and viral pathogens in the soil have the potential for transmission through plants to new hosts. Prions are a protein-based pathogen that has a high N content and can also enter the soil environment. The potential internalization into plants and subsequent transmission to new hosts needs to be investigated for prion diseases to determine factors in disease spread.

PRIONS

Prion diseases, also termed transmissible spongiform encephalopathies (TSE), are a class of fatal neurodegenerative infections that currently have no cure. These diseases affect different mammals including cattle (bovine spongiform encephalopathy, BSE), sheep (scrapie), cervids (chronic wasting disease, CWD) and humans (Creutzfeldt-Jakob disease, CJD). The unifying feature of prion diseases is that they all involve cellular prion protein (PrP^{C}) encoded by the *PRNP* gene (Brandner et al. 1996). PrP^{C} is found in many parts of the body but it is most highly concentrated in the nervous system where disease pathogenesis progresses (Brandner et al. 1996). The native function of membrane GPI-anchored PrP^{C} has been investigated for years and interestingly goats naturally lacking PrP^{C} and transgenic rodents without PrP^{C} do not exhibit significant health problems or cognitive deficits (Benestad et al. 2012; Bueler et al. 1992). A number of studies have shown that PrP^{C} has the capacity to bind copper molecules, suggesting that it may play a role in metal homeostasis (Jackson et al. 2001). PrP^{C} also has been shown to play a role in signal transduction in neurons affecting circadian rhythm and olfactory sensing (Le Pichon et al. 2009; Tobler et al. 1996). A main feature of prion disease is that misfolded prion protein (PrP^{TSE}) accumulates in large aggregates in areas of the central nervous system (Prusiner 1982). The secondary structure of PrP^{TSE} is mainly composed of beta sheets, while PrP^{C} is alpha helical (Pan et al. 1993). The misfolded conformation, PrP^{TSE}, is resistant to proteinase K

(PK) digestion and this feature is used to identify prions in infected neural tissue post-mortem (Prusiner 1982).

Cyclic Misfolding and the Infectious Agent

Progression of prion disease occurs through template misfolding where PrP^{C} changes conformation to PrP^{TSE} (Fig. 1-2) (Pan et al. 1993; Saborio et al. 2001). This proceeds in an autocatalytic manner where aggregates of PrP^{TSE} accumulate in the intercellular spaces of neural tissue (Prusiner 1998). The specifics of the mechanism of misfolding are largely unknown but the use of laboratory methods such as protein misfolding cyclic amplification (PMCA) has led to a better understanding of the components necessary for conversion (Saborio et al. 2001). Initial work showed that the presence of purified hamster PrP^{C} template and a scrapie seed, Sc237 PrP27-30, was not sufficient alone to consistently facilitate cyclic misfolding (Deleault et al. 2007). Using PMCA, the addition of poly (A) RNA to the PrP^{C} and Sc237 PrP27-30 reaction mixture, facilitated the conversion of PrP^{C} to PrP^{TSE} (Deleault et al. 2007). Later, *de novo* infectious prions from bacterially-expressed PrP^{C} in the presence of RNA cofactors and a synthetic anionic phospholipid, POPG (1-palmitoyl-2-oleoylphosphatidylglycerol), were formed during PMCA (Wang et al. 2010). A recent study has shown that the cofactor phosphatidylethanolamine alone is sufficient to propagate *de novo* generated prions from recombinant PrP^{C} using PMCA (Deleault et al. 2012)

Many of the experiments investigating the conversion of PrP^{C} to PrP^{TSE} have advanced our understanding of the role of this infectious agent in prion disease. Early studies proposed that prion diseases were viral or bacterial

mediated. The protein-only hypothesis was unique among infectious diseases as nucleic acids were invariably associated with other disease causing agents (Prusiner 1982). In addition to the prion replication studies involving PMCA, other work with yeast, fungi and other mammalian systems supported the protein-only hypothesis (Legname et al. 2004; Maddelein et al. 2002; Sparrer et al. 2000). Although PrP^{TSE} is now largely accepted as the only infectious agent required to transmit the disease, the specific factor responsible for neurodegeneration has still not been determined. It was initially proposed that large aggregates of PrP^{TSE} were responsible for neurodegeneration, ultimately leading to death of the infected host. Instances where clinical disease was confirmed without detectable PK-resistant PrP^{TSE} or where PK-resistant PrP^{TSE} was present with no clinical indications of disease made it questionable that PrP^{TSE} was responsible for neurodegeneration (Chesebro et al. 2005; Lasmezas et al. 1997). Other work has also shown that accumulation of misfolded PrP^{TSE} and neurodegeneration do not occur simultaneously during pathogenesis (Sandberg et al. 2011). Misfolded PrP^{TSE} in diseased hosts contains both PK-resistant and PK-sensitive populations of PrP^{TSE} (Safar et al. 1998). Further work showed that smaller oligomers of PrP^{TSE} were more toxic than larger fibrils and that these toxic oligomers were also more PK-sensitive (Simoneau et al. 2007). In infected mice with splenic injury, the spleen contains only protease sensitive prions capable of infecting new hosts to again demonstrate that protease resistant prions are not necessary to cause disease (Krasemann et al. 2013). Another study isolated a monomeric alpha-helix rich PrP^{TSE} molecule that was toxic to neurons and caused neurodegeneration *in*

vivo (Zhou et al. 2012). These studies show that the neurodegenerative agent in prion diseases is likely a smaller by-product of the misfolding process rather than the large fibrils first identified (Fig. 1-2).

Oral Transmission

Prion infections in a natural setting are largely considered to occur through oral transmission of the infectious agent. This applies to the animal prion diseases CWD, BSE, scrapie as well as human variant CJD (vCJD) and kuru. These two human classifications are linked to consumption of BSE-infected meat and cannibalism, respectively (Bruce et al. 1997; Prusiner 1998). Consumed PrP^{TSE} is able to survive the harsh environment of the digestive system and reach the intestinal system (Kruger et al. 2009). At the ileum, PrP^{TSE} interacts with lymphoid tissue and enters M-cells in the Peyer's patch (Donaldson et al. 2012). From M-cells, PrP^{TSE} moves to follicular dendritic cells (FDCs) which possess high levels of PrP^{C} and are thought to be the initial site of PrP^{TSE} formation in the host (Brown et al. 1999; McCulloch et al. 2011). PrP^{TSE} then infects neurons of the enteric nervous system near the Peyer's patch and spreads to the central nervous system (McBride et al. 2001; Seelig et al. 2011). Other mechanisms independent of M-cells have been identified where PrP^{TSE} is detected in vesicles of enterocytes and macrophages before replication on FDCs (Kujala et al. 2011). The involvement of the complement immune system in the spread of PrP^{TSE} has also been demonstrated in work showing that depletion of complement receptors in transgenic mice slows PrP^{TSE} accumulation and extends survival (Michel et al. 2012). These studies show how lymphoid/immune tissue is involved in spread of

PrP^{TSE} in a new host. In contrast, macrophages have also been implicated in reducing the accumulation of PrP^{TSE} in Peyer's patches (Maignien et al. 2005). Further investigation is needed to determine the overall role, positive or negative, of the immune system in prion diseases.

Prion Disease in Animals

Prion diseases affect a number of different mammalian species with each disease having different characteristics. Scrapie, in sheep, was the first characterized prion disease and has been a significant model for characterizing prion pathogenesis (Prusiner 1982). Scrapie has been present for centuries in sheep herds but current selective breeding for resistance to the disease has reduced its prevalence (Hagenaars et al. 2010). Different prion diseases have subsequently been identified in humans including kuru (cannibalism), fatal familial insomnia (genetic), Gerstmann–Sträussler–Scheinker syndrome (genetic) and CJD. Creutzfeldt-Jakob disease in humans has been classified as sporadic (unknown), iatrogenic (surgical contamination) and variant (consumption of BSE agent) (Tranulis et al. 2011). The link between vCJD and BSE in cattle had widespread socioeconomic impact in the United Kingdom and North America (Bruce et al. 1997). The relatively low number of vCJD cases worldwide (227) compared to the high number of confirmed BSE cases (>190000) demonstrates that transmission to humans is inefficient. The number of BSE cases has been in decline worldwide since the introduction of a feed ban that prevented the feeding of specified risk materials to livestock (Canadian Food Inspection Agency 2013). Initially, meat and bone meal was removed from all feed in 1997 but later the

defining of specified risk material (SRM) in 2004 increased the breadth of banned tissues (Canadian Food Inspection Agency 2013). SRMs are areas with high titres of PrP^{TSE} in tissues like the brain, spinal cord, dorsal root ganglia, trigeminal ganglia, eyes and tonsils for ruminants 30 months or older and the distal ileum of all ruminants (Canadian Food Inspection Agency 2013).

Chronic wasting disease affects a number of cervids including white-tailed and mule deer as well as moose and elk in both captive and free-ranging populations. Experimental transmission has also been demonstrated in reindeer (Mitchell et al. 2012). CWD is unique among prion diseases because it is prevalent in wild populations of deer and elk. The disease has been identified in free-ranging animals in 15 states of the USA and 2 provinces in Canada (Saunders et al. 2012). Prevalence in certain areas in the United States is high and can include 50% of deer, but the prevalence in elk is usually much lower (Saunders et al. 2012). Compared to BSE where transmission has been exclusively linked to feeding of animal by-products to cattle, CWD appears to spread through other means. Transmission of CWD has been shown to share characteristics in common with scrapie, with both animal-to-animal contact and indirect transmission through the environment possibly playing a role (Hoinville 1996; Mathiason et al. 2009; Miller and Williams 2003; Miller et al. 2004). The continued spread of CWD through wild and captive herds of different cervids represents a significant health risk to these cervids (Saunders et al. 2012).

The species barrier associated with all prion diseases describes the ability of the disease to transmit from one species to another species (Bartz et al. 1994).

Transmission of a prion strain to a new species results in a more lengthy incubation period and a low attack rate (Bartz et al. 1994; Scott et al. 2005). However, consecutive passages of the disease into the same species decreases the IP and AR increase, a process known as adaptation (Bartz et al. 1994; Scott et al. 2005). There has not been any evidence to this point that CWD can be directly transmitted to humans but the potential for transmission through an intermediate host has been hypothesized (Barria et al. 2011; Bartz et al. 1998). The presence of different strains within a prion disease has been established based on the identification of atypical and typical diseases in BSE, scrapie and CJD (Tranulis et al. 2011). These strains have different characteristics such as altered species barrier and IP (Collinge and Clarke 2007; Tranulis et al. 2011). Recently, the existence of different strains in CWD has been confirmed (Perrott et al. 2012). The existence of these strains requires more investigation in order to determine how infectious properties are altered and how this may influence spread of the disease.

CWD Transmission

Evidence for indirect transmission of CWD between cervids is a unique aspect of this prion disease. Mule deer exposed to pens with CWD-infected deer carcasses or pens that previously held CWD-infected deer acquire CWD at a low frequency (Miller et al. 2004). Similar findings were reported when white-tail deer were exposed to environments that previously harboured CWD-infected animals (Mathiason et al. 2009). A number of different tissues in the central nervous system and lymphatic system have been shown to contain PrP^{TSE} in CWD

infected animals (Fox et al. 2006). The spread of PrP^{TSE} in the tissues of infected deer is quite extensive as it has been shown outside of the lymphatic and nervous system tissue including skeletal muscle and the velvet of antlers (Angers et al. 2006; Angers et al. 2009; Daus et al. 2011). Indirect transmission of CWD is facilitated by the release of PrP^{TSE} into the environment by an infected host through shedding of bodily fluids. PrP^{TSE} has been detected in the feces and urine of CWD-infected deer (Haley et al. 2011; Tamguney et al. 2009). Elk with CWD have also been confirmed to shed CWD in feces (Pulford et al. 2012). Other bodily fluids from CWD infected deer also have been shown to have PrP^{TSE} including blood and saliva (Haley et al. 2011; Mathiason et al. 2006). The shedding of PrP^{TSE} by cervids infected with CWD leads to deposition of these infectious particles in the surrounding environment. The resistance of PrP^{TSE} to physiochemical degradation allows the infectious agent to survive in the environment for extended periods of time. For example, scrapie prions have been shown to remain infectious in the environment for sixteen years as shown by re-infection of new sheep to a previously contaminated housing area (Georgsson et al. 2006). CWD prions have also been shown to remain infectious in a paddock for at least two years (Miller et al. 2004). PrP^{TSE} remains stable in water environments such as in seawater where it required over 5 weeks for scrapie prions to decline by one log, indicating that water may serve as a medium for transmission (Maluquer de Motes et al. 2008).

Prions in the Environment

The entry of PrP^{TSE} into soil and their ability to remain infectious for extended periods of time makes it likely that they interact with both the organic and inorganic components of this environment. It has been shown that hamster-adapted transmissible mink encephalopathy (TME) PrP^{TSE} has a strong affinity for soil minerals such as montmorillonite (Mte), kaolinite and quartz (Johnson et al. 2006). Mte clay bound TME PrP^{TSE} is still infectious and other work has shown that these prions bound to minerals can be even more infectious than their unbound counterparts (Johnson et al. 2006; Johnson et al. 2007). PrP^{TSE} desorbed from Mte lacks the N-terminus portion of the prion protein, suggesting that binding of TME PrP^{TSE} to Mte involves this region (Johnson et al. 2006). Soil samples collected from four different regions in the USA also displayed an affinity to TME PrP^{TSE} and two of these complexes also resulted in increased infectivity (Johnson et al. 2006; Johnson et al. 2007). Other studies have looked at the kinetics of soil binding as a means of predicting the movement of PrP^{TSE} in soils. When using a brain homogenate solution as opposed to purified TME PrP^{TSE}, both the rate and extent of binding decreased (Saunders et al. 2009b). The rate of binding time depended on soil type but saturation of TME PrP^{TSE} binding sites required as long as two months in some cases (Saunders et al. 2009b). The type of PrP^{TSE} examined also had an effect on affinity kinetics as hamster HY TME exhibited maximal absorption to a sandy soil after 30 d whereas it required 60 d with CWD (Saunders et al. 2009a). N-terminally truncated PrP^{TSE} displayed variable affinity to different soils, with enhanced binding to sandy soils and decreased binding to clay soils (Saunders et al. 2009a). When looking at

degradation in a brain homogenate, CWD is more resistant to degradation compared to HY TME but the N-terminus for both strains degrades in approximately one month (Saunders et al. 2008). This suggests that in many situations PrP^{TSE} may enter the environment lacking an N-terminus which could increase (clay soil) or decrease (sandy soil) its movement through the environment. Using PMCA as a measure of conversion properties, PrP^{TSE} maintains converting ability after being bound to minerals for one year (Saunders et al. 2011). Suspension of soil in dH_2O reduces its affinity for PrP^{TSE} as compared to soil that is suspended in phosphate buffered saline (Saunders et al. 2011). These studies outline that prions may be mobile in the environment longer than initially thought given that adsorption to soil minerals is not immediate and survival in water would aid spread within the environment. The interaction with soils is also important to consider in characterizing disease transmission as this can affect disease properties.

Prion Degradation

Prions survive in the environment for a number of years for both CWD and scrapie (Georgsson et al. 2006; Miller et al. 2004). As well, survival of scrapie for approximately twice as long as BSE in sewage and PBS shows that prion diseases have different environmental stabilities (Maluquer de Motes et al. 2008). Degradation of prions outside of a host, although sometimes slow, does occur and can be enhanced under certain conditions. There is evidence that some soil components like the strong oxidant, manganese oxide, are capable of degrading PrP^{TSE} in the course of 24 h (Russo et al. 2009). There is also evidence that

earthworms and some of the proteases present in them have the ability to degrade PrP^{TSE} (Aiken & Schneider 2010). Earthworm protein extract is efficient at degrading PrP^{TSE} for both CWD and hamster adapted scrapie HY (Aiken & Schneider 2010). Incubation period of hamster HY also increased from 77 d to 110 d when digested with earthworm extract (Aiken & Schneider 2010). However, when using recombinant PrP^{C} as a surrogate for PrP^{TSE}, proteases extracted from earthworms did not degrade the protein (Nechitaylo et al. 2010). This shows that the ability for earthworms to degrade prion protein may be specific to infectious prions only. Other work has looked at specific enzymes derived from *Bacillus spp.* for their ability to degrade PrP^{TSE} (McLeod et al. 2004; Pilon et al. 2009). Degradation on PrP^{TSE} based on mouse bioassay was not complete for either subtilisin 309 or properase but significant extensions in mouse survival were seen for both (McLeod et al. 2004; Pilon et al. 2009). Although these experiments were using purified protease extracts, the presence of enzymes able to degrade prions from bacterial species indicates the potential for prion degradation outside of a mammalian host.

Composting has been investigated as a method for killing different pathogens like avian influenza, *E. coli* O157:H7 and *Salmonella* (Ceustermans et al. 2007; Elving et al. 2012; Xu et al. 2009). Degradation of PrP^{TSE} has also been suggested in composting studies (Huang et al. 2007; Xu et al. 2013a). Due to the complex nature of the compost matrix, the decreased PrP^{TSE} signal over time from compost samples may be due, in part, to interference of compost materials (Huang et al. 2007; Xu et al. 2013a). The ability of compost to reduce PrP^{TSE} is suggested

to involve thermophilic microbes that populate composts (Huang et al. 2007). As well, inclusion of feathers in compost is suggested to increase degradation of PrP^{TSE} and a number of bacterial and fungal genera have been identified as candidates for this enhanced degradation (Xu et al. 2013a; Xu et al. 2013b). Sewage has also been shown to degrade PrP^{TSE} material quicker than a neutral buffer owing to the presence of microbes in the sewage (Maluquer de Motes et al. 2008). These studies indicate that although PrP^{TSE} survives in the environment for extend periods of time, there are environmental conditions that result in its degradation.

PRION UPTAKE BY PLANTS

It is likely that PrP^{TSE} is disseminated in the environment through a variety of different processes. Examples would include the application of manure or possibly compost onto arable land, carcass decay or direct shedding from bodily fluids (Saunders et al. 2012; Xu et al. 2013a). The persistence and initial mobility of PrP^{TSE} in soil raises the possibility that it may come in contact with plant roots (Saunders et al. 2009b; Saunders et al. 2011). It has been established that plants have the ability to take up large organic molecules including proteins and even living microorganisms such as bacteria and viruses (Paungfoo-Lonhienne et al. 2008; Paungfoo-Lonhienne et al. 2010). One hypothesis for this phenomenon is that it is a means for the plant to acquire N (Paungfoo-Lonhienne et al. 2008; Paungfoo-Lonhienne et al. 2010). Entry of PrP^{TSE} into the root system is a possibility based on the high probability that PrP^{TSE} will contact roots and on the

ability of roots to take up large proteins of similar molecular weight. The ability of the PrP^{TSE} to remain infectious in soil and water while remaining mobile upon initial entry into soil increases the probability that it could interact with plant roots. This also will influence the spread of PrP^{TSE} through the environment by abiotic factors. The differential affinity of PrP^{TSE} for soil particles likely also influences the potential of this interaction. Once PrP^{TSE} has entered the roots, movement to stem and leaves of the plant through the xylem could occur given evidence that ovalbumin is transported in the plant through this mechanism (Neumann et al. 2010). The possibility for uptake of PrP^{TSE} into plant roots and transport to other areas could potentially pose a health risk for animals and humans. If consumption of PrP^{TSE} contaminated plants resulted in infection this would add a new dimension to the epidemiology of prion diseases. Such a discovery would be particularly relevant to the spread of CWD in wild cervids where transmission occurs through the environment (Mathiason et al. 2009; Miller et al. 2004).

Plant Uptake Models

To determine the ability for PrP^{TSE} uptake into plants a number of factors need to be considered. Although the most relevant environmental situation would be investigating the plant-PrP^{TSE} interaction in soil, this approach introduces multitude of compounding factors. For example, the interaction of PrP^{TSE} with soil particles and its persistence in soil are likely to affect the probability of it interacting with plant roots. Initially, it is important to determine if uptake of PrP^{TSE} is possible in the absence of soil particles, conditions that should be more

conducive for this event to occur. If uptake under these conditions is confirmed, additional studies using soil particles in a controlled environment could be undertaken. Another important consideration is the form of PrP^{TSE} during contact with plant roots. Purified PrP^{TSE} from tissues does not represent the total population of infectious prions and purification processes alter the aggregation of PrP^{TSE} in a manner that could influence its absorption into roots. Homogenized tissue containing PrP^{TSE} represents the most physiologically relevant form of PrP^{TSE} that would likely be applied to crop land. The presence of CWD in the environment and the suggested indirect transmission of this disease within wild animal populations make this prion disease a suitable option for initial studies on prion uptake by plants. Different plants including tomatoes, cereals, and *A. thaliana* have been discussed above in studies for uptake of bacteria and protein. This indicates that, based on uptake of larger organic molecules, a number of plants may be suitable to model prion uptake studies. Wheat (*Triticum aestivum* L.) is a common cereal crop that is relevant to situations where prions could come into contact of roots through animal shedding or application of compost and manure to arable land. Root damage affects the internalization of bacteria and viruses (Bernstein et al. 2007; Urbanucci et al. 2009). Given that root damage is likely to occur in the environment it is important to investigate this may affect uptake of PrP^{TSE} into plants.

OBJECTIVES

This project used wheat (*Triticum aestivum* L. cv. AC Andrew) as a model crop for the Canadian prairies to determine its interaction with model proteins and prions. Initial experiments looked at the N nutritional value of bovine serum albumin for wheat to determine the nutritional value of an animal protein and gain knowledge on N cycling. Concurrent work built a model for protein uptake in plants using ovalbumin as another model protein. These experiments served as proof-of-concept studies to confirm uptake of protein in wheat before uptake experiments were undertaken with infectious prions. In addition to serving as a model, these experiments answered questions on N cycling of animal protein in soil. Finally, the model for uptake of protein in wheat was applied to CWD PrP^{TSE} to determine if uptake and transport within the plant occurred. If uptake of prions from plants was demonstrated it would have important regulatory implications for the practices that are presently used to control and prevent the transmission of prion diseases.

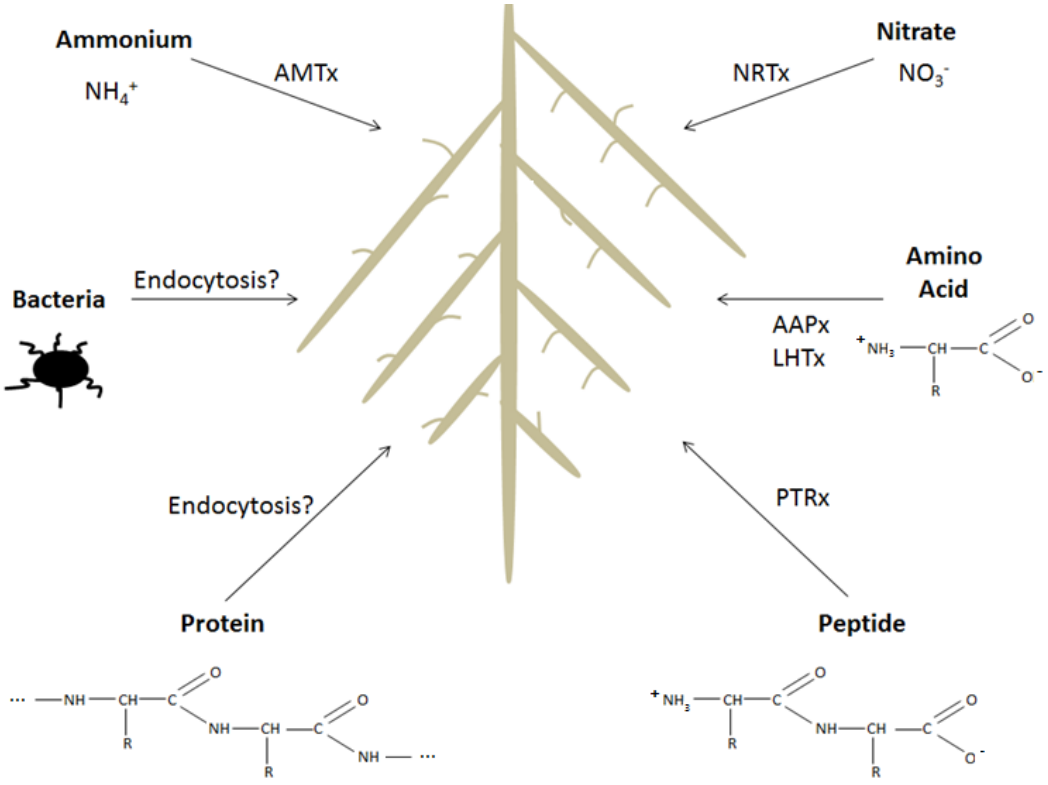

Fig. 1-1. Diagram outlining different nitrogen sources used by plants and associated mechanisms of uptake. AMTx=ammonium transporter family; NRTx=nitrate transporter family; AAPx=amino acid permease family; LHTx=lysine/histidine transporter family; PTRx=peptide transporter family.

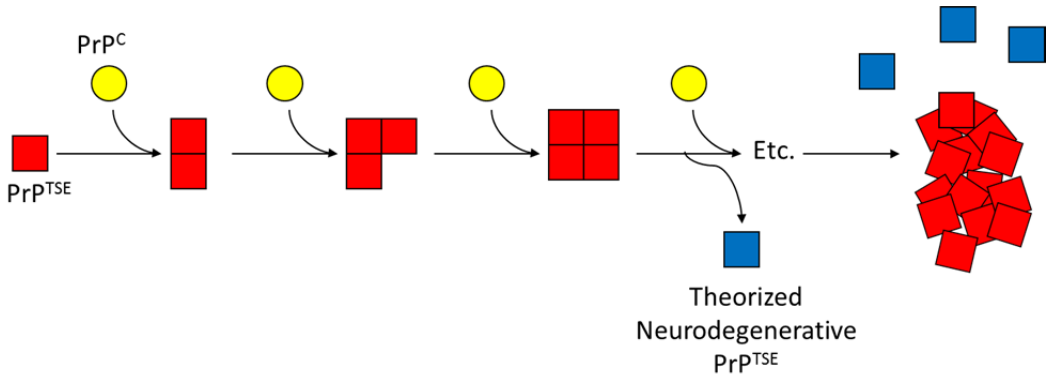

Fig. 1-2. General schematic of prion misfolding. Infectious PrP^{TSE} causes cellular PrP^{C} to misfold into PrP^{TSE} which leads to an accumulation of aggregated PrP^{TSE} overtime. Another PrP^{TSE} particle is theorized as the neurodegenerative agent in prion disease produced as a by-product of misfolding.

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Chapter 2: Efficiency of protein as a nitrogen source for wheat and morphological changes in roots exposed to high protein concentrations

INTRODUCTION

Application of agricultural by-products, such as compost onto arable or pasture land is an environmentally friendly practice. Proteins of animal origin are present in these materials and their introduction to agricultural soil results in their potential contact with crop plants. This situation raises questions of protein turnover in soil and the nutritional value of protein for crop plants.

In most soil environments, nitrogen (N) is present in both organic and inorganic forms. Plants have the ability to assimilate N from the surrounding environment in different ways. Inorganic N uptake is facilitated by transporters with high and low substrate affinity and is encoded by nitrate (NRTx) and ammonium transport proteins (AMTx) (Gutierrez et al. 2011). Organic N acquisition is achieved in part by uptake of amino acids through lysine histidine transporters and amino acid permeases (Hirner et al. 2006; Lee et al. 2007; Svennerstam et al. 2007). Plants can also form symbiotic relationships with soil microorganisms where the N assimilated by microorganisms is transferred to the plant host. Mycorrhizal fungi are frequently associated with plants and within this symbiosis, fungi provide N from outside the root zone to the host (Govindarajulu et al. 2005). Indeed, varying amounts of N (21-75%) in plants have been reported as being acquired through symbiosis with mycorrhizae (Govindarajulu et al. 2005; Jin et al. 2005; Tanaka and Yano 2005). Mycorrhizal fungi are also able to access

organic N sources and while most of the acquired N is used by the fungus, a small amount (< 4%) is transferred to the host plant (Hodge and Fitter 2010; Leigh et al. 2009). The ability to access different N sources alone or in conjunction with the soil microbial community enables plants to survive and grow under varying soil N conditions.

Utilization of organic N by plants and microorganisms involves breakdown of large proteins by proteases into peptides that are in turn hydrolyzed by peptidases into small peptides and amino acids (Adamczyk et al. 2010; Hill et al. 2012; Jan et al. 2009). A number of plants release proteases into the rhizosphere (Adamczyk and Godlewski 2007; Adamczyk et al. 2009) and it has been proposed that this enables wheat (*Triticum aestivum* cv. Tacher) to utilize casein as the sole N source when grown hydroponically (Adamczyk et al. 2008).

Competition for the same N source by plants and microbes demonstrates the importance of diverse N acquisition strategies to meet plant nutritional requirements (Owen and Jones 2001). Both soil microbes and cereals preferentially utilize similar peptide isomers (L-enantiomers over D-enantiomers) and amino acids to satisfy nutritional requirements (Hill et al. 2011; Hill et al. 2012; Jamtgard et al. 2008). The natural cycling of N in the environment creates a pool of smaller molecules that all soil microorganisms and plants compete for. The potential for plants to absorb larger proteins and have exclusive use of the N they contain has obvious advantages over competing with soil microbes for N.

Although it is generally believed that plants cannot directly utilize larger, more complex nitrogenous compounds, some studies have suggested otherwise.

For example, *Arabidopsis thaliana* grown with protein as an N source had higher dry mass as compared to plants grown without N, suggesting that protein was utilized for N nutrition (Paungfoo-Lonhienne et al. 2008). In a subsequent experiment, it was demonstrated that *A. thaliana* was able to absorb and degrade whole microbes for nutrients (Paungfoo-Lonhienne et al. 2010). These studies demonstrate that *A. thaliana* is able to take up larger, N containing molecules without prior degradation for subsequent utilization.

The goal of this study was to compare the ability of wheat (*T. aestivum* L. cv. AC Andrew) to utilize the animal protein, bovine serum albumin (BSA), vs. ammonium nitrate as N sources over a range of N concentrations when it is grown in a solid medium.

MATERIALS AND METHODS

Plant Growth Conditions

Triticum aestivum L. (cv. AC Andrew) seeds were surface sterilized with 70% ethanol and 3% hypochlorite and germinated for 5 d prior to transfer into Magenta® boxes (5 seeds per box). Magenta boxes contained 50 mL of modified N-free Murashige and Skoog media containing 29.21 mM sucrose, 2.99 mM $CaCl_2 \cdot 2H_2O$, 1.50 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM KH_2PO_4, 0.11 mM ethylenediaminetetraacetic acid ferric sodium salt, 0.10 mM H_3BO_3, 0.10 mM $MnSO_4 \cdot H_2O$, 29.91 nM $ZnSO_4 \cdot 7H_2O$, 5.00 nM KI, 1.03 nM $Na_2MoO_4 \cdot 2H_2O$, 0.11 nM $CoCl_2 \cdot 6H_2O$, and 0.10 nM $CuSO_4 \cdot 5H_2O$ (Murashige and Skoog 1962; Paungfoo-Lonhienne et al. 2008). N was added to the media either in the form of

NH_4NO_3 [IN; 35% N by mass], BSA [P; 15% N by mass, A3059, Sigma Aldrich, Oakville, Canada] or a combination of both [C] where each of the N sources supplied an equal amount of N. Thus, the inorganic nitrogen concentration in the combination treatment was half of the inorganic only treatment at iso-nitrogenous levels. Final N concentrations were 214.13 mM (High; H), 71.38 mM (Medium; M) or 21.41 mM (Low; L) with an additional low level of 2.14 mM (very Low; vL) for NH_4NO_3 only (Table 2-1). The L nitrogen level was determined using nitrogen application rates in southern Alberta (35-46 kg N ha^{-1}) (Huffman et al. 2008) and the M level was based off previous studies on protein nitrogen nutrition (Paungfoo-Lonhienne et al. 2008). The N concentration in the L treatment is likely an underestimation of N concentration in the soil after fertilizer application considering N already contained in the soil, however, the N concentration for the L treatment fits well with the range of concentrations for our study. Negative control media had no N added and positive control media contained the following components: 87.64 mM sucrose, 20.61 mM NH_4NO_3, 18.79 mM KNO_3, 2.99 mM $CaCl_2 \cdot 2H_2O$, 1.50 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM KH_2PO_4, 0.11 mM ethylenediaminetetraacetic acid ferric sodium salt, 0.10 mM H_3BO_3, 0.10 mM $MnSO_4 \cdot H_2O$, 29.91 nM $ZnSO_4 \cdot 7H_2O$, 5.00 nM KI, 1.03 nM $Na_2MoO_4 \cdot 2H_2O$, 0.11 nM $CoCl_2 \cdot 6H_2O$, and 0.10 nM $CuSO_4 \cdot 5H_2O$, 0.56 mM *myo*-inositol, 26.64 nM glycine, 4.06 nM niacin, 2.43 nM pyridoxine-HCl, 0.30 nM thiamine-HCl (Murashige and Skoog 1962). N concentration of positive control media was ~60 mM, similar to the medium N treatment level (71.38 mM). Positive control media was chosen based on the widely accepted use of complete Murashige and Skoog

(MS) media as a standard for plant growth. Media was adjusted to pH 5.8 and filter-sterilized (0.2 µm) prior to combining with autoclaved phytagel (P8169, Sigma Aldrich) to achieve a final concentration of 0.3%. Five germinated seeds were transferred to each magenta box using sterile technique. Plants were cultivated in a growth room under 16 h d/8 h night light conditions (150 µmole m^{-2} s^{-1}) at 20°C ± 2°C for 33-34 d before analysis. Plants with different protein sources were cultivated in the same growth room with the same media components as the P-L,M and H treatments for 33 d on 6.16 mg/mL (65.95 mM N) BSA, casein or ovalbumin to look at the effect of different proteins on root morphology (n=3).

Plant Analysis

After 33-34 d, plants were removed from magenta boxes and rinsed with MilliQ® water to remove any excess media before being blotted dry with paper towels. Root and stem lengths were measured as well as the wet mass for separated plant parts (root, stem and leaves) for all five plants in each of six independently replicated Magenta® boxes (n=6) (Appendix 1). Samples were then incubated in a drying oven at 60°C for a minimum of 48 h. Dry mass was then measured for the different plant parts of each of three independently replicated Magenta® boxes (n=3) (Appendix 1). Shoot mass refers to the combined mass of the stem and leaves.

Nitrogen Content Analysis

Dried plant samples were pulverized using a Mixer Mill MM200 (Retsch, Newtown, USA) for 1 min at 26.0 Hz. N content was determined using a Carlo

Erba NA2100 combustion analyzer (Carlo Erba Strumentazoine, Milan, Italy) and modified Dumas analysis (Tabatabai and Smith 2003). Data was collected for six independently replicated Magenta® boxes (n=6).

Abnormal Root Morphology

Gross morphology images were taken of plant roots using a Leica MZFLIII dissecting microscope using samples from all three BSA concentrations as well as positive and negative MS media controls at various time points. Images were also taken of plant roots after 33 d of growth on 6.16 mg/mL BSA, casein or ovalbumin. Roots from the highest protein (P-H) and positive control (+) treatments were embedded in Tissue-Tek O.C.T Compound (Canemco & Marivac, Quebec, Canada) and incubated at -20°C until frozen. Portions with abnormal (P-H) and normal (+) root morphology were cryo-sectioned (16 µm thickness) using a CTF Microtome-Cryostat (Thermo Scientific-IEC, Waltham, USA) and placed on glass slides. Sections were stained using safranin O for lignin & suberin and Astra Blue for cellulose following sectioning. Images were taken with a 10X objective lens using a Zeiss Primo Star (Zeiss, Jena, Germany) compound microscope.

Statistics

Statistical analysis was performed using R software (v2.13.1) to carry out ANOVA and Tukey's HSD tests.

RESULTS

Images of wheat exposed to the different treatments give an overview of plant appearance (Fig. 2-1). Wheat seedlings supplied with BSA at the low and medium (P-L [21 mM] and P-M [71mM]) concentrations displayed chlorosis and reduced growth similar to the N-free negative control (-) while the high BSA concentration (P-H [214 mM]) exhibited less chlorosis (Fig. 2-1). Inorganic N treatments IN-H and IN-M induced a toxic N response, while IN-vL (2 mM) plants were reduced in size and exhibited increased chlorosis in leaves (Fig. 2-1). Plants from the high and low combination treatments (C-H and C-L) showed more chlorosis in their leaves as compared to C-M (Fig. 2-1). Plants from C-M and IN-L displayed less chlorosis than other experimental treatments and were most similar to plants grown on complete MS media (+) (Fig. 2-1).

Shoot (leaves and stem) wet mass for all three levels of protein were not different ($P > 0.05$) from the negative control (Fig. 2-2a). Shoot mass of wheat plants in IN-vL and IN-H, were also not different ($P > 0.05$) from the negative control (Fig. 2-2a). IN-L, IN-M, and all combination levels, had higher ($P < 0.01$) shoot wet mass as compared to the negative control (Fig. 2-2a). The inorganic and combination treatments supplied with the low and medium N levels did not differ ($P > 0.05$) from each other (Fig. 2-2a). Comparing different treatments at equivalent N concentrations, only IN-H did not display ($P > 0.05$) increased wet shoot mass compared to protein N treatment which contrasted to IN-M and IN-L (Fig. 2-2a). The positive control had a larger shoot wet mass than all other treatments (Fig. 2-2a). Dry mass of shoots also displayed similar trends to the shoot wet mass data, but there were no differences ($P > 0.05$) between

experimental treatments (Fig. 2-2b). The IN-L, IN-M and all the combination treatments had a larger (P <0.01) dry shoot mass compared to the negative control (Fig. 2-2b). Similar to shoot wet mass, the positive control had larger (P <0.01) dry mass than all other treatments (Fig. 2-2b).

All protein treatments resulted in a high root:shoot biomass ratio that was similar (P >0.05) to the negative control (Fig. 2-3). IN-vL displayed a higher (P <0.05) root:shoot ratio than all other inorganic treatments (Fig. 2-3). The combination treatments resulted in a response similar to the inorganic treatments where C-L had the highest (P <0.05) root:shoot biomass ratio among combination treatments (Fig. 2-3). There was no difference (P >0.05) in the root:shoot ratio among treatments when N was supplied at the low concentration (Fig. 2-3). This is in contrast to the medium and high concentrations of N where plants subjected to protein treatments had a larger (P <0.01) root:shoot biomass than those supplied with inorganic or combination treatments (Fig. 2-3).

There were no differences (P >0.05) in N content among the different levels of protein supplied in any of the three tissues sampled and in the stem and leaves, all protein treatments did not differ (P >0.05) from the N-free negative control (Fig. 2-4). Conversely, N content in the roots of P-M and P-H were higher (P <0.01) than the negative control (Fig. 2-4). Plant N content in all tissues increased (P <0.05) with increasing N supplied in the inorganic and combination treatments (Fig. 2-4). At equivalent N concentrations, combination treatments consistently resulted in a lower (P >0.05) N content in the leaf and stem than inorganic N treatments (Fig. 2-4).

P-H had a larger (P <0.05) root dry mass/root length as compared to P-L (Fig. 2-5a). This contrasted with inorganic N treatments where IN-vL exhibited a larger (P <0.05) root dry mass/root length as compared to other IN treatments (Fig. 2-5a). In the combination treatments, C-L had larger (P <0.05) root dry mass/root length when compared to C-M, but did not differ from C-H (Fig. 2-5a). There were no differences (P >0.05) among treatments at the low N level. At the medium N concentration, the protein treatment had (P <0.01) larger root dry mass/root length compared to the combination treatment (Fig. 2-5a). The root dry mass/root length for P-H was larger (P >0.05) than both IN-H and C-H (Fig. 2-5a). The positive control treatment had a similar (P >0.05) root dry mass/root length to the P-M, P-H, IN-vL and C-L treatments with high values (Fig. 2-5a).

Gross structural images of root morphology from the protein treatments exhibited an increased frequency of root branches compared to both the positive and negative control (Fig. 2-5b). As protein was increased the abnormal root structure became more pronounced with thicker and more bulbous root branches (Fig. 2-5b). After 54 days, the abnormal morphology became even more apparent in P-M and P-H (Fig. 2-5b). Similar increased root branching and less severe knob-like structures were seen in C-H (data not shown).

Cross-sections of the bulbous and branched portions of the roots exposed to P-H treatment when compared to the positive control showed that there was hyperplasia in tissue layers, resulting in a thicker cross-section (Fig. 2-5c). The abnormal root branches of P-H stained with safranin O indicate the presence of lignin/suberin in the cortex, whereas the positive control was primarily stained

with Astra Blue in the cortex indicating the presence of cellulose (Fig. 2-5c). The roots of wheat plants grown with the proteins: BSA, casein and ovalbumin as the only N source show similar knob-like outgrowths (Fig. 2-5d).

A summary of the root:shoot biomass ratio, root dry mass/root length and N content data provides an overview of general trends and relationships (Fig. 2-6). Arbitrary groups were assigned to clustering data points to highlight plant responses (Fig. 2-6). There was a positive linear relationship between root:shoot dry mass and root dry mass/root length in the combination and inorganic treatments, whereas, the protein treatments lacked this positive linear relationship (Fig. 2-6). Within the linear relationship of inorganic treatments, as nitrogen increased the root dry mass/root length and root:shoot mass decreased (Fig. 2-6). This differed from the combination treatments where C-H did not have the lowest root dry mass/length and root:shoot mass values (Fig. 2-6).

DISCUSSION

The data presented shows that AC Andrew wheat is unable to use BSA as efficiently as NH_4NO_3 for N nutrition. While changing the N concentration in the inorganic N treatments resulted in a corresponding increase in plant growth and N content, this was not the case for protein. The lack of a change in the shoot mass or N content measurements taken from wheat plantlets (*T. aestivum* L. cv. AC Andrew) treated with different concentrations of protein suggests that protein N sources provide little nutritional value for the cultivar of wheat studied here. Our methodology was altered compared to previous studies in order to equally

evaluate protein vs. inorganic N at similar N concentrations (Adamczyk et al. 2008; Paungfoo-Lonhienne et al. 2008).

Shoot mass did not change significantly for all three protein N concentrations and were similar to the negative control that contained no N (Fig. 2-2a). This shows that when using shoot mass as an indicator of plant growth, protein was unable to provide adequate N nutrition at the concentrations tested. This contrasted with the plant images where the highest protein treatment (P-H) displayed less chlorosis in the leaves as compared to the other protein treatments (Fig. 2-1). Although plant growth did not respond to increased protein N, the health of the plant as indicated by leaf colour did appear to be improved (Fig. 2-1,2). The fact that IN-L and C-M resulted in shoot wet masses similar to the positive control without apparent chlorosis shows that the range of N concentrations selected were adequate for normal plant growth (Fig. 2-1,2a). The similarity between IN-L and C-M is likely due to them having similar NH_4NO_3 concentrations as protein N had little nutritional value in C-M. The significant differences observed between experimental treatments in the shoot wet mass data were due to water content when dry mass was considered (Fig. 2-2a,b). This is likely due to leaf tissue in the treatments with lower nutritional value (P-L, M, IN-vL and H) becoming necrotic. The significantly larger shoot mass in the positive control is likely due to the inclusion of vitamins that were removed from other treatments in order to make a completely N deficient media (Fig. 2-2a,b). Based on this result it suggests that vitamins are likely having an effect on the growth of plants but the comparisons made between experimental treatments where N was

manipulated are still valid as vitamins were removed from all treatments other than the positive control.

Partitioning of mass within wheat is a gauge of the physiological response of the plant to its environment. The negative control showed that root:shoot mass increased under N limiting conditions (Fig. 2-3). Likely, the increased allocation of mass to the roots represents an attempt to enhance N uptake, and the accompanying decreased mass in the shoot was a reflection of limited N availability (Nagel et al. 2001). The root:shoot mass displayed by the protein treatments suggests that, under these conditions, the wheat plants were also deficient in N based on the high amount of biomass allocated to the roots (Fig. 2-3). Another explanation for the larger allocation of biomass to roots is that only these tissues are able to successfully utilize protein N. The lack of an expected decrease in root:shoot mass with an increase in protein N levels indicates that the amount of protein had little effect on altering the allocation of plant biomass (Fig. 2-3) and that wheat was unable to use protein as a source of N for growth and development in the stem and leaves.

N content of the leaves and stems also did not show any response to changes in N concentration in the protein treatments (Fig. 2-4). Noteworthy is the significant increase in the root N content of P-M and P-H compared to the negative control (Fig. 2-4). It is possible that protein entered the root system, either intact or partially degraded by exuded proteases. Uptake of full length green fluorescent protein into the roots of *Arabidopsis thaliana* and *Hakea actites* has been documented (Paungfoo-Lonhienne et al. 2008) and the ability of different

plants, including wheat, to degrade protein by exuding proteases from the root has also been demonstrated (Adamczyk and Godlewski 2007; Adamczyk et al. 2008; Adamczyk et al. 2010). A possible explanation for the results from the shoot and root is that BSA may have entered the roots at which point it was immobilized and not further utilized for N nutrition in other plant areas. The combination treatments contained half of the effective N (NH_4NO_3) as the inorganic treatments at the same total N concentration. This explains the decreased N content of all combination treatments when compared at the iso-nitrogenous concentration of the inorganic treatments. This suggests that only inorganic N had a vital and measurable effect on the amount of N contained in the stem and leaves (Fig. 2-4).

A review of the literature reveals different findings on whether or not plants can utilize N provided as a high molecular weight protein directly for nutrition. Larger organic molecules containing N can be accessed by mycorrhizal symbiosis and from soil microorganisms, but the ability to utilize protein N without microbial assistance is contentious. In a study by Adamczyk *et al.* (2008), wheat grown in a hydroponic system with casein protein increased in plant biomass (root and shoot) when compared to a N-free control. The shoot mass results from our study are in disagreement when we used an agar based growth system with BSA protein (Fig. 2-2). Our results for root mass agree with Adamczyk *et al.* (2008) where we observed a significant increase in root dry mass/root length in the high protein treatment (P-H) as compared to the N-free (-) control (Fig. 2-5a). The increased root mass along with the increased N content of roots in P-H may suggest that protein N was being utilized only by the roots.

Using an agar system with *Arabidopsis*, a significantly larger biomass was measured when BSA was provided as a N source, but with the woody shrub *Hakea actites*, provision of BSA resulted in no difference in shoot biomass as compared with N-free controls (Paungfoo-Lonhienne et al. 2008). Using the same agar system, we found that varying BSA concentrations had no significant effect on the growth of wheat in the stem or leaf areas. We used high purity BSA (99%) to reduce the presence of free amino acids in the medium, while the purity of casein in previous studies was not reported and the presence of exuded proteases suggests free amino acids may have been present in the media (Adamczyk et al. 2008).

The different size of BSA (~66 kDa) compared to casein (<25 kDa) and altered size when cleaved by potential exuded proteases could also affect uptake of proteins by wheat. The difference in hydrophobicity between BSA (hydrophilic) and casein (hydrophobic) is also a characteristic that could affect the interaction of these proteins with roots. These properties could affect the ability of the roots to uptake whole proteins and utilize them as a N source.

Another explanation for the opposing results is the different growth systems comparing an agar based system in our study to the hydroponic system, which is likely to affect the behaviour of root exudate and protein movement in the growth medium (Adamczyk et al. 2008). In an agar based system, movement of proteins in the media and exuded proteases would be restricted more than in a hydroponic system where diffusion would promote dispersion of these particles. In addition, the wheat cultivar (AC Andrew) used in our study is a commercial

variety bred to respond to inorganic N which differs from *A. thaliana* and *H. actites* used in previous studies (Paungfoo-Lonhienne et al. 2008). Wheat is a monocot whereas *A. thaliana* and *H. actites* are both eudicots, possibly another reason for the contrasting findings related to the nutritional value of protein to plants.

With increasing N saturation, plants allocate less biomass to the roots to seek out N. The low root dry mass/root length in the negative control shows that under complete N deficiency the plant is unable to allocate enough resources to the root to seek out N. The high root dry mass/root length of the positive control shows that the roots were well developed and this could be attributed to the vitamins contained in the complete MS media. The high root dry mass/root length in P-M and P-H reflects the abnormal branching morphology and formation of bulbous structures with increased lignin/suberin deposition in the cortex. In the treatments exposed to protein only (BSA, Ovalbumin and Casein) and the C-H treatment (not shown) roots were characterized as having an increased number of branches. These root branches were in general thicker and knob-like in structure, something not seen in any treatment not containing protein. This unexpected root morphology suggests the plant is responding to a protein environment very similarly regardless of specific protein. The absence of this unique root morphology response in the negative control suggests that it is not related to N deficiency, but rather to the presence of protein. It cannot be ruled out that the abnormal knob-like outgrowths and branching of roots in a protein environment could be a cumulative effect of the presence of protein and the lack of vitamins.

The process of sensing and responding to N contained in the surrounding environment is vital for plant survival. Local proliferation of roots through the emergence of lateral roots is a plant response to inorganic N (Drew et al. 1973). The physiological response of lateral root initiation and elongation in plants involves a network of hormones, receptors and signalling molecules (Fukaki and Tasaka 2009; Krouk et al. 2010; Zhang and Forde 2000). In previous research both nitrate and plant hormones such as auxin have been shown to induce lateral root initiation (Francisco and Akao 1993; Zhang and Forde 2000). Using synthetic auxin, wheat plants had a root response referred to as *para*-nodules, characterized by increased frequency of irregularly lobed root structures (Francisco and Akao 1993; Gantar and Elhai 1999). The authors found that upon inoculation with the soil bacterium *Rhizobium trifolii* these *para*-nodules were no longer irregularly lobed, and this was attributed to an interaction between the plant and *R. trifolii* (Francisco and Akao 1993). Further investigation showed that N_2-fixing activity in cyanobacteria associated with *para*-nodules in wheat was increased when plants were treated with synthetic auxin (Gantar and Elhai 1999). The knob-like outgrowths seen in BSA, casein and ovalbumin treatments do look morphologically similar to these lobed *para*-nodules and this suggests it may also be inducing hormonal signalling within lateral root architecture (Francisco and Akao 1993). The general increased size of root branches in these protein treatments show that the roots are responding to protein by having a more bulbous structure. The consistent altered morphology seen with all three proteins suggests it is not specific to one protein. The increased amount of safranin O staining in the

wheat roots exposed to high protein is the result of lignified or suberized cell walls (Fig. 2-5c). Chemical analysis of nodules in a legume (*Vicia faba* L. cv. Troy) revealed that the nodule epidermal cell wall had increased lignin and suberin content (Hartmann et al. 2002). Our study differed from this where we observed increased lignin/suberin content in the cortex of roots from P-H. The similar general increase of lignin/suberin deposition in a true nodule found in legumes and the abnormal root branches in P-H suggests that this may be a nodule-like response (Hartmann et al. 2002). The mechanism of this response, although undetermined, could be a due to growth of the plant in a high protein environment or the protein entering the root and directly altering the signalling pathway. Regardless of the response, there was little evidence that it enhanced the ability of wheat to utilize protein as a N source outside of the root tissue.

The results of this study show that commercial wheat variety AC Andrew is unable to directly utilize BSA efficiently as a N source when compared to NH_4NO_3 in a sterile agar environment. Protein seems to be having an effect on plant root morphology that can be explained by either protein N only being utilized by root tissue or proteins altering signalling pathways involved in root branching. The altered root architecture found in wheat exposed to protein is a finding that requires further investigation to fully characterize and determine its significance. In agricultural fields, animal production by-products may not be directly utilized by crop plants like wheat. However, proteins contained in these by-products may be further degraded and accessible with support from soil microorganisms.

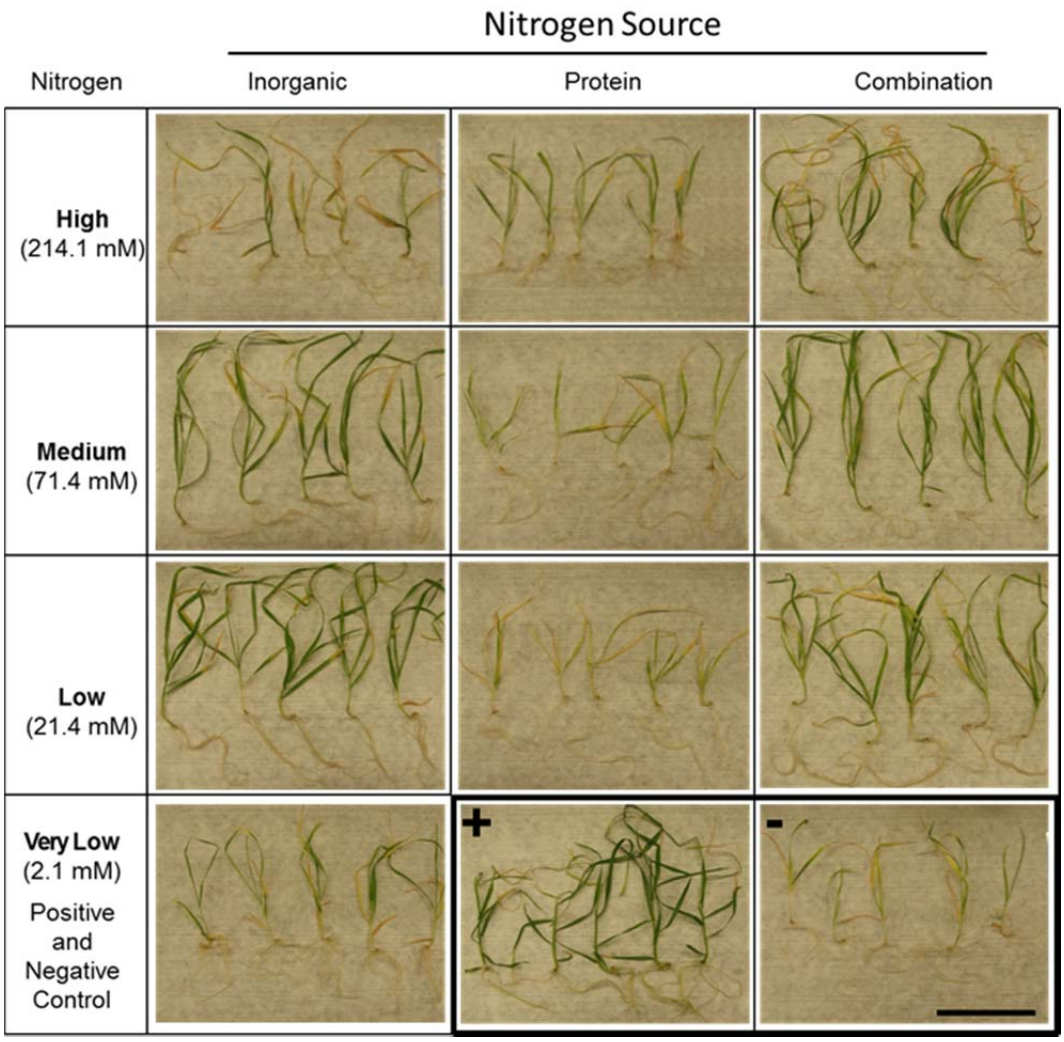


Fig. 2-1. Representative images of wheat plants from different N treatments after 34 d of axenic growth. Pictures show relative health when supplied with different N concentrations as either bovine serum albumin (P), ammonium nitrate (IN), or a 50:50 combination (C) (n=6). Positive (+) and negative (-) control treatments are in the bottom right corner of the figure. Scale bar in negative control (10cm) is consistent with all pictures.

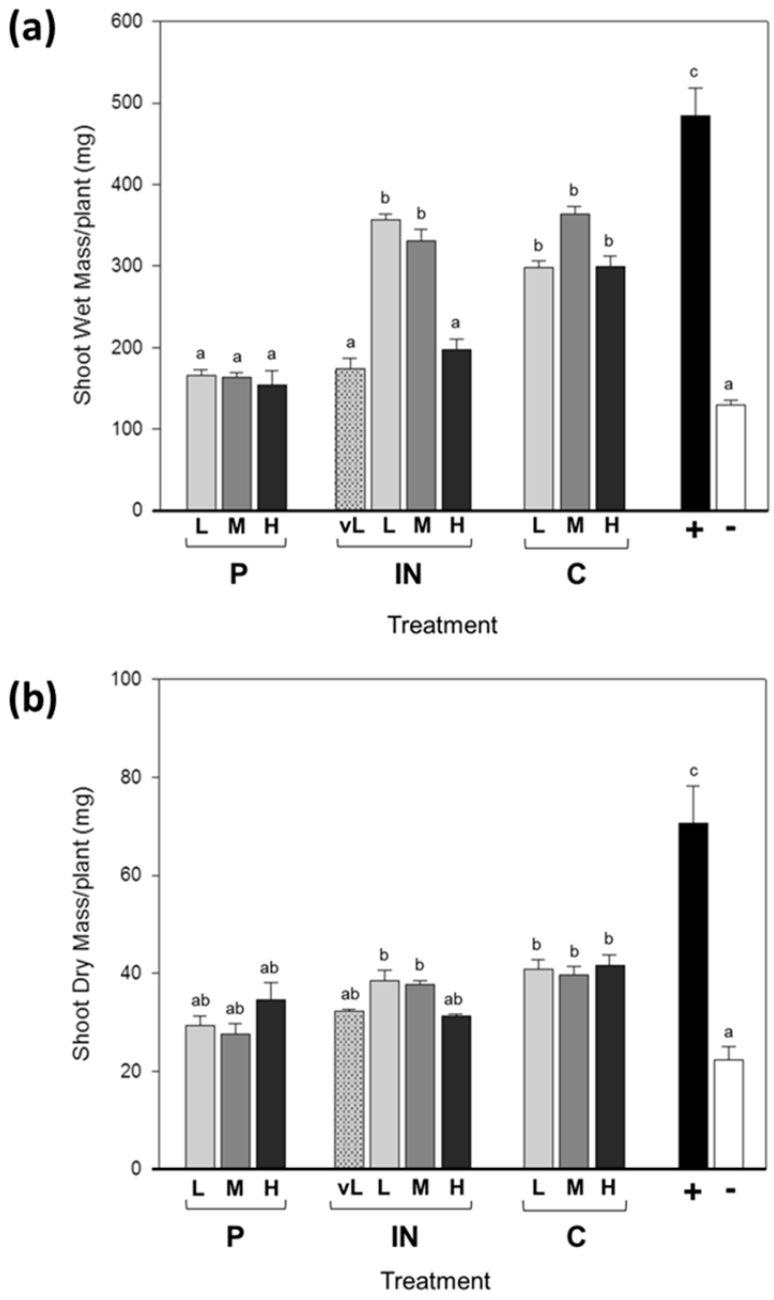


Fig. 2-2. Shoot masses of wheat plants after 33-34 d of axenic growth. (a) Wet (n=6) and (b) dry (n=3) shoot masses were recorded for wheat plants and significance was determined by ANOVA followed by Tukey's HSD tests and is represented by lower case letters. P=BSA, IN=Ammonium Nitrate, C=Combination, +=positive control and -=negative control. Error bars represent standard error of the mean.

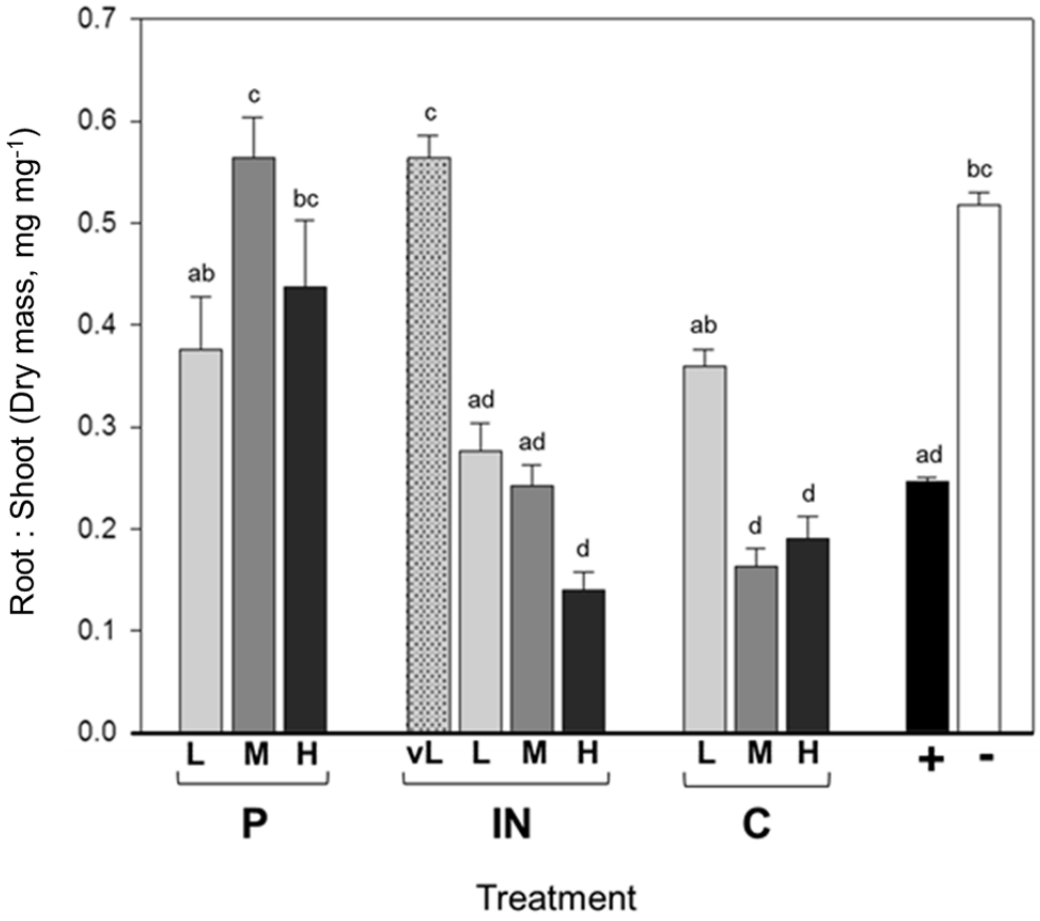


Fig. 2-3. Ratio of root to shoot dry mass for wheat plants grown axenically for 33-34 d. Ratios were calculated from separated dry masses of the shoots (leaf and stem) and roots. Significance was determined using ANOVA followed by Tukey's HSD tests and is represented by lower case letters. Error bars represent standard error of the mean (n=6).

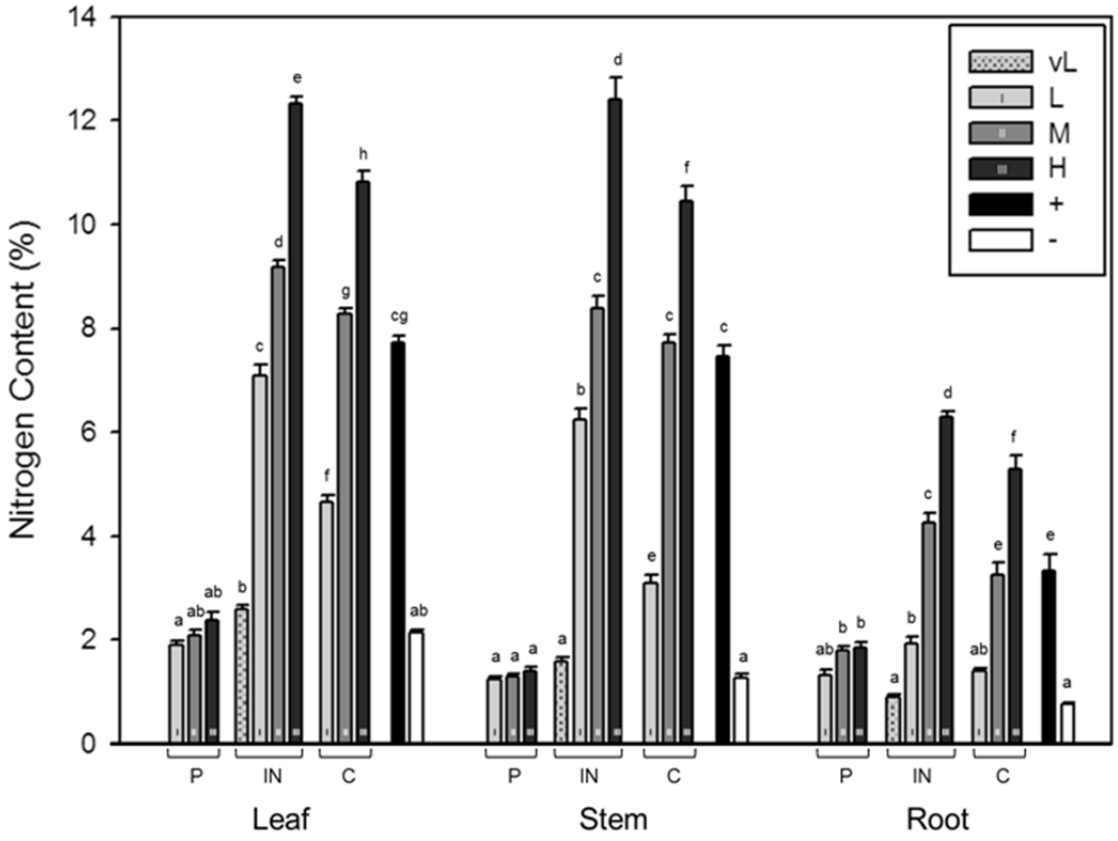
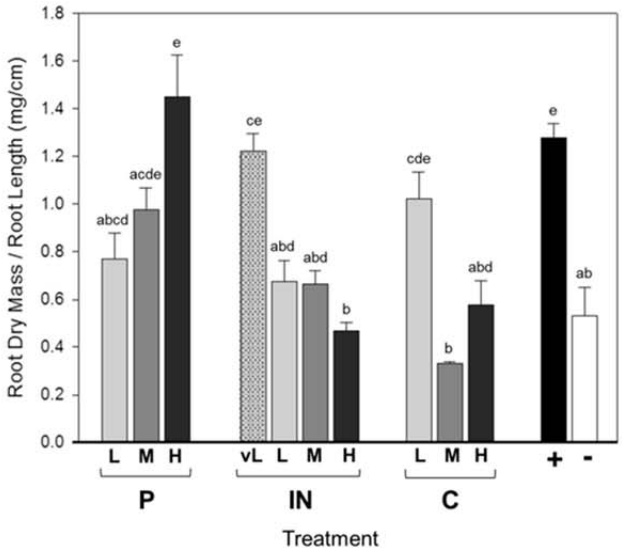
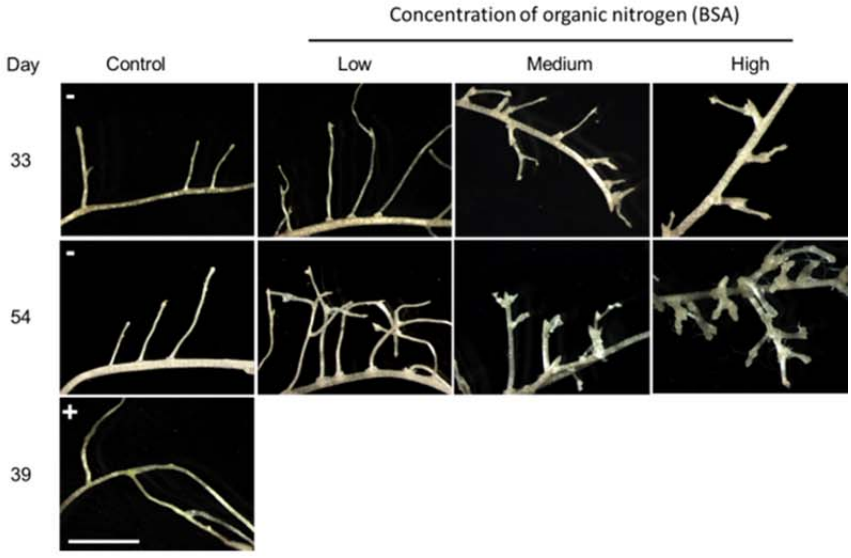


Fig. 2-4. N content (%, by weight) of wheat plant parts after 33-34 d of axenic growth under different N treatments. Significance was determined within each plant part using ANOVA and Tukey's HSD tests and is represented by lower case letters. Error bars represent standard error of the mean (n=6).

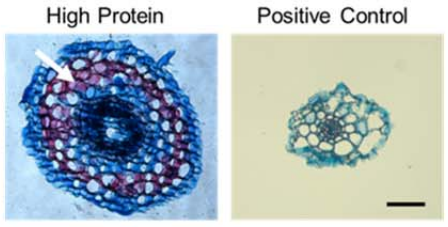
(a)


Root Dry Mass / Root Length (mg/cm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
abcd
acde
e
ce
abd
abd
b
cde
b
abd
e
ab
L M H
vL L M H
L M H
+ -
P
IN
C
Treatment

(b)


Concentration of organic nitrogen (BSA)
Day
Control
Low
Medium
High
33
54
39

(c)


High Protein
Positive Control

(d)

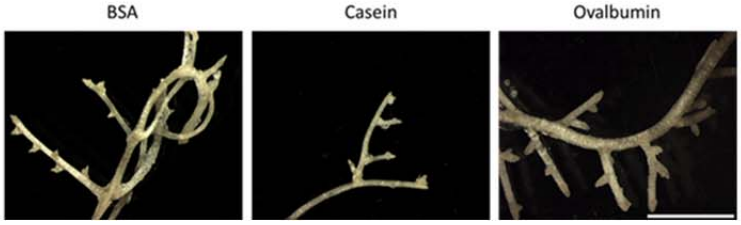

BSA
Casein
Ovalbumin

Fig. 2-5. Effects of protein on root morphology of wheat. (a) Root dry mass/root length of wheat after 33-34 d of growth under different treatments. Lower case letters denote significance determined by ANOVA and Tukey's HSD tests. Error bars represents standard error of the mean (n=3). (b) Gross structural images of wheat roots under different levels of BSA exposure. Scale bar in positive control (4 mm) is consistent for all images. (c) Cross-sectioned and stained wheat roots grown on high protein or positive control media for 39 d. Background colour in high protein section is a result of residual Astra Blue stain in mounting media. Arrow in high protein panel shows cortex with high Safranin O staining. Scale bar in positive control (0.1 mm) is consistent for both images. (d) Gross structural images of wheat roots grown for 33 d with 6.16 mg/mL of different proteins as the only N source. Scale bar in ovalbumin (4 mm) is consistent for three images.

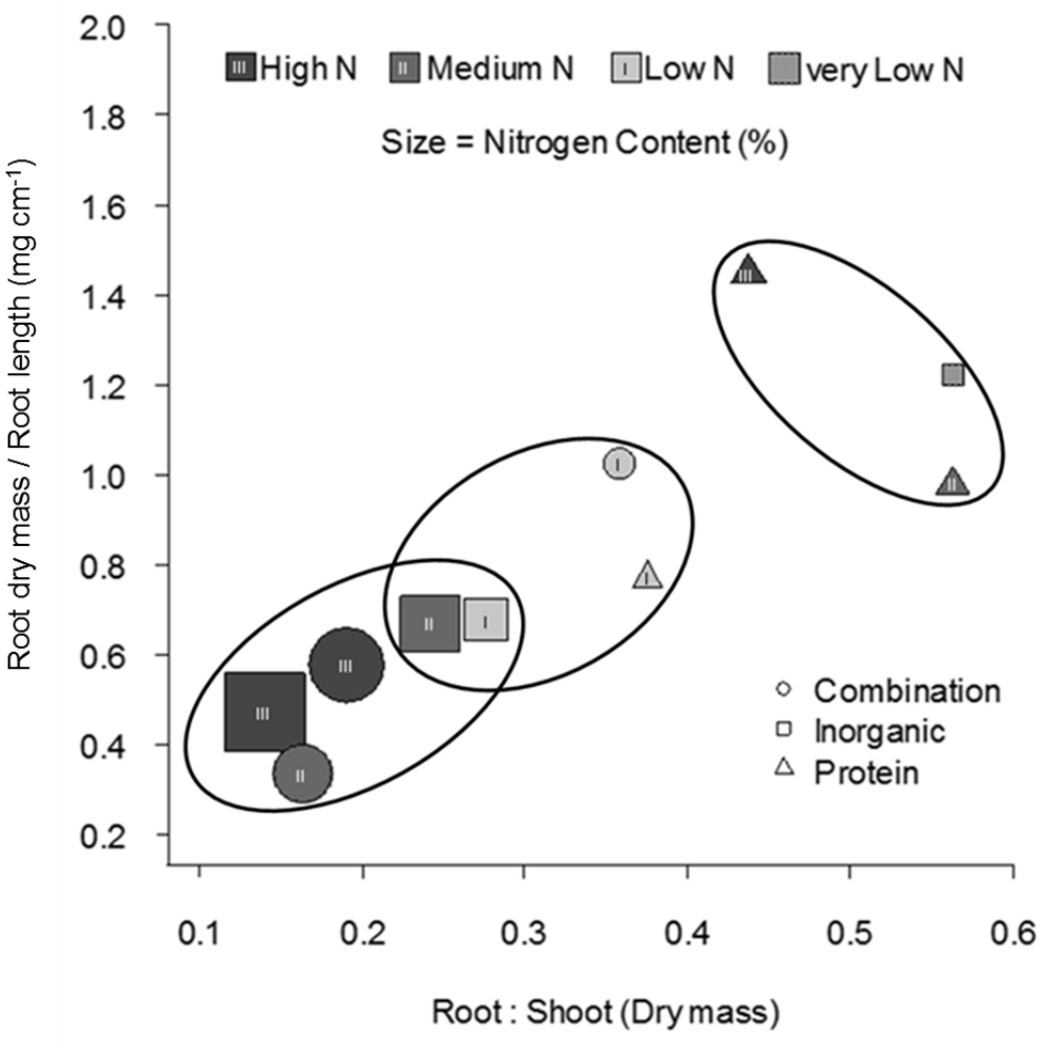


Fig. 2-6. Clustering patterns of root dry mass/root length to root /shoot biomass of wheat grown with different N sources. Measurements were taken after 33-34 d of axenic growth. Circles to define clustering of treatments were assigned arbitrarily.

Table 2-1. Summary of abbreviations for N treatments.

Treatment [Nitrogen]	Inorganic ammonium nitrate (NH_4NO_3)	Protein bovine serum albumin (BSA)	Combination 50:50 (BSA:NH_4NO_3)
High [214mM]	IN-H	P-H	C-H
Medium [71mM]	IN-M	P-M	C-M
Low [21mM]	IN-L	P-L	C-L
very Low [2mM]	IN-vL	-----	-----

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Chapter 3: Ovalbumin protein can be taken up by damaged wheat roots and transported to the stem

INTRODUCTION

Nitrogen (N) is present as organic and inorganic forms in the soil environment and animal proteins which constitute a wide faunal diversity can be part of this overall pool. The release of animal proteins into the soil environment raises the possibility that they may interact with plant roots.

Symbiosis of plants with microorganisms increases available N. Plant species like legumes are able to form nodules which are colonized by N fixing *Rhizobia spp.* (Oldroyd and Downie 2008). Other plants are able to form symbioses with mycorrhizal fungi which acquire N for the host plant and allow plants to access N outside of the root zone (Govindarajulu et al. 2005; Jin et al. 2005; Parniske 2008; Tanaka and Yano 2005).

In order to acquire sufficient N, plants have uptake mechanisms specific to different forms of N. Inorganic nitrate and ammonium have been extensively studied as primary sources of N for plants (Jahn et al. 2010; Miller and Cramer 2005; Paponov et al. 1999; Vonk et al. 2008). Transporters with high and low target affinities are conserved among plants and are responsible for internalization of these molecules. Specifically, the nitrate (NRTx) and ammonium transporters (AMTx) represent the major inorganic N transport mechanisms (Girin et al. 2010; Ludewig et al. 2002; Tsay et al. 2007). Organic N such as amino acids and shorter peptides are also vital plant nutrients. The amino acid permease (AAPx) and

lysine histidine transporter (LHTx) proteins facilitate the uptake of specific amino acids such as glutamate, phenylalanine, lysine and histidine (Hirner et al. 2006; Lee et al. 2007; Svennerstam et al. 2007). The uptake of di- and tri-peptides is facilitated by PTRx transporters (Dietrich et al. 2004; Komarova et al. 2008). These active uptake mechanisms allow plants to acquire different forms of N from the surrounding environment.

Recent studies on larger organic molecules have shown that plants can take up more complex and diverse N-containing molecules than previously realized. The internalization of green fluorescent protein into the root system of *Arabidopsis thaliana* and the woody shrub *Hakea actites* has been reported (Paungfoo-Lonhienne et al. 2008). The mechanism for uptake of protein into the roots has been hypothesized to involve endocytosis (Paungfoo-Lonhienne et al. 2008). In a separate study, ovalbumin was shown to move through the xylem of wheat, suggesting that proteins can likely move through plant vasculature if they enter it (Neumann et al. 2010). Furthermore, the uptake of entire bacteria has been documented in a number of plant species including tomatoes and sweet basil (Gorbatsevich et al. 2013; Paungfoo-Lonhienne et al. 2010). The mechanism of internalization for bacteria involves restructuring of the cell wall, and has been hypothesized to involve endocytosis (Paungfoo-Lonhienne et al. 2010). The extent to which these molecules represent an alternative N source for plants remains to be determined.

In this study we investigated the ability of wheat (*Triticum aestivum* L.) to take up ovalbumin into the roots and transport it through the plant. In addition, the

potential of individual cells to uptake ovalbumin was investigated using wheat mesophyll protoplasts. Findings from this work add knowledge to the potential movement of proteins to aerial plant structures and provide insight in to the likelihood that intact proteins could be consumed by grazers.

MATERIALS AND METHODS

Plant Growth

Wheat (cv. AC Andrew) seeds were germinated for 5 d after surface sterilization with 70% ethanol and 3% hypochlorite. Germinated seeds were transferred to Magenta® boxes containing sterile Murashige and Skoog (MS) agarose media adjusted to pH 5.8 (Murashige and Skoog 1962). Media was filter-sterilized (0.2 µm) prior to combining with autoclaved phytagel (P8169, Sigma Aldrich, Oakville, Canada) to achieve a final concentration of 0.3% (Murashige and Skoog 1962). For hydroponic growth experiments, a second set of germinated seeds was transferred to RITA® boxes (CIRAD, Montpellier, France) where roots were bathed on a pulse cycle of 1 min every h with sterile Murashige and Skoog media. All plants were grown axenically for 30-45 d prior to uptake experiments. Growth boxes were placed in a growth room with light conditions of 16 h d/8 h night (150 µmole m^{-2} s^{-1}) at 20°C ± 2°C.

Protein Uptake in Wheat

Alexa Fluor 555 conjugated Ovalbumin (OVAF555, Life Technologies, Burlington, Canada), was chosen to study protein uptake in wheat based on its previous use in experiments with tobacco and olive (Neumann et al. 2010). Plants

were removed from growth media and roots were rinsed with dH_2O and blotted dry before being exposed to 50 µg/mL OVAF555 solution containing nitrogen-free Murashige and Skoog media: 29.21 mM sucrose, 2.99 mM $CaCl_2 \cdot 2H_2O$, 1.50 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM KH_2PO_4, 0.11 mM EDTA ferric sodium salt, 0.10 mM H_3BO_3, 0.10 mM $MnSO_4 \cdot H_2O$, 29.91 nM $ZnSO_4 \cdot 7H_2O$, 5.00 nM KI, 1.03 nM $Na_2MoO_4 \cdot 2H_2O$, 0.11 nM $CoCl_2 \cdot 6H_2O$, and 0.10 nM $CuSO_4 \cdot 5H_2O$ (Murashige and Skoog 1962). Plant roots were exposed to OVAF555 for 24 h. After exposure, roots were treated with 10 µg/mL proteinase K for 5 min and washed for 5 min under running tap water to remove protein adhering to the outer surface of the root. This proteinase K treatment was confirmed to degrade OVAF555 in 50 µg/mL solution under the described conditions. Plant parts (roots [zone of maturation], stems and leaves) were embedded in Tissue-Tek® O.C.T™ (Sakura Finetek, Inc., Torrance, USA), frozen and sectioned (8 µm) with a microtome-cryostat (IEC, Inc., Chattanooga, USA). Tissues were imaged using a Zeiss Axiovert 40 CFL fluorescent microscope with a 10X objective lens using consistent exposure times with a Zeiss Axiocam MRm camera (296 ms, Jena, Germany). Whole protein extract was obtained using a Plant Total Protein Extraction Kit (Sigma Aldrich). The amount of extraction buffer used depended on the tissue mass in the protein extraction protocol. Protein extracts were analyzed by Western blotting (WB) to detect for the presence of ovalbumin in roots, stems and leaves using an Advanced One-Hour Western kit (GenScript, Piscataway, USA) and OVA-14 ovalbumin mAb (1:10000, Sigma Aldrich). Presence of protein in the stem extracts was also confirmed using a Typhoon 9400

Variable Mode Imager (Cy3 laser, Amersham Biosciences, Baie d'Urfe, Canada) to detect Alexa Fluor 555 fluorescence after extracts were ran on NuPAGE® Novex® 4-12% Bis-Tris gels (Life Technologies).

In analogous experiments using the hydroponic RITA® temporary immersion system (CIRAD), plant roots were exposed to the same nitrogen-free MS media containing 50 µg/mL OVAF555 for 24 h. Similar to experiments described above, roots were treated with 10 µg/mL proteinase K for 5 min followed by washing under running tap water for 7 min to remove protein adhering to the outside of the root. Roots, stems and leaves were analyzed by fluorescent microscopy and WB as described above to determine the location of protein. The RITA system (Liquid) was employed to avoid mechanical damage to the root system that may have occurred while removing plants from the growth media in the solid system. Root damage evident in the solid system was not apparent in the liquid growth system. Uptake experiments were independently replicated three times for both Solid and Liquid growth systems (n=3).

Protoplast culture and ovalbumin uptake

Protoplast mesophyll cells from *T. aestivum* L. (cv. AC Andrew) were harvested as previously described (Chugh and Eudes 2007). The cuticle was removed from the leaves of 14-21 d old wheat plants and longitudinally sectioned using a scalpel. Cell and protoplast washing (CPW) solution (0.6 M D-mannitol, 10 mM $CaCl_2$, 10 mM MES hydrate, 1 mM KNO_3, 1 mM $MgSO_4$, 0.2 mM KH_2PO_4 and 1 µM $CuSO_4$, pH 5.6) was used as the buffer in all extractions and incubations (Chugh and Eudes 2007). Protoplasts were incubated in CPW containing 2%

Maceroenzyme & 2% Cellulase (Yakult Honsha Co. Ltd, Tokyo, Japan) for 16 h at 28°C after 30 min of incubation in a desiccation chamber with enzyme solution. Protoplasts were then purified on a 17% sucrose gradient and split into 500 µL aliquots that each contained ~200 000 cells. Fractions were incubated with 400 µL of 50 µg/mL Alexa Fluor 488 ovalbumin or Alexa Fluor 647 ovalbumin (OVAF488 or OVAF647, Life Technologies) in a manner similar to that previously described (Chugh and Eudes 2008). After exposure to ovalbumin, protoplasts were washed with CPW and treated with 0.0625% Trypsin-EDTA for 5 min, and washed again with CPW to remove proteins not within cells. Fluorescent protoplast populations (10000/data point) were enumerated using a FACSCanto II flow cytometer (488 or 633 nm laser, BD Biosciences, Mississauga, Canada). In the third treatment, protoplasts were incubated in 400 µL high purity unlabelled ovalbumin at 50 µg/mL (OV, A5503, Sigma Aldrich). For all three treatments, WB were performed using lysed protoplasts and an Advanced One-Hour Western Kit (GenScript) with OVA-14 ovalbumin mAb (1:10000, Sigma Aldrich). Each treatment (OVAF488, OVAF657 and OV) was independently replicated three times (n=3). Protoplasts were also incubated for 30 min with the macropinocytosis inhibitor, cytochalasin D (10µM) prior to incubation with OVAF488 and flow cytometry analysis (Chugh and Eudes 2008). Cytochalasin D slightly reduces protoplast viability possibly increasing the likelihood of OVAF488 entering the protoplast. Viability of protoplasts was determined by both fluorescein diacetate (10 µg/mL) and SYTOX® Green (0.5 nM, Life Technologies) staining.

RESULTS

Protein Uptake in wheat

Solid and Liquid growth systems were used to compare the impact of mechanical damage to intact roots on uptake of ovalbumin. Removing wheat roots from solid agarose (Solid) resulted in mechanical damage to the roots as visualized by some regions being devoid of root hairs. However, major damage as result of the cracking or severance of root branches was not visible after close inspection of the roots under a stereomicroscope. Roots from the RITA hydroponic system (Liquid) displayed no visual root damage.

After 24 h exposure to Alexa Fluor 555 tagged ovalbumin (OVAF555) in the solid system, fluorescence was detected in roots as observed in the intercellular spaces of the epidermis, the cellular junctions of the endodermis and the cortex (Fig. 3-1a). With the liquid system, root fluorescent signal was primarily localized to the epidermis (Fig. 3-1a). Signals in the intercellular spaces of the cortex were less intense and did not extend to the endodermis (Fig. 3-1a). Sections from the base of the stem exhibited auto-fluorescence, compromising the ability to differentiate OVAF555 from natural fluorescence in both the solid and liquid growth systems (Fig. 3-1a). Similar to the lower stem, fluorescent images of the upper half of the stem and leaves were inconclusive due to auto-fluorescence in these tissues (data not shown).

Western blots of root protein extracts showed an ovalbumin band in both the solid and liquid systems at ~43kDa (Fig. 3-1b). Protein extracts from the

control sample of the lower stem consistently had two cross-reaction bands at ~40kDa and ~46kDa (Fig. 3-1b). The lower stem from the solid system had an ovalbumin band at ~43 kDa in-between the two cross-reaction bands that was not present in the control (Fig. 3-1b). Furthermore, this ovalbumin band was absent from the lower stem extract in the liquid system (Fig. 3-1b). The described ~43 kDa ovalbumin band in roots and stem aligned with the smaller band in the OVAF555 lane and confirmed the presence of ovalbumin (Fig. 3-1b). Upper stem and leaf extracts did not show ovalbumin bands (data not shown). Due to ovalbumin cross-reaction bands in the lower stem, extracts were also analyzed for fluorescence after PAGE. This approach confirmed the presence of OVAF555 at the correct size in the lower stem from the solid system with weak fluorescence observed in the liquid system (Fig. 3-1c).

Protoplast culture and ovalbumin uptake

Protoplasts were used to determine if individual cells were capable of ovalbumin uptake. Uptake of ovalbumin was analyzed for two different Alexa Fluor labelled species (OVAF647 and OVAF488) as well as unlabelled ovalbumin. Protoplast viability was 65.3% ± 3.65% after extraction and processing for experiments with OVAF488 and OVAF647 (Table 3-1). Flow cytometry revealed conflicting results for OVAF488 and OVAF647, with 15.1% ± 2.64% of protoplasts fluorescing after contact with OVAF488, but a fluorescing population was absent in protoplasts exposed to OVAF647 (Fig. 3-2a & Table 3-1). Western blotting was used to further characterize the interaction of ovalbumin with protoplasts. Western blots of lysed protoplasts showed that ovalbumin was associated with

protoplasts for OVAF488 with a negligible signal for OVAF647 (Fig. 3-2b). A cross-reaction band at ~29 kDa was also seen in all protoplast lanes but only the OVAF488 treated protoplasts had two bands at ~40 kDa and ~44 kDa that aligned with the two ovalbumin bands in controls (Fig. 3-2b). In the unlabelled ovalbumin treatment (OV) there was no ovalbumin protein bands detected in protoplasts (Fig. 3-2b). When protoplasts were pre-treated with cytochalasin D and then incubated with OVAF488, 47.9% ± 0.61% of protoplasts fluoresced and viability was reduced to 42.6% ± 7.09% (Table 3-1).

DISCUSSION

This study shows that wheat cultivar AC Andrew has the ability to internalize protein and further transport it within plant organs. The findings using liquid vs. solid growth systems coupled with a protoplast culture assay shed light on the mechanism of ovalbumin uptake and transport in wheat.

Solid and liquid growth systems showed that uptake by roots and transport of ovalbumin in wheat appears to depend on the degree of root damage. The fluorescent signal at the epidermis in the root and lack of detection of a fluorescent or antibody signal in the stem in the liquid vs the solid system illustrates this phenomenon (Fig. 3-1). Based on weak signals in the stem after PAGE our data suggests that negligible ovalbumin is being transported into the lower stem in the liquid system (Fig. 3-1). The absence of fluorescence in the cortex region of roots grown in the liquid system suggests that protein was unable to gain entrance to the interior of the plant roots and be transported to the stem

(Fig. 3-1a). Given that Alex Flour labelled ovalbumin can move within the xylem of olive and tobacco plants, this outcome suggests that ovalbumin did not reach the plant vasculature (Neumann et al. 2010). The lack of protein in the stem for the liquid system compared to the solid system suggests that mechanical damage to the roots in the solid system opened pathways for ovalbumin uptake into the vascular system and its transport to the stem. The concentration of fluorescence at cell junctions of the endodermis in the roots indicates protein movement was likely impeded by the Casparian strip. This pattern suggests that some of the protein is moving apoplastically with water and stops at this impermeable point, and would only reach the vasculature in the event that the Casparian strip barrier was breached.

Uptake of large organic molecules into plants has been investigated using both fluorescent and radioactive labels. The ability of different proteins such as lysozyme and hemoglobin to enter excised roots of barley plants has been clearly demonstrated (Bradfute and McLaren 1964; McLaren et al. 1960; Seear et al. 1968). Excised roots from maize, onion, tomato and vetch can also absorb lysozyme (Seear et al. 1968). These studies demonstrate that, when removed from the rest of the plant, the root organ retains the ability to take up protein to support our studies where protein enters roots in a whole plant system. More recent work documented the uptake of green fluorescent protein into the roots of *Arabidopsis thaliana* and *Hakea actites* (Paungfoo-Lonhienne et al. 2008). Another study used radioactively labelled lysozyme, ovalbumin and hemoglobin to track movement of protein through the roots to other areas in tomato plants (Ulrich and McLaren

1965). Degraded hemoglobin as well as intact lysozyme and ovalbumin were detected in the leaves of the plant (Ulrich and McLaren 1965). The authors suggested that for lysozyme and ovalbumin the presence of protein in the leaves was due to mechanical damage of the roots and physiological stress (Ulrich and McLaren 1965). This physiological stress is from changes in humidity that effected movement of water through the plant. Our observation that ovalbumin was transported to the stem only in the solid system suggests that minor root damage may have given rise to this observation. In another study, uptake of *Escherichia coli* TG1 was enhanced if the root tips of corn were severed (Bernstein et al. 2007). Root damage occurs in the environment through a variety of interactions with plant feeding organisms (van Dam 2009). Together, these findings suggest that in environments where root damage occurs, entry of large organic molecules including proteins into roots and their transport to stems and leaves can occur.

Protoplasts have been used in many plant studies to characterize cell penetrating peptide (CPP) domains as a means of delivering bioactive molecules to plant cells (Chugh et al. 2010). Work with *Petunia hybrida* protoplasts showed that core histones have CPP domain properties that enable their passage through the plasma membrane whereas bovine serum albumin lacks these domains (Rosenbluh et al. 2004). Conversely, in studies fusing GFP to *Brome mosaic virus* capsid protein, it was revealed that GFP was unable to enter barley mesophyll protoplasts in the absence of the capsid protein (Qi et al. 2011). This outlines how protein translocation into protoplasts is dependent on the specific protein and the

presence of CPP domains. Ovalbumin does contain a sequence of amino acids (274-291) weakly similar (33%) to a known CPP domain. The lack of unlabelled ovalbumin uptake by wheat protoplasts in the current study suggests the putative CPP domain in ovalbumin does not facilitate translocation of this protein into protoplasts. The inability of ovalbumin to translocate across the plasma membrane suggests the uptake and transport seen in the plant growth systems described above is primarily due to apoplastic movement and root damage rather than direct entry into cells.

Our results showed that Alexa Fluor 488 but not Alexa Fluor 647 labelled ovalbumin associated with protoplasts, suggesting that the nature of the fluorescent label itself effects interaction with a plant cell. Alexa Fluor 647 is approximately twice the size of Alexa Fluor 488, a size difference which could possibly account for this differential affinity. There was an increase in the number of fluorescent protoplasts when they were exposed to the macropinocytosis inhibitor cytochalasin D, prior to interaction with OVAF488 (Table 3-1). We also observed a decrease in the percentage of viable cells in protoplasts exposed to cytochalasin D (Table 3-1). It is confusing that an inhibitor of macropinocytosis would increase the number of fluorescing protoplasts upon exposure to a fluorescent protein. Based on this unexpected finding we suggest fluorescence in OVAF488 exposed protoplasts is due to an interaction between the fluorescent label and dead cells rather than uptake by viable cells. This interaction with dead cells could be due to specific electrostatic or hydrophobic properties of the Alexa

Fluor 488 molecule that differ from Alexa Fluor 647 and are absent from unlabelled ovalbumin.

The protoplast experiments reveal that the nature of the Alexa Fluor label for ovalbumin influences the interactions of this protein with plant cells. For whole plant studies, a different Alexa Fluor tagged ovalbumin (OVAF555) was used based on reduced auto-fluorescence in this wavelength for plant tissues (Fig. 3-1). Given the differential responses in the affinity of Alexa Fluors with protoplasts, we cannot rule out the possibility that the nature of Alexa Fluor 555 influenced the interaction of ovalbumin with wheat roots. The benefit of being able to track the protein by fluorescence in the root added to the findings of these experiments. Consistent cross-reaction between wheat proteome and OVA-14 mAb was likely due to the high similarity, based on BLAST analysis, between ovalbumin and the SERPIN family of plant proteins (>92%).

This study shows that AC Andrew has the ability to take up ovalbumin into roots and, when roots are damaged, transport the protein to the stem. In comparison, wheat mesophyll protoplasts lacked the ability to translocate ovalbumin protein across the plasma membrane. Taken together these results suggest that uptake of ovalbumin into wheat plants proceeds by apoplastic movement in-between cells. In environmental situations where root damage occurs, it is possible that protein may enter wheat roots and be transported to the stem. This gives insight into the potential of animal protein moving above the soil horizon through plants and being consumed and dispersed by grazers given that root damage in the environment occurs naturally.

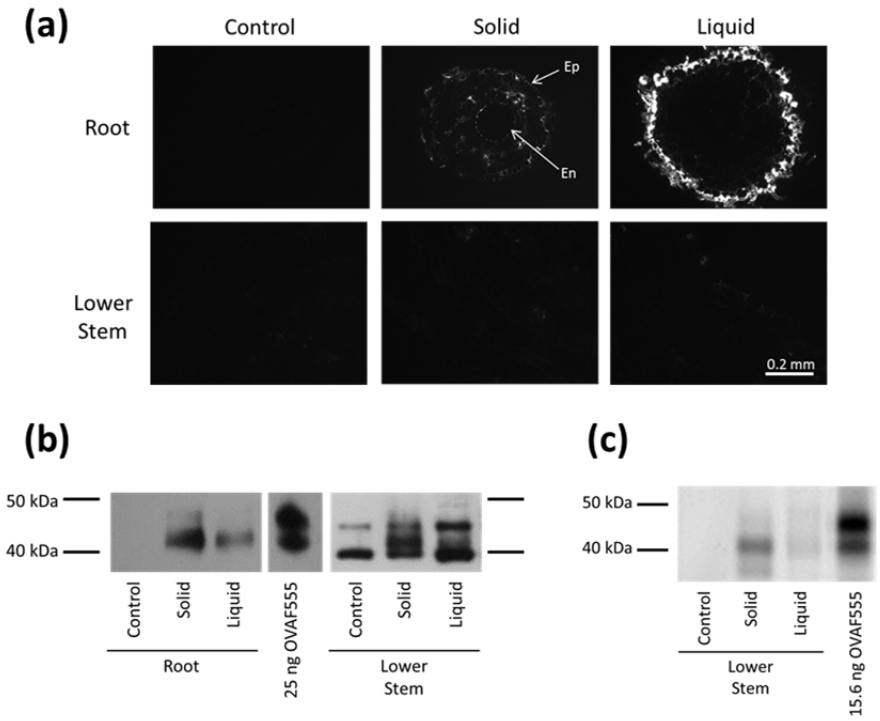

Fig. 3-1. Uptake and transport of ovalbumin to the stem of wheat plants with minor root damage. (a) Fluorescent images of cryo-sectioned wheat roots and stems from plants exposed to Alexa Fluor 555 tagged ovalbumin (OVAF555) in wheat plants grown in solid or liquid medium. Plant roots (zone of maturation) and stems were cryo-sectioned (8um) and imaged on a Zeiss Axiovert CFL 40 fluorescent microscope. Images represent three independent replications (n=3). Scale bar is consistent for all images. Ep=Epidermis, En=Endodermis. (b) Western blotting of total protein extracts to detect ovalbumin in plants exposed to Alexa Fluor 555 tagged ovalbumin (OVAF555). OVA-14 mAb (1:10000) was used with Advance One-Hour Western Kit (GenScript) for detection. Blot results are representative of three independent replicates (n=3). Root and stem extracts were from different blots developed under the same conditions (25ng OVAF555 control lane is from the same blot as root extracts with irrelevant lanes omitted). (c) Fluorescent detection of ovalbumin in stems of plant protein extracts. Typhoon 9400 imager (Amersham Biosciences) was used to detect presence of Alexa Fluor 555 tag on ovalbumin after PAGE.

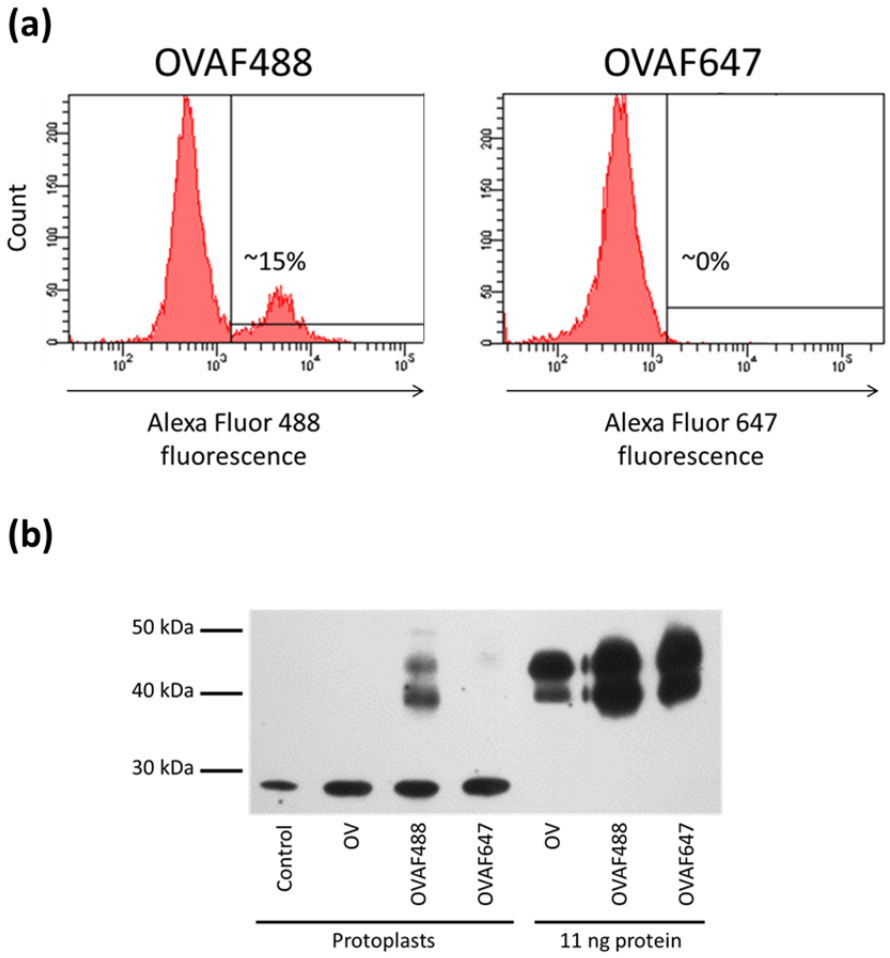

Fig. 3-2. Wheat protoplasts were unable to take up ovalbumin and Alexa Fluor 488 tag interacts with cells. (a) Flow cytometry data from protoplasts exposed to Alexa Fluor 488 tagged and Alexa Fluor 647 tagged ovalbumin. Representative of three independent replicates (n=3). (b) Western blot of protoplasts exposed to untagged ovalbumin (OV), Alexa Fluor 488 tagged ovalbumin (OVAF488) or Alexa Fluor 647 tagged ovalbumin (OVAF647). Western blotting was done using OVA-14 mAb (1:10000) to detect ovalbumin using Advanced One-Hour Western Kit (GenScript). Blot is representative of three independent replicates (n=3).

Table 3-1. Protoplast association with Alexa Fluor tagged ovalbumin (OVAF488) alone or after exposure to cytochalasin D.

	OVAF488	Cytochalasin D-OVAF488
Fluorescing Protoplasts (%)	15.1 ± 2.64	47.9 ± 0.61
Protoplast Viability (%)	65.3 ± 3.65	42.6 ± 7.09

Values are averages of three independent replicates (n=3) with standard error of the mean.

OVAF647 treated protoplasts did not have a distinct high fluorescing population.

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Chapter 4: Can plants serve as a vector for prions causing Chronic Wasting Disease?

INTRODUCTION

Chronic Wasting Disease (CWD) is a fatal neurodegenerative prion disease affecting wild and farmed cervids. Specifically, CWD has been found in deer (mule and white-tail), elk and moose (Saunders et al. 2012). Experimental transmission has also been demonstrated in reindeer (Mitchell et al. 2012). Prion diseases, also termed Transmissible Spongiform Encephalopathies (TSE), are caused by mis-folding of the cellular prion protein, PrP^{C}, into the disease-causing conformation designated PrP^{TSE}. Misfolded PrP^{TSE} is broadly accepted as the disease causing agent responsible for cyclic misfolding of PrP^{C} into PrP^{TSE} (Deleault et al. 2007; Wang et al. 2010).

Prevalence of CWD is high in endemic areas like Wyoming (USA) where, in a number of herds, 50% of wild mule deer are CWD positive (Saunders et al. 2012). Indirect transmission of CWD has been confirmed in studies where deer became infected following exposure to areas which previously held CWD-positive deer (Mathiason et al. 2009; Miller et al. 2004). CWD PrP^{TSE} remains infectious for more than two years *ex vivo* in the environment representing a long term hazard for transmission (Miller et al. 2004). Infected cervids have been reported to shed PrP^{TSE} in bodily fluids, disseminating infectious prions in urine, feces, blood and saliva (Haley et al. 2011; Mathiason et al. 2006; Pulford et al. 2012; Tamguney et al. 2009).

PrP^{TSE} binds to whole soil and interaction with some soil minerals has been shown to increase the infectivity of PrP^{TSE} (Johnson et al. 2006; Johnson et al. 2007). Although PrP^{TSE} binds to soil, CWD PrP^{TSE} takes 60 d to reach maximal binding in sandy soil compared to 30 d for hamster adapted transmissible mink encephalopathy (TME) (Saunders et al. 2009). In seawater, scrapie PrP^{TSE} remains stable with a one log reduction only observed after 30 d, suggesting unbound PrP^{TSE} may persist and flow with ground water (Maluquer de Motes et al. 2008). Taken together, these factors suggest PrP^{TSE} may remain unbound from soil for a substantial period of time and be mobile after entering the environment.

Plants acquire nutrients from the surrounding soil environment. Nitrogen (N) is one of the main macronutrients vital for growth and reproduction. There are multiple forms of N in the soil environment and the importance of organic N, including amino acids and peptides, to plant health is well documented (Hill et al. 2011; Jamtgard et al. 2008). The nutritional value of protein N to plants is contentious but, the potential for uptake has been demonstrated (Adamczyk et al. 2008; Paungfoo-Lonhienne et al. 2008; Ulrich and McLaren 1965). Uptake of green fluorescent protein into roots has been shown for *Arabidopsis thaliana* and *Hakea actites* (Paungfoo-Lonhienne et al. 2008). Earlier work suggested ovalbumin is not only taken up by roots, but is subsequently transported to upper regions of tomato plants when root damage is present (Ulrich and McLaren 1965).

The ability of plants to take up larger organic particles, raises the possibility that PrP^{TSE} may enter plants through the roots. This study used wheat

as a model to investigate the potential uptake of CWD PrP^{TSE} into the roots and its subsequent transport to the stem. If such a phenomenon was confirmed it would suggest the potential for plants to act as a vector for the transmission of CWD in cervids.

MATERIALS AND METHODS

Plant Growth

Triticum aestivum L. (cv. AC Andrew) seeds were germinated for five days after surface sterilization with 70% ethanol and 3% hypochlorite. Germinated seeds were transferred to Magenta® boxes containing sterile Murashige and Skoog (MS) agarose media adjusted to pH 5.8 (Murashige and Skoog 1962). Filter-sterilized media (0.2 µm) was combined with autoclaved phytagel (P8169, Sigma Aldrich, Oakville, Canada) to a final concentration of 0.3%. All plants were grown axenically for 30-45 d prior to uptake experiments. Growth boxes were placed in a growth room with light conditions of 16 h d/8 h night (150 µmol m^{-2} s^{-1}) at 20°C ± 2°C.

PrP^C Exposures

Recombinant mouse PrP^C (amino acids 23-230) was expressed in BL21 *E. coli* (Stratagene, Mississauga, Canada) and purified as previously described (Baral et al. 2012). The PrP^C gene contained a His_6 affinity tag at the C-terminus that was removed by a thrombin cleavage site after purification on a Ni-NTA agarose column (Qiagen, Toronto, Canada). After re-folding, PrP^C was dialyzed against dH_2O and concentrated using Amicon Ultra centrifugal filters (3kDa, Millipore,

Billerica, USA). Purity was confirmed by SDS-PAGE and concentration was determined by Bradford assay (Bradford 1976). Wheat plants were removed from growth boxes, roots were rinsed with dH_2O and patted dry with paper towel. Roots were exposed to 4 mL of 50 µg/mL PrP^C for 24 h in nitrogen-free MS media: 29.21 mM sucrose, 2.99 mM $CaCl_2 \cdot 2H_2O$, 1.50 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM KH_2PO_4, 0.11 mM ethylenediaminetetraacetic acid ferric sodium salt, 0.10 mM H_3BO_3, 0.10 mM $MnSO_4 \cdot H_2O$, 29.91 nM $ZnSO_4 \cdot 7H_2O$, 5.00 nM KI, 1.03 nM $Na_2MoO_4 \cdot 2H_2O$, 0.11 nM $CoCl_2 \cdot 6H_2O$, and 0.10 nM $CuSO_4 \cdot 5H_2O$ (Murashige and Skoog 1962). Plants were removed from the PrP^C solution and roots were rinsed for 1 min in dH_2O. Roots from one half of the plants were exposed to 10 µg/mL proteinase K (0.7 U/s L, PK, Life Technologies, Burlington, Canada) for 5 min followed by another rinse with dH_2O to remove loosely adherent protein on the outer surface. Plants were separated into roots, stems and leaves and each fraction was flash frozen in liquid nitrogen. Separated fractions were pulverized with a mortar and pestle and protein was extracted using a plant total protein extraction kit (Sigma Aldrich). Protein samples were heated at 96°C for 5 min in buffer (2.2% SDS, 48 µM Tris, 10% glycerol, 1% 2-mercaptoethanol, pH 6.8) prior to loading on a polyacrylamide gel. Samples were electrophoresed using a 4-12% NuPAGE® Bis-Tris polyacrylamide gels at 200 V for 50 min in 1X MOPS buffer (Life Technologies). Gels were transferred (wet) to nitrocellulose membranes (0.2 µm pore, Genscript, Piscataway, USA) at 100V for 60 min in 1X NuPAGE® Transfer buffer (Life Technologies) with 10% methanol.

Western blotting was done with 8H4 mAb (1:10000, Sigma Aldrich) and an Advanced One-Hour Western Kit (Genscript).

Prion source

All experiments using infectious material were completed in enhanced BSL 2 containment laboratories at the National Centre for Animal Disease Lethbridge Laboratory, Canadian Food Inspection Agency and material was also obtained through this collaboration. Elk (*Cervus canadensis*) brain stems positive for CWD were homogenized in PBS (CWD positive) or dH_2O (CWD negative) with a Dounce tissue grinder to make 20% solutions. The titre of CWD positive elk homogenate used in this study was $10^{7.2}$ i.c. ID_{50}/g as determined by bioassay in Tg(CerPrP)$1536^{+/-}$ transgenic mice expressing elk PrP. For experiments with purified PrP^{TSE}, both CWD positive and negative brain homogenates (BH) were processed using TeSeE® purification kit (Bio-Rad, Mississauga, Canada) following manufactures specifications. Pelleted PrP^{TSE} was re-suspended in Dulbecco's phosphate-buffered saline (PBS, Sigma Aldrich), vortexed, and subject to 8, 10 sec rounds of sonication at maximum intensity using a Misonix Sonicator 3000 Ultrasonic Cell Disruptor (Misonix, Farmingdale, USA). Solutions containing purified PrP^{TSE} were diluted to be equivalent to 1% (w/v) of the BH input material. For BH experiments, digestion of homogenates before plant exposures were done on the homogenized 20% brain solutions at 37°C for 30 min with 50 µg/mL PK (Life Technologies). BH were then diluted to 1% (w/v) with dH_2O for plant exposure.

PrP^{TSE} Exposure and Extraction

Wheat plants were removed from growth boxes, roots were rinsed in dH_2O and patted dry with paper towels. Plants with roots were placed in conical tubes containing 4 mL of either CWD positive or negative 1% BH or 1% purified solutions so as to ensure the roots were immersed in the solution for 24 h. Exposures were independently replicated three times (n=3). Roots and stems were separated with a razor blade immediately following exposure and handled independently to avoid cross-contamination with a dedicated blade used for each treatment. Roots and the lower half of stems were then rinsed in dH_2O for 1 min and flash frozen in liquid nitrogen. Frozen tissue was pulverized using a tissue TUBE™ Impactor (Covaris Inc., Woburn, USA). Protein was extracted either using a plant total protein extraction kit (Sigma Aldrich) following manufactures specifications or by incubation of tissue with 4 µL 1% sodium dodecyl sulfate (SDS) / mg tissue for 15 min at room temperature. For both the kit and 1% SDS extraction, the protein concentration was kept consistent for roots and stems by using the same volume of extraction buffer per mg of tissue.

Detection

Protease digestion of protein extracts was done with 10 µg/mL PK at 37°C for 30 min. Samples were heated at 96°C for 5 min in sample buffer (2.2% SDS, 48 µM Tris, 10% glycerol, 1% 2-mercaptoethanol, pH 6.8) prior to loading onto a polyacrylamide gel. Samples were electrophoresed using a 12% NuPAGE® Bis-Tris polyacrylamide gels at 150 V for 60 min in 1X MOPS buffer with NuPAGE® antioxidant (Life Technologies). Gels were transferred (wet) to polyvinyl difluoride membranes (PVDF, 0.45 µm pore, Millipore) at 150V for 60

min in 1X NuPAGE® Transfer buffer (Life Technologies) with 10% methanol. Western blotting was done using P4 mAb (1:5000, R-biopharm, Darmstadt, Germany), Prionics®-Check WESTERN kit (Prionics, Wagistrasse, Switzerland) and were developed with CDP-Star (Roche, Mississauga, Canada).

Samples extracted with 1% SDS were subjected to diagnostic testing with Bio-Rad TeSeE® and IDEXX HerdChek®. Sample analyses using Bio-Rad TeSeE® were done in duplicate, with one replicate following manufacture's specifications and the other without the addition of PK (undigested). Samples were also analysed using Seprion-capture technology with the IDEXX HerdChek® Ag Test and were performed according to manufacturer's specifications.

RESULTS

PrP^{C} Exposures

Wheat plants with roots exposed to recombinant mouse PrP^{C} contained a prion band in the root protein extract as identified with western blotting (Fig. 4-1). The prion band in the root extract had a slightly smaller molecular weight than PrP^{C} used in the exposure solution (Fig. 4-1). When the outer surface of roots was digested with 10 µg/mL proteinase K (PK) for 5 min at room temperature, no prion signal in the root protein extract was observed (Fig. 4-1). No prion signal was seen in stem or leaf protein extracts (data not shown).

PrP^{TSE} Exposures

In wheat plants exposed to purified PrP^{TSE}, the root extract had three prion reactive bands <30 kDa not present in control extracts (Fig. 4-2). Plant proteins cross-reacted with P4 mAb in both the root and stem extracts with prominent bands at ~40 kDa and ~29 kDa (Fig. 4-2). When root extracts were digested with PK (10 µg/mL, 37°C, 30 min) there were no PK-resistant proteins present in either the control or CWD exposed samples (Fig. 4-2). Only PK-sensitive cross reaction bands were present in the lower stem extract (Fig. 4-2).

Wheat plants were also exposed to either infectious or non-infectious elk brain homogenates (BH), which were either digested with PK to leave only PK-resistant PrP^{TSE} protein, or not digested, to represent all brain proteins (Fig. 4-3). The root extract from plants exposed to PK-digested CWD BH had prion reactivity with bands <30 kDa not present in the control extract (Fig. 4-3a). Similarly, the lower stem extract from the same PK-digested CWD BH treatment had three prion reactive bands which were not present in the control lower stem extract when the outside of the stem was not rinsed with dH_2O (Fig. 4-3a). When the outside of the stem was rinsed with dH_2O following exposure, the suspected PrP^{TSE} bands were no longer visible in the lower stem extract, leaving only cross-reaction bands (Fig. 4-3b). There were no definitive bands in the root or stem extracts of the other treatments (normal BH-digested and undigested; and CWD BH-undigested) which differed from the control protein extracts (Fig. 4-3a).

The plant total protein extraction kit (Sigma Aldrich) made PrP^{TSE} sensitive to a mild PK digestion (10 µg/mL, 30min, 37°C) (data not shown). An alternate extraction with 1% sodium dodecyl sulfate (SDS) was used in order to

preserve PK-resistance of any PrP^{TSE} present in plant tissue for the PK-digested CWD BH treatment (Fig. 4-4). With the 1% SDS extraction, no cross-reaction bands were observed in the control root extract (Fig. 4-4). Prion protein in the root extract was resistant to mild PK treatment (10 µg/mL, 30min, 37°C) (Fig. 4-4). There were no PK-resistant proteins in the lower stem and only cross-reaction bands were visible when extracts were not digested (Fig. 4-4).

Protein extracted with 1% SDS from plants exposed to PK-digested CWD BH was analyzed with the diagnostic tests, Bio-Rad TeSeE® and IDEXX HerdChek® (Table 4-1). TeSeE® analysis revealed all root and stem extracts were negative (Table 4-1). When PK digestion was omitted from TeSeE® protocol, the root extract from the PK-digested CWD BH treatment was positive and control root extract was negative (Table 4-1). Stem extracts were negative when PK treatment was omitted from the TeSeE® protocol (Table 4-1). All samples were negative when tested with IDEXX HerdChek® (Table 4-1).

DISCUSSION

This study shows that protease-digested CWD PrP^{TSE} interacts with wheat roots, both when exposed in a brain homogenate (BH) and purified solution. Similarly, when in a purified solution, recombinant PrP^{C} interacts with the outside of wheat roots. There was no detectable PrP^{TSE} or PrP^{C} transported from roots into the stem in the treatments investigated. This suggests the risk associated with plant uptake of PrP^{TSE} may be lower than initially hypothesized.

One interesting observation was that only PK-digested PrP^{TSE} associated with roots, while undigested PrP^{TSE} did not (Fig. 4-3). Proteinase K digestion is known to alter the structure of PrP^{TSE} by removing the N-terminus (Saunders et al. 2008). With this altered structure, PrP^{TSE} may have exhibited an enhanced affinity for wheat roots. When considering the strong interaction between recombinant PrP^{C} and the roots, it is worth noting that this prion protein is also N-terminally truncated when compared to mammalian PrP^{C} (Fig. 4-1). The fact that PrP^{TSE} and PrP^{C} lacking the N-terminus interact with roots is supported by studies showing the N-terminus plays a role in the binding of PrP^{TSE} to soil components (Johnson et al. 2006; Saunders et al. 2009). Alternatively, in an undigested BH it is possible that other brain proteins may coat the roots, blocking any interactions between PrP^{TSE} and the root surface. Degradation of PrP^{TSE} by microbes has been suggested in composting studies indicating partial degradation in the soil environment is possible (Xu et al. 2013). Considering our data, this suggests partially degraded PrP^{TSE} in the soil may be more likely to interact with plant roots.

Initially, extractions were done using a plant total protein extraction kit to maximize sensitivity of protein detection (Fig. 4-2, 3). The high concentration of detergents in the extraction buffer affected the PK-resistance of prions, as other studies have confirmed (Breyer et al. 2012). A 1% SDS solution was used as an alternate extraction procedure to preserve PK-resistance of any PrP^{TSE} based on other studies that extracted PrP^{TSE} from compost samples (Xu et al. 2013) (Fig. 4-4). It should be noted that 1% SDS extracted protein less efficiently than the plant

total protein extraction kit and likely lowered our detection limit in plant tissue as demonstrated by the decreased intensity of cross-reaction bands in the root and stem extract (Fig. 4-4). The identity of cross-reaction bands in the root and stem is difficult to determine given the low similarity of the P4 antibody epitope and the wheat proteome based on BLAST analysis. The closest match for the cross reaction bands at ~40 kDa and ~29 kDa were the Cysteine Proteinase protein (71% coverage, 80% similarity) and R2R3-MYB protein (57% coverage, 100% similarity), respectively.

When extracted with 1% SDS, the PrP^{TSE} signal from the roots was reduced with a mild PK digestion (10 µg/mL, 30 min, 37°C) (Fig. 4-4). The PrP^{TSE} signal from the exposure solution also decreased when in the presence of SDS and digested under the same conditions (Fig. 4-4). There was a larger decrease in PrP^{TSE} signal from digestion when in the root extract as compared to the PrP^{TSE} still in solution (Fig. 4-4). Together, these results suggest PrP^{TSE} was more PK-sensitive when associated with wheat roots than when still in solution (Fig. 4-4). The interaction with roots could have altered PrP^{TSE} conformation making it more PK-sensitive. This hypothesis is supported by the negative reading obtained from the IDEXX HerdChek® test, which identifies PrP^{TSE} based on misfolded conformation by Seprion-capture (Table 4-1). The lack of any positive samples from IDEXX HerdChek® suggests even though there are PK-resistant prions in the roots, the conformation of the prion species is no longer identifiable as CWD PrP^{TSE}. This finding is also supported by Bio-Rad TeSeE® which did not identify any tissue as positive for PrP^{TSE}. Conversely, when Bio-Rad TeSeE®

protocol was altered by removing PK-treatment of samples, roots from the PK-digested CWD BH treatment were positive for prions, while control roots were negative (Table 4-1). The Bio-Rad TeSeE® manufacturer's protocol was altered by removing PK-digestion in order to determine if more PK-sensitive prion species could be identified. Given that in the PK-digested CWD BH treatment the only prion reactive material by P4 mAb was PK-resistant PrP^{TSE}, it confirms these roots contained PrP^{TSE} (Table 4-1). These results suggest the PrP^{TSE} in the root extract underwent significant changes and the infectious properties need to be determined.

The loss of suspected PrP^{TSE} signal in the unwashed stem of plants exposed to PK-digested CWD BH after a simple dH_2O wash was surprising (Fig. 4-3). Although unlikely, this observation may be due to external contamination from the media solution even though care was taken to have only roots submerged in the media. It was unexpected that the other treatments with prion reactive protein in the media (undigested CWD and Normal BH) (n=3) did not result in a prion signal in unwashed stems (Fig. 4-3a). A possible explanation for external contamination occurring only with PK-digested PrP^{TSE} is, similar to findings in the roots, this prion species more readily binds to the plant surface. In the event that the solution came in contact with the stem, this would result in PK-digested PrP^{TSE}, but not other prion species being associated with the stem.

The interaction of protease-digested PrP^{TSE} with wheat roots may have implications for the behaviour of prions in the environment. Previous studies have shown CWD can survive for over two years outside of the host (Miller et al.

2004). Other work has also outlined that CWD is relatively resistant to degradation in a brain homogenate but loses the N-terminus quickly (Saunders et al. 2008). In subsequent work, the role of the N-terminus in soil interactions was investigated (Saunders et al. 2009). In that study, truncated hamster adapted transmissible mink encephalopathy (TME) HY PrP^{TSE} bound more to sand and sandy loam soil, but less to silty clay loam soil as compared to undigested TME HY PrP^{TSE} (Saunders et al. 2009). This finding suggests that in situations of carcass decay or microbial degradation where PrP^{TSE} is lacking the N-terminus, the affinity to soil may be affected. Our work shows PK-digested CWD PrP^{TSE} binds to wheat roots when soil is not present, but the effect of soil particles on this system are unknown. Based on the documented affinity of PrP^{TSE} to different soils and soil minerals, like montmorillonite, it is likely there would be competition between wheat roots and soil for binding of PrP^{TSE} (Johnson et al. 2006; Johnson et al. 2007). We predict N-terminally truncated CWD PrP^{TSE} would have the best possibility of binding to wheat roots when in a silty clay loam soil, since PK-digested PrP^{TSE} has a lower affinity for this soil (Saunders et al. 2009).

The lack of detectable PrP^{TSE} inside the stem of wheat plants suggests there is little transport from the roots to above ground tissues. It is possible trace amounts of PrP^{TSE}, requiring a more sensitive detection method, are transported to the stem. The Prionics®-Check WESTERN kit had a detection limit on the elk PrP^{TSE} source used in this study of approximately 10^2 mg-/mg+ brain tissue (based on di-glycosylated band) similar to other work on BSE with the same kit (data not shown) (Gray et al. 2011). Given the bioassay titration was $10^{7.2}$ i.c.

ID_{50}/g in transgenic mice it shows infectious material could be contained in tissue that was not identified with our western blotting. Cervids grazing on plants with trace PrP^{TSE} could potentially replicate PrP^{TSE} *in vivo* with a sub-clinical infection to further shed PrP^{TSE} in the surrounding environment. This would represent a cycle of prion disease persistence involving plants indicating that further investigation of this topic is needed.

Early studies have suggested transport of lysozyme and ovalbumin proteins to aerial portions of tomato plants occurs and is aided by root damage (Ulrich and McLaren 1965). Recently, uptake of whole bacteria into plants and transport distal from the roots was shown as pathogenic *Salmonella enterica* entered the roots of sweet basil plants and moved internally to the stem and leaves (Gorbatsevich et al. 2013). This phenomenon has been reported in another study where leeks took up *E. coli* O157:H7 and *S. enterica* into the roots and transported the bacteria to the stem (Gurtler et al. 2013). Similar to protein uptake, root damage has also been identified as a factor enhancing the uptake of viruses and bacteria into plants (Bernstein et al. 2007; Urbanucci et al. 2009). In soil, the potential for root damage as a result of grazing is possible and the possibility that root lesions could serve as a port of entry into plants should be considered (van Dam 2009). In our study, after plants were grown in solid agarose media they were removed and then exposed to PrP^{TSE} solution. No major damage in the form of cracked or severed root branches was observed, but damage to root hairs could be visualized as a result of removal from solid media. Previous work done in our lab, has shown that ovalbumin protein enters wheat roots and can be transported

to the stem when root damage was present (Chapter 3). The ports of entry caused by root damage in the wheat plants used for this study may not have been large enough to allow uptake of aggregated PrP^{TSE}. Another explanation for the lack of detectable PrP^{TSE} uptake into roots is that protein properties such as electrostatic charge and hydrophobicity of PrP^{TSE} may inhibit uptake. This may suggest the signal of PrP^{TSE} from root extract was from PrP^{TSE} adhering to the outer surface but there was no internalization into the root system. The adherence of PK-digested PrP^{TSE} to the outside of roots likely involves the disruption of the N-terminus, given the importance this region has on interactions with soil.

Interestingly, uptake of *E. coli* O157:H7 and *S. enterica* bacteria into leeks is increased by the presence of arbuscular mycorrhizal fungi, showing an interaction with soil organisms enhances the uptake of large organic molecules (Gurtler et al. 2013). It is likely both biotic and abiotic factors will affect uptake of PrP^{TSE} into plants. This highlights that although circumstances were optimized for uptake in this study, there may be unexpected factors which could result in uptake of PrP^{TSE}. Further investigation should determine if other plants and environmental factors trigger PrP^{TSE} uptake.

The absence of detectable PrP^{TSE} in the stem of wheat plants by western blotting shows that, at most, trace amounts of PrP^{TSE} are transported within plants to above-ground areas. With little to no PrP^{TSE} inside stems, it suggests that internalization of this infective agent into plants does not serve as a vector for transmission of CWD as a result of grazing. However, it is still possible that the surface of plant leaves or stems could be contaminated with PrP^{TSE} shed from

infected animals and serve as a source of the disease during grazing. The significant interaction between partially digested CWD PrP^{TSE} and wheat roots suggests that PrP^{TSE} may concentrate on roots. These findings indicate that transmission of CWD by horizontal means through animal interaction and contact with bodily fluids is likely the major pathway. Another mechanism could be due to consumption of PrP^{TSE} bound to soil or other materials. In determining future regulations for the food industry it will be important to consider how plants may play a role in prion diseases and this study provides the first investigation of the topic. These findings show that wheat plants may not be a significant risk in food safety for humans or disease transmission in animals, but the involvement of root vegetables such as carrots in transmission is important to consider. The investigation of pasture grasses for uptake of PrP^{TSE} would identify if plants important in the cervid diet have this ability and could explain the transmission of CWD. It is also important to consider how other prion diseases interact with plants given the potential for prion diseases to cross the species barrier. Future work needs to address the infectious properties of PrP^{TSE} on wheat roots and how soil will affect this system.

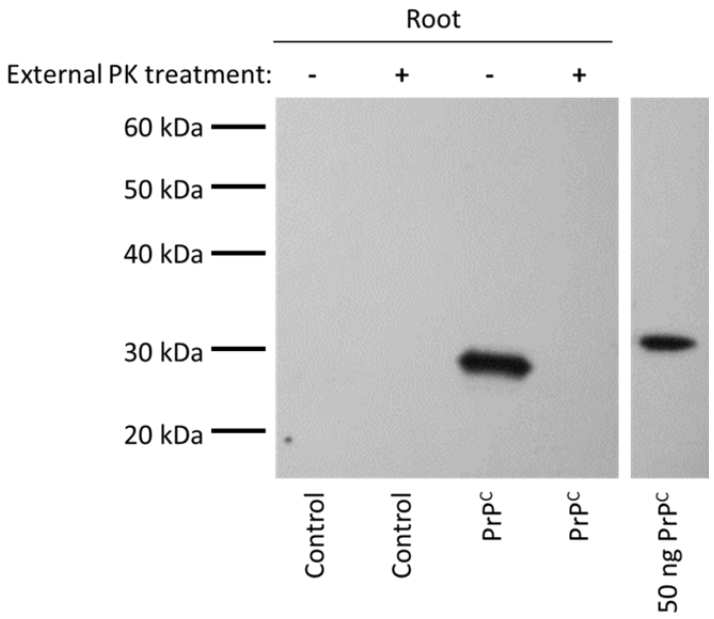

Fig. 4-1. Recombinant PrP^C binds to the outside of wheat roots. Wheat plant roots were exposed to 50 µg/mL PrP^C for 24 h then roots were either digested with (10 µg/mL) proteinase K (PK) for 5 min or left undigested. Control plants were exposed to the same solution lacking PrP^C. Western Blotting on root protein extracts used the 8H4 mAb (1:10000). The last lane confirming antibody specificity is from the same blot with irrelevant lanes omitted. Results are representative of three independent replicates (n=3).

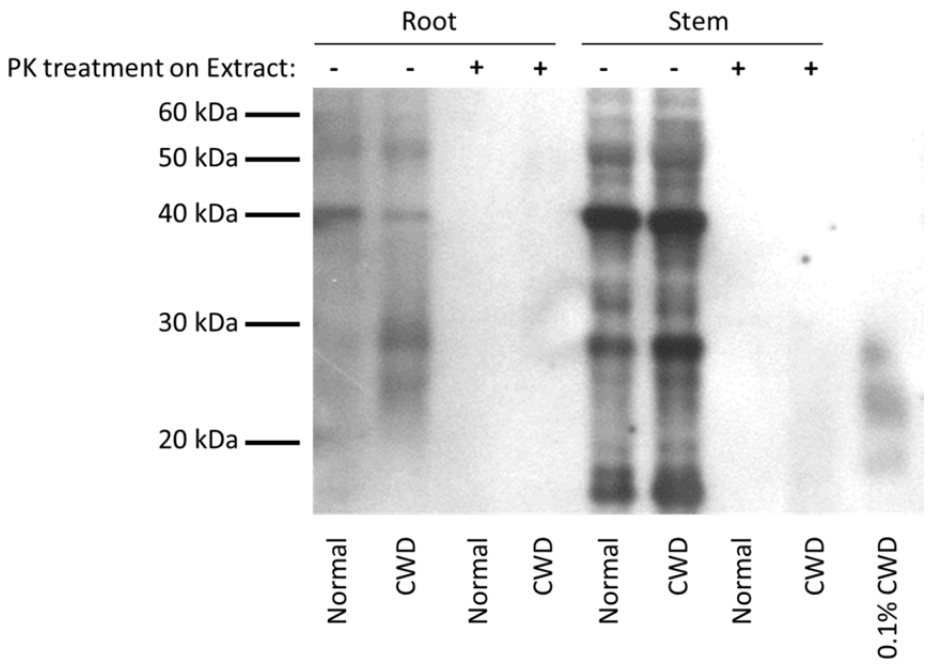

Fig. 4-2. Prion signal found in wheat roots exposed to purified CWD PrP^{TSE} is protease sensitive but no visible prion signal in lower stem (Stem) extract. CWD PrP^{TSE} was purified from brain homogenate (BH) with Bio Rad-TeSeE® purification and re-suspended in phosphate buffered saline to a 1% solution (w/v) based on the initial BH solution. Wheat plant roots were exposed to the purified solution for 24 h. Normal BH processed with TeSeE® served as a negative control. Plant protein extracts were digested with proteinase K (PK) (10 µg/mL, 30 min, 37°C) to determine PK-resistance of any proteins. Western blotting of plant protein extracts (plant total protein extraction kit was done using P4 mAb (1:5000) and Prionics®-Check Western kit. Results are representative of three independent replicates (n=3).

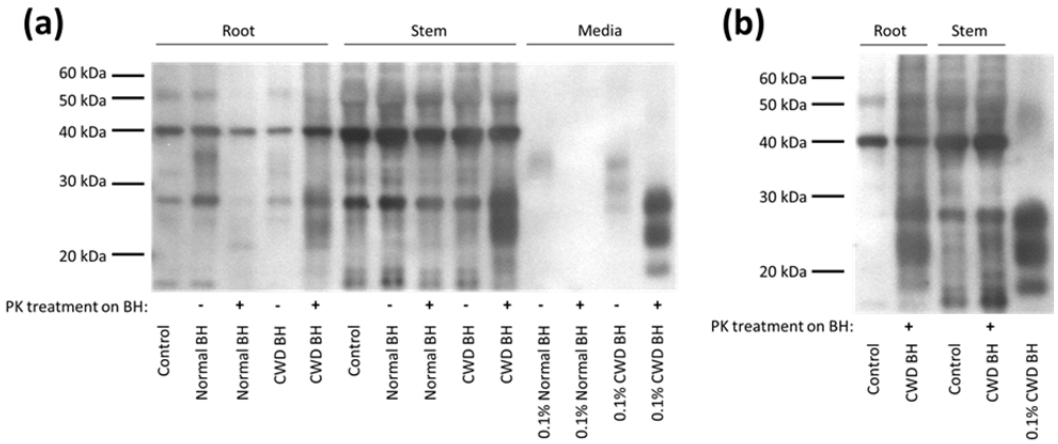

Fig. 4-3. Proteinase K (PK) digested CWD PrP^{TSE} in a brain homogenate (BH) solution interacts with wheat roots but signal from lower stem (Stem) disappears when the stem is rinsed. (a) Wheat plant roots were exposed for 24 h to BH solutions either CWD positive (CWD BH) or CWD negative (Normal BH) and both digested with PK (50 µg/mL, 30 min, 37°C) or not. Control plants were exposed to dH_2O for 24 h. Only the roots were rinsed for 1 min in dH_2O, with protein extracted from both the root and lower stem. Western blotting of plant extracts was done using P4 mAb (1:5000) and Prionics®-Check Western kit. (b) Wheat plant roots were exposed to PK-digested (50 µg/mL, 30 min, 37°C) CWD BH for 24 h then both the roots and stem were rinsed in dH_2O for 1 min. Western blotting of extracted protein used P4 mAb (1:5000) and Prionics®-Check Western kit . Images are from the same blot with non-relevant lanes omitted. Results are representative of three independent replicates (n=3).

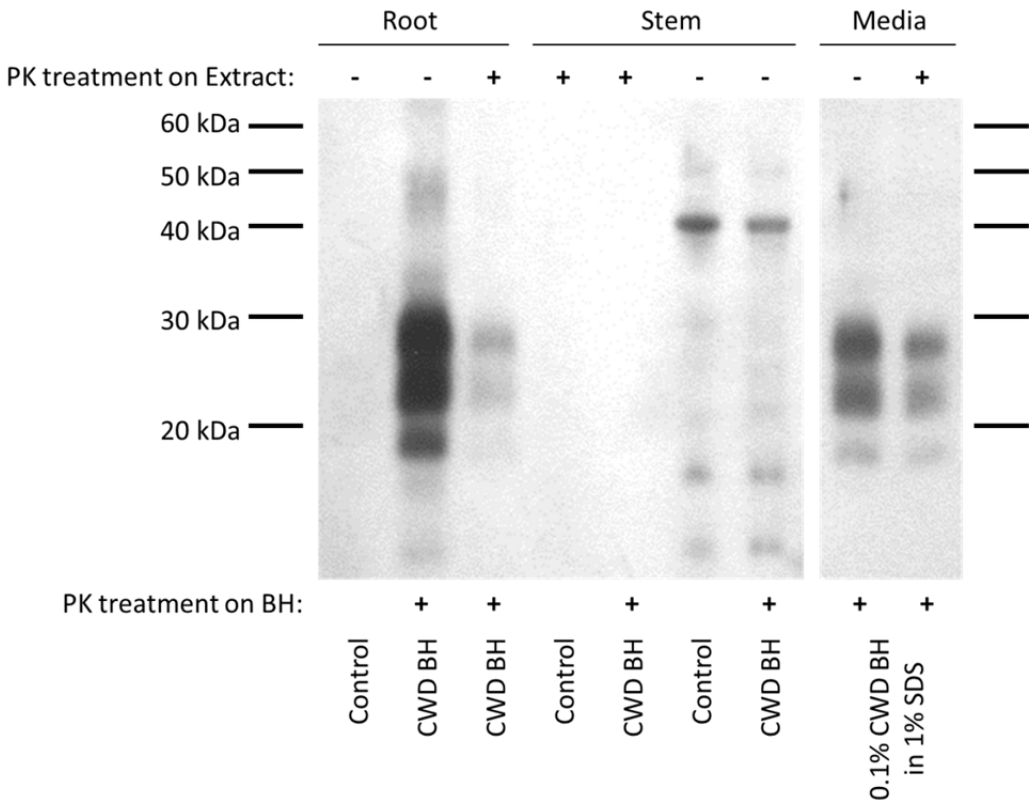

Fig. 4-4. Proteinase K (PK) digested CWD PrP^{TSE} interacts with wheat roots and remains slightly PK resistant after extraction while no CWD PrP^{TSE} was detected in the lower stem (Stem). CWD positive brain homogenates (BH) were digested with PK (50 µg/mL, 30 min, 37°C) prior to being exposed to wheat roots for 24 h. Wheat roots and stems were rinsed with dH_2O for 1 min then protein was extracted with 1% SDS. Extracts were digested with PK (10µg/mL, 30 min, 37°C) to determine any PK-resistant bands prior to blotting. Western blotting on protein extracts was done using P4 mAb (1:5000) and Prionics®-Check Western kit (Prionics). Images are from the same blot with irrelevant lanes omitted. Results are representative of three independent replicates (n=3).

Table 4-1. Analysis of plant protein extracts (1% SDS) from proteinase K-digested (PK) CWD BH treatment with Bio-Rad and IDEXX diagnostic tests.

	Root		Stem	
	Control	PK-digested CWD BH	Control	PK-digested CWD BH
Bio-Rad TeSeE® (no PK)	-	+	-	-
Bio-Rad TeSeE®	-	-	-	-
IDEXX HerdChek®	-	-	-	-

Results are representative of three independent replicates (n=3).

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Chapter 5: General Discussion and Conclusions

SIGNIFICANCE

Agriculture has become a globally integrated industry involving extensive trading networks. There is increasing pressure to meet the food demand of a growing human population, expected to exceed 9 billion by the year 2050 (Food and Agriculture Organization of the United Nations 2009). Shrinking availability of land for food production requires crop and pasture land use to intensify, resulting in crowding of both wild and farmed animals in local environments. The close proximity between both farmed and wild animals promotes the transmission of disease from animals into the human food chain. Prion diseases represent a risk to animal health given the abilities of Scrapie and Chronic Wasting Disease (CWD) to transmit horizontally (Hoinville 1996; Miller et al. 2004). Human health is also in jeopardy, as documented by the zoonotic property of bovine spongiform encephalopathy (BSE) in causing variant Creutzfeldt-Jakob Disease (Bruce et al. 1997). The ability of prion diseases to cross the species barrier and infect new hosts through indirect or horizontal transmission, highlights the risk of having wild and farmed animals in close proximity (Miller et al. 2004). In particular, this is a risk for Chronic Wasting Disease (CWD) given the disease is spreading through wild cervid populations and transmission to farmed animals could occur if the species barrier is broken by an intermediate host (Saunders et al. 2012). Movement of animals across the world to meet food requirements in this intensive production framework represents a health risk given that the range of affected

areas expands rapidly. The spread of CWD has been documented in elk exported from Canada to South Korea. The fact that CWD is not currently under control and is spreading through cervid populations requires a better knowledge of disease transmission and more comprehensive legislation to deal with infected animals to mitigate risk to food products (Saunders et al. 2012). The push to increase agricultural production increases the strain on the industry and can lead to relaxation of safety standards in favour of increasing output. The spread of pathogens through food products requires important consideration and regulations to mitigate disease spread.

Recent work has identified that plants can internalize whole proteins into the root system as a suspected method of nitrogen (N) acquisition (Paungfoo-Lonhienne et al. 2008). Earlier work using radioactive labeling provided evidence that protein entering through the roots could also be detected in leaves (Ulrich and McLaren 1965). In addition, different groups have demonstrated that pathogenic bacteria and viruses also enter plant roots and are transported internally to the leaves (Bernstein et al. 2007; Dicaprio et al. 2012; Esseili et al. 2012; Gorbatsevich et al. 2013). These findings not only broaden our knowledge of plant biology but reveal potential problems this could pose to disease transmission. The proteinaceous nature of the prion disease particle shows that there is the potential for prions to enter plants. This would represent a previously unconsidered vector for transmission of the disease from the environment back to mammalian hosts. The implications this would have on human health and understanding disease transmission of CWD in cervids are substantial.

FINDINGS AND COMPARISONS

This project provided insight into aspects of N nutrition sources for wheat and its interaction with prions. Initial work looked to contribute to the relatively new idea of N nutrition provided by whole protein to plants (Paungfoo-Lonhienne et al. 2008). These experiments investigated the value of protein N to wheat using an axenic solid agarose growth system to compare different iso-nitrogenous concentrations of bovine serum albumin (BSA) and ammonium nitrate. Interpretations were based on plant weights and N content. Using wheat as the model plant in all of our studies we found that BSA was not an efficient N source when compared to inorganic ammonium nitrate at the same N concentration. This research suggested that plants require protein to be broken down into smaller peptides and amino acids outside of the root for efficient utilization. This also suggests wheat competes for the limited pool of free N in the environment with other soil organisms. An unexpected finding was observed in wheat plants exposed to BSA with roots exhibiting a unique morphology characterized by bulbous branches. A similar root morphology has been described previously in wheat with bulbous root branches termed *para*-nodules being induced by synthetic auxin (Francisco and Akao 1993). Auxin is known to play an intricate role in the emergence of root branches (Fukaki and Tasaka 2009). Based on this similarity, it would appear that BSA and synthetic auxin have the same effect on wheat roots and we speculate the mechanism as BSA inducing an auxin signal cascade in wheat roots either directly or indirectly. This could be a response of the

wheat plant to external BSA or possibly by BSA entering wheat roots and directly affecting the auxin signal. Further investigation is needed to discern the exact mechanism of this phenomenon.

Concurrent work served as a proof-of-concept study and used wheat plants grown in agarose or hydroponic culture before exposure to a protein solution to determine uptake of the protein. Wheat roots and stems were analyzed by fluorescence and western blotting to determine location of ovalbumin protein. We identified that ovalbumin is able to enter wheat roots and that minor root damage enables transport to the stem. Root damage was an important consideration in building the model of protein uptake given that root herbivory occurs in the soil environment from nematodes and other organisms (van Dam 2009). The use of protoplast cell assays revealed that ovalbumin is unable to translocate the cell membrane, suggesting that movement of ovalbumin in the whole plant model followed an apoplastic route.

The final study built upon earlier findings with ovalbumin to determine the ability of CWD infectious prions (PrP^{TSE}) to enter wheat roots and translocate to the stem. Wheat plants were grown in agarose culture prior to PrP^{TSE} exposures and contained minor root damage similar to experiments with ovalbumin. Prions in plant tissues were detected using western blotting and diagnostic tests from Bio-Rad and IDEXX. We found that CWD PrP^{TSE} interacted with roots only when it was partially digested with proteinase K, but it was not detected in the stem. There was no interaction when PrP^{TSE} was undigested and only recombinant PrP^{C} missing the N-terminus interacted with roots. It is important to mention the

detection methods used in this study may not have been sensitive enough to detect trace amounts of PrP^{TSE}. This shows that CWD likely is not transmitted through wheat as a vector and demonstrates this is not likely a crucial factor in the dissemination of CWD prions among cervids. Wheat was a suitable initial model given its importance to agriculture and the potential for introduction of prions to the local soil environment through application of compost material and manure to arable land. It will be useful to investigate other plant species in order to determine if they take up PrP^{TSE} as the background knowledge on protein uptake alone in plants is lacking.

The low nutritional value of BSA to wheat plants in our study is interesting given that we found uptake of ovalbumin when exposed in liquid solution. This shows that uptake of protein is likely an inadvertent, passive process that follows the path of water rather than being driven by a process of active N acquisition. The absence of protein translocation into protoplasts suggests that uptake by a plant is not by active transport. It is important to note that BSA and ovalbumin may behave differently in terms of plant uptake given we know proteins do not universally translocate membranes (Rosenbluh et al. 2004). Another factor to consider is that there was no root damage in the nutrition study with BSA compared to the uptake study with ovalbumin. Our work and that of others has identified root damage as a key variable for uptake of large organic molecules (Bernstein et al. 2007; Ulrich and McLaren 1965; Urbanucci et al. 2009). With no root damage there may have been little internalization of BSA.

Based on our work it can be hypothesized that the uptake of protein is unrelated to any attempt by wheat to acquire N in this model.

The lack of significant PrP^{TSE} uptake into the stem of wheat plants demonstrates that in determining prion interaction with plants, other proteins may not be suitable surrogates. The lack of any uptake of PrP^{TSE} into the stem of the wheat plant is different from ovalbumin, but could indicate that molecular weight is a key factor. Previous work showed that movement of ovalbumin monomers (~45 kDa) through the xylem is possible; however, there is a portion of protein retained at the pit membranes (Neumann et al. 2010). Given that PrP^{TSE} naturally forms aggregates larger than an ovalbumin monomer, PrP^{TSE} may not move efficiently through the xylem based on size limitations. This difference between CWD PrP^{TSE} and ovalbumin demonstrates that uptake of PrP^{TSE} cannot be predicted based on results from other proteins.

The model system used in this study was basic as to involve only interactions between wheat plants and PrP^{TSE} given the lack of knowledge about this process. There are numerous other environmental factors that will likely influence interactions between PrP^{TSE} and roots. The well documented affinity of PrP^{TSE} for soil minerals represents a competing binding surface for PrP^{TSE} in the presence of roots that would likely affect the system (Johnson et al. 2006). Other biotic factors such as the presence of bacteria would also be important to investigate. Microbial degradation of PrP^{TSE} outside of the host could alter interactions seen in our model (Xu et al. 2013). Plant roots would also be exposed to PrP^{TSE} for extended time periods in the environment and our model system may

not have allowed sufficient time for uptake to occur. The model used here provided valuable knowledge to a novel process but introduction of other factors will give a better idea of its environmental relevance.

FUTURE DIRECTIONS

The unexpected root morphology in wheat when exposed to BSA similar to previous studies with auxin, needs further investigation. It is well known that interactions between plant roots and bacteria occur, whether in symbiosis or an uptake process (Oldroyd and Downie 2008; Paungfoo-Lonhienne et al. 2010). It would be interesting to determine if a significant interaction between N-fixing microorganisms and the bulbous roots on wheat plants would occur given their unique morphology. Studying this root morphology more in-depth will identify if it has a specific function. It would also be interesting to investigate if these protein-exposed plant roots behave differently, in terms of protein uptake. This speculation is based on a situation where the plant may be adapting to a high protein environment, in order to better acquire protein N from it.

An aspect lacking from our study was the use of animal bioassay to determine the infectious properties of PrP^{TSE} associated with plants. It will be important for future studies to investigate if any plant tissue contains a significant amount of infectious material. The amount of PrP^{TSE} material detected in the root extract from our study, although detectable by western blotting, may not be that significant in infecting a cervid. It needs to be remembered that the amount of infectious material needed to infect a cervid orally is higher than the amounts

identified as containing PrP^{TSE} by transgenic rodent intracranial bioassay. PrP^{TSE} entering the soil environment through carcass decay, shedding or application of mortality composts to arable land will encounter a number of interactions before contacting a plant root. Given these complicating factors, even if trace amounts of CWD PrP^{TSE} reach above ground areas of plants, a cervid may need to consume large quantities to become infected.

This study only used CWD when determining prion uptake into plants; however, the behaviour of other prion proteins is unknown. It will be important to determine how other infectious prion proteins from diseases like BSE and Scrapie will behave when interacting with wheat plants given their respective link to human health and indirect transmission (Bruce et al. 1997; Hoinville 1996). The variable affinity of infectious prion proteins to soil particles for diseases such as CWD and transmissible mink encephalopathy highlight that further investigation is needed to determine the extent of root binding (Saunders et al. 2009). The next studies should also address the influence of soil particles on interactions between PrP^{TSE} and wheat roots. Addition of the soil mineral montmorillonite into a PK-digested CWD brain homogenate (BH) solution in the same exposure system described above, could determine if there is any competition for PrP^{TSE} binding. The apparent importance of PK-digestion to PrP^{TSE} interaction with plants also requires further characterization. To determine if this is due to interference of other brain proteins or the effect that digestion has on PrP^{TSE}, purified PK-digested PrP^{TSE} could be spiked into an undigested normal BH solution for exposure to plants. This would reveal if PK-digested PrP^{TSE} still interacts with

roots in the presence of other brain proteins. As mentioned above, future studies should also utilize more sensitive detection methods such as PMCA or RT-QuIC to determine if trace amounts of PrP^{TSE} can be detected inside plant tissues. The utilization of rodent bioassays to determine if the material is still infectious or if the process of uptake has altered the prion protein rendering it non-infectious would also be an asset. The use of bioassay will be particularly useful in determining if plants are a risk factor for disease spread or potentially a mitigation strategy. Another important factor to consider is the type of plant used as well as the extent of root damage in the system. In particular, tomatoes would be a suitable option given its proven ability to take up bacteria in the absence of root damage and protein when minor root damage is present (Paungfoo-Lonhienne et al. 2010; Ulrich and McLaren 1965). Other plants of interest would include root vegetables, given that binding of PrP^{TSE} to the roots could have direct implications on disease transmission to humans. The findings of this study suggest that plants are not a vector for CWD disease transmission. This would imply that horizontal transmission through bodily fluids and the inadvertent consumption of PrP^{TSE} adhering to soil particles during grazing are more likely routes of spread in wild cervid populations. If this hypothesis is true, the possibility of controlling CWD in wild cervids will be a challenge. The risk the unhindered spread of prion diseases puts on other animals and humans is important to consider.

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Appendix 1

Table A1. Measurements of wheat plantlets under different N treatments

Treatment	Root length (cm)	Root wet mass (mg plant^{-1})	Stem wet mass (mg plant^{-1})	Leaf wet mass (mg plant^{-1})	Root dry mass (mg plant^{-1})	Stem dry mass (mg plant^{-1})	Leaf dry mass (mg plant^{-1})	Leaf nitrogen (%)	Stem nitrogen (%)	Root nitrogen (%)
+	12.1 ± 1.17	233.1 ± 31.95	192.5 ± 19.85	291.5 ± 19.73	17.3 ± 1.68	18.4 ± 5.59	52.2 ± 2.26	7.7 ± 0.12	7.5 ± 0.21	3.3 ± 0.30
-	19.2 ± 4.02	65.4 ± 4.54	49.7 ± 4.30	80.1 ± 4.79	11.6 ± 1.64	3.6 ± 1.33	18.6 ± 1.42	2.1 ± 0.06	1.3 ± 0.06	0.8 ± 0.02
P-L	16.7 ± 2.05	149.4 ± 31.60	59.8 ± 6.05	106.2 ± 2.79	10.9 ± 1.34	4.1 ± 0.95	25.2 ± 1.70	1.9 ± 0.10	1.2 ± 0.06	1.3 ± 0.08
P-M	13.2 ± 1.43	186.4 ± 22.12	59.3 ± 4.01	104.6 ± 4.26	15.7 ± 2.36	5.8 ± 1.04	21.7 ± 1.40	2.1 ± 0.11	1.3 ± 0.06	1.8 ± 0.08
P-H	10.1 ± 0.90	105.4 ± 18.06	60.0 ± 6.20	94.2 ± 13.21	15.5 ± 3.47	8.5 ± 2.15	26.2 ± 1.82	2.4 ± 0.16	1.4 ± 0.10	1.8 ± 0.12
IN-vL	14.9 ± 0.90	148.9 ± 3.49	64.8 ± 1.14	136.2 ± 2.85	18.1 ± 0.55	9.4 ± 0.25	22.8 ± 0.26	2.6 ± 0.09	1.6 ± 0.02	0.9 ± 0.01
IN-L	15.8 ± 0.71	202.0 ± 10.01	85.8 ± 2.02	271.1 ± 6.66	10.7 ± 1.59	4.4 ± 0.88	34.0 ± 1.43	7.1 ± 0.21	6.2 ± 0.23	1.9 ± 0.12
IN-M	13.0 ± 0.53	125.4 ± 7.17	90.0 ± 3.38	240.9 ± 11.45	9.1 ± 0.63	5.3 ± 0.17	32.4 ± 0.75	9.2 ± 0.14	8.4 ± 0.24	4.3 ± 0.19
IN-H	8.6 ± 0.59	52.0 ± 5.39	66.5 ± 5.79	131.0 ± 7.84	4.4 ± 0.53	4.6 ± 0.26	26.8 ± 0.57	12.3 ± 0.13	12.4 ± 0.41	6.3 ± 0.10
C-L	13.1 ± 0.93	229.2 ± 22.44	77.0 ± 3.82	221.6 ± 5.73	14.7 ± 0.60	7.4 ± 1.21	33.5 ± 0.74	4.7 ± 0.14	3.1 ± 0.17	1.4 ± 0.04
C-M	16.1 ± 1.52	123.4 ± 8.89	83.0 ± 3.06	280.4 ± 9.04	6.4 ± 0.40	4.6 ± 0.39	35.1 ± 1.77	8.3 ± 0.11	7.7 ± 0.15	3.3 ± 0.21
C-H	12.5 ± 0.74	92.7 ± 6.67	84.3 ± 3.95	215.1 ± 8.81	8.0 ± 1.36	5.4 ± 0.30	36.2 ± 2.25	10.8 ± 0.21	10.5 ± 0.28	5.3 ± 0.25