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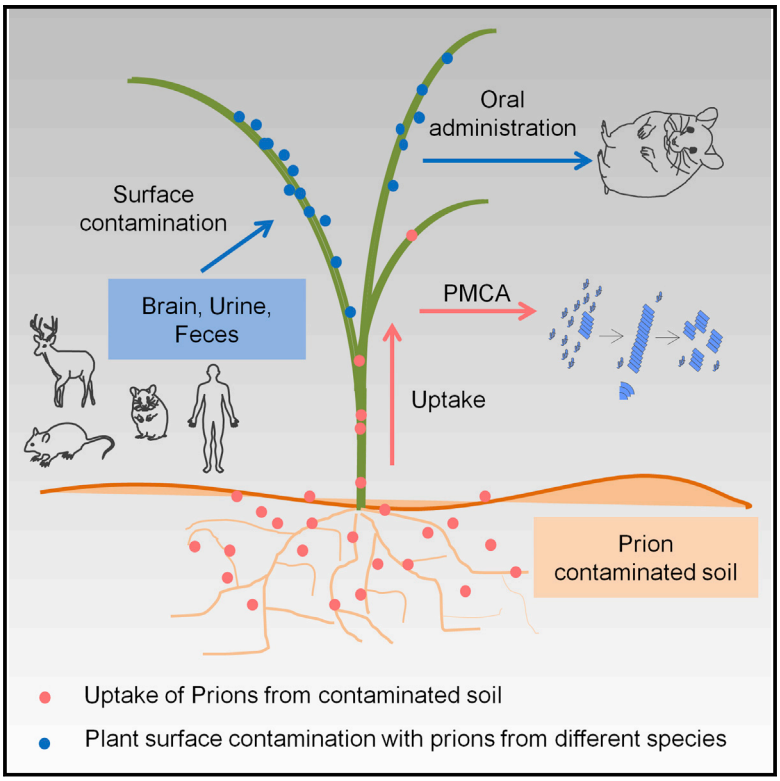
Grass Plants Bind, Retain, Uptake, and Transport Infectious Prions

Graphical Abstract



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In Brief

Prions are the proteinaceous infectious agents responsible for prion diseases. Pritzkow et al. report that prions from brain and excreta can bind grass plants and remain attached to living plants for a long time and that contaminated plants can infect animals. In addition, grass plants can uptake and transport prions from infected soil.

Highlights

- Grass plants bind prions from contaminated brain and excreta
- Prions from different strains and species remain bound to living plants
- Hamsters fed with prion-contaminated plant samples develop prion disease
- Stems and leaves from grass plants grown in infected soil contain prions

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Grass Plants Bind, Retain, Uptake, and Transport Infectious Prions

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SUMMARY

Prions are the protein-based infectious agents responsible for prion diseases. Environmental prion contamination has been implicated in disease transmission. Here, we analyzed the binding and retention of infectious prion protein (PrP^{Sc}) to plants. Small quantities of PrP^{Sc} contained in diluted brain homogenate or in excretory materials (urine and feces) can bind to wheat grass roots and leaves. Wild-type hamsters were efficiently infected by ingestion of prion-contaminated plants. The prion-plant interaction occurs with prions from diverse origins, including chronic wasting disease. Furthermore, leaves contaminated by spraying with a prion-containing preparation retained PrP^{Sc} for several weeks in the living plant. Finally, plants can uptake prions from contaminated soil and transport them to aerial parts of the plant (stem and leaves). These findings demonstrate that plants can efficiently bind infectious prions and act as carriers of infectivity, suggesting a possible role of environmental prion contamination in the horizontal transmission of the disease.

INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of fatal, infectious neurodegenerative disorders that affect humans and other mammals (Collinge, 2001; Prusiner, 2001). The most common animal TSE is scrapie, a disorder of sheep and goats that was first recognized almost 200 years ago and has become an endemic problem. However, the most recent and worrisome animal prion outbreaks are bovine spongiform encephalopathy (BSE) affecting cattle and chronic wasting disease (CWD) affecting cervids (deer, elk, moose). BSE, because of its proven transmission to humans, generating a fatal new disease, termed variant Creutzfeldt-Jakob disease (vCJD) (Collinge, 1999) and CWD, due to its uncontrolled spread among wild and captive cervids in North America and its uncertain transmissibility to humans and/or domestic animals (Miller and Williams, 2004; Sigurdson and Aguzzi, 2007; Gilch et al., 2011). The nature of the infectious agent in TSEs has been the center of passionate controversy (Soto and Castilla, 2004). The most accepted hypothesis proposes that the misfolded form of the prion protein (PrP^{Sc}) is the sole component of the infectious agent that replicates in infected individuals by transforming the normal version of the prion protein (PrP^{C}) into the misfolded isoform (Prusiner, 2001; Soto, 2011).

Prion diseases are transmissible between animal-to-animal, animal-to-human, and human-to-human; however, we still do not understand completely the mechanisms, factors, and biological processes that control the transmission of this unique infectious agent. The transmission of some of the naturally acquired forms of TSEs (such as vCJD, kuru, BSE) has been linked to the consumption of meat or meat-derived products from individuals affected by the disease (Collinge, 2001; Prusiner, 2001). On the other hand, some of the most prevalent and horizontally transmissible animal TSEs, including scrapie and CWD, have implicated environmental contamination with prions as a putative mode of transmission (Mathiason et al., 2009; Gough and Maddison, 2010; Bartelt-Hunt and Bartz, 2013). Various studies have shown that infectious prions can enter the environment through saliva, feces, urine, blood, or placenta from infected animals, as well as by decaying carcasses (Mathiason et al., 2006; Haley et al., 2009, 2011; Tamgüney et al., 2009; Maddison et al., 2010; Terry et al., 2011). It has been shown that infectious prions bind tightly to soil and remain infectious for years in this material, suggesting that environmental contamination of soil may play a role in TSE spreading (Johnson et al., 2006, 2007; Seidel et al., 2007). Since the main natural hosts for animal TSEs (sheep, cattle, and cervids) are herbivores, it is surprising that the interaction between prions and plants and the putative role of these organisms as carriers of prion infectivity have not been studied in detail. The main goal of this study was to evaluate whether plants can bind, retain, uptake, and transport prions in an experimental setting. Overall, our findings show that grass plants efficiently interact with prions, suggesting that they may play an important role in natural prion transmission, particularly in wild animals.

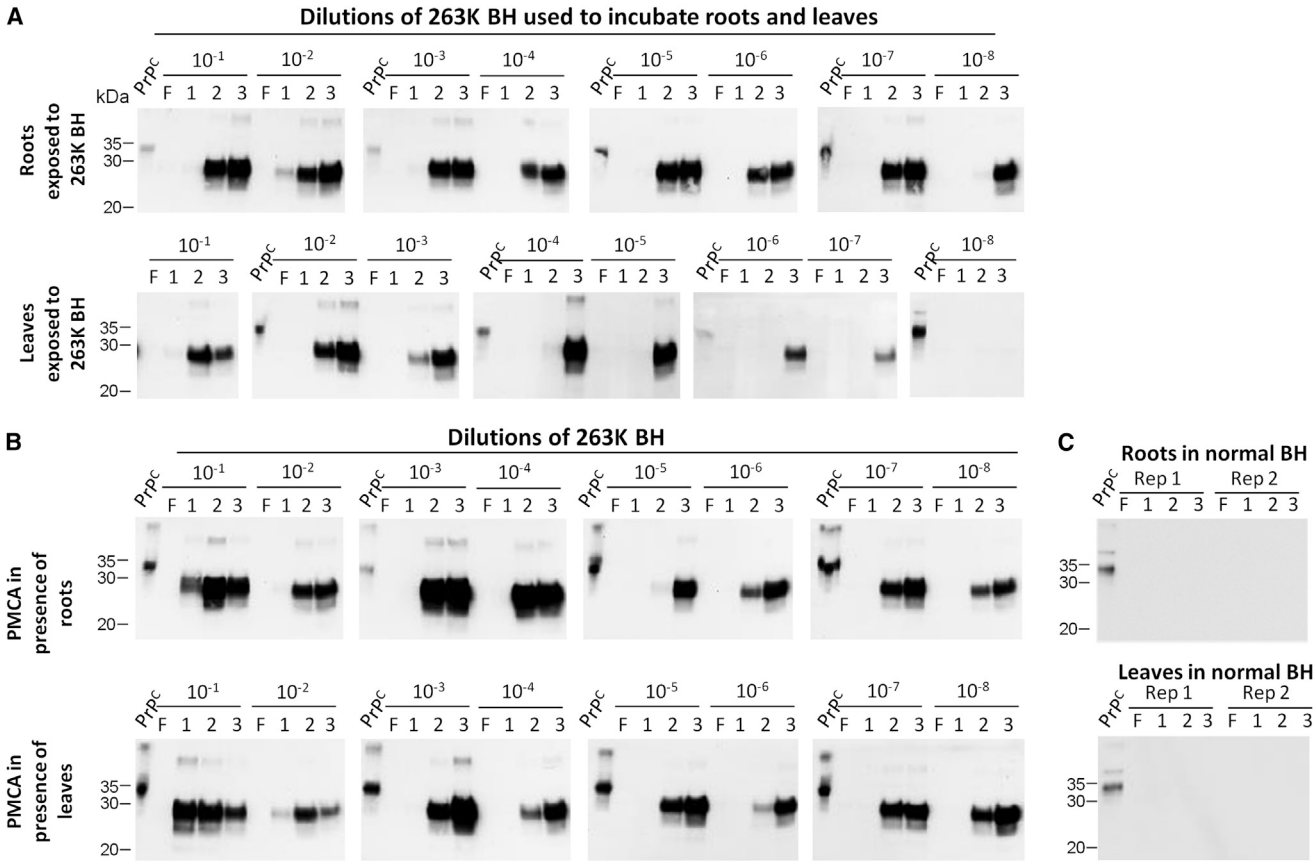

Figure 1. Detection of PrP^{Sc} Bound to Leaves or Roots by PMCA

(A) Serial dilutions of 263K brain homogenate (BH, 10^{-1} to 10^{-8}) done in PBS were incubated with either wheat grass roots (15 mg weight) or leaves (2 cm^2) during 16 hr at room temperature. Thereafter, unbound material was discarded, and leaves and roots were thoroughly washed five times with water and deposited into tubes containing 120 μl of 10% normal hamster brain homogenate. The presence of plant-attached PrP^{Sc} was detected by serial rounds of PMCA, as described in Experimental Procedures. Positive PrP^{Sc} signal was detected by western blot after proteinase K (PK) digestion.

(B) Serial dilutions of 263K brain homogenate (10^{-1} to 10^{-8}) were directly loaded into tubes containing NBH PMCA substrate and wheat grass roots and leaves not previously exposed to PrP^{Sc}. The purpose of this experiment was to study the level of amplification expected for the total amount of PrP^{Sc} contained in each dilution of sick brain homogenate.

(C) To investigate the possible induction of PrP^{Sc} formation by plant material and to rule out cross-contamination, we exposed leaves and roots to 10% normal brain homogenates and subjected the material to several rounds of PMCA as described in (A). The figure shows two replicates of the same experiment (Rep 1 and 2). No PMCA amplification was detected for any of the samples. F, non-amplified control. 1, 2, and 3, number of PMCA rounds performed. Each round consisted of 96 PMCA cycles (2 days). All samples were digested with PK, except the normal brain homogenate (NBH, PrP^{C}) used as a migration control.

RESULTS

Prions Bind to Plants and Bound-PrP^{Sc} Efficiently Sustain Prion Replication

To study whether plants can interact with prions, we exposed wheat grass roots and leaves to brain homogenate from hamsters that have succumbed to prion disease induced by experimental inoculation with the 263K prion strain. The presence of PrP^{Sc} and infectivity attached to the plants was studied in vitro using the protein misfolding cyclic amplification (PMCA) technique and in vivo by infectivity bioassays. For in vitro analyses, the plant tissues (roots and leaves) were incubated for 16 hr with serial dilutions of 263K-brain homogenate ranging from 10^{-1} to 10^{-8}. Roots and leaves were washed thoroughly and analyzed for the presence of PrP^{Sc} by serial PMCA (Morales et al., 2012). The results show that even highly diluted PrP^{Sc} can bind to roots and leaves and sustain PrP^{C} conversion (Figure 1A). Although a direct comparison cannot be made, because of differences on the effective surface, roots appear to retain PrP^{Sc} better than leaves. However, both roots and leaves capture PrP^{Sc} efficiently, even at very small concentrations, equivalent to those present in biological fluids, such as blood and urine (Chen et al., 2010). By comparing the detection of PrP^{Sc}-bound to plants (Figure 1A) with an experiment in which the same dilutions of 263K brain homogenate were added directly to the tubes containing normal brain homogenate and an equivalent piece of leaves or roots (Figure 1B), we can estimate that a high proportion of PrP^{Sc} present in the sample was attached to the plant tissue. Importantly, no detection of PrP^{Sc} was observed when leaves and roots were

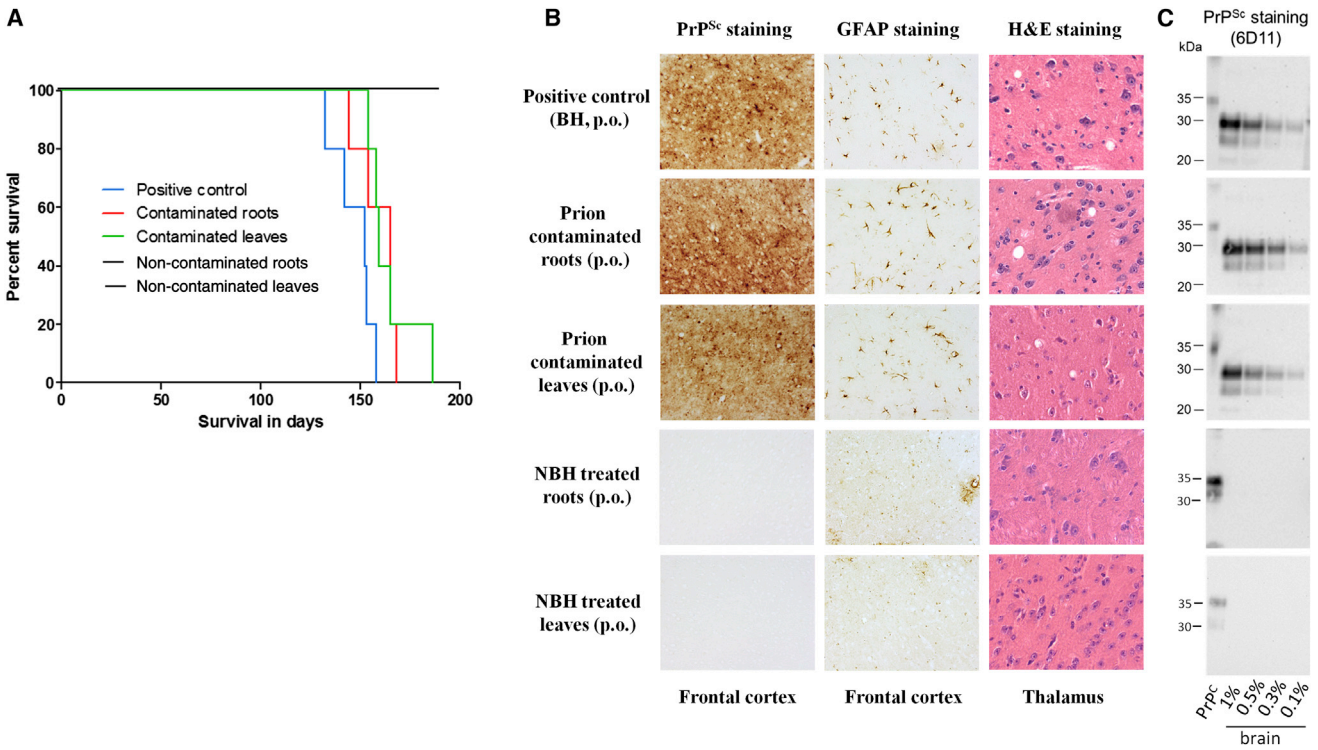

Figure 2. PrP^{Sc} Contaminated Plants Induce Prion Disease by Oral Ingestion

(A) Survival curve of hamsters orally inoculated with leaves or roots exposed to 263K BH. Plant tissue was exposed to prions as described in Figure 1 and in Experimental Procedures. Three units of leaves and roots were used to orally inoculate healthy hamsters. The positive control group consisted on hamsters orally inoculated with 750 μl of 5% 263K BH. Negative control groups were inoculated with leaves and roots incubated with normal brain homogenate. All sick animals exhibited the typical 263K clinical signs, including ataxia, hyperactivity, aggressiveness, and sensitivity to noise, and were sacrificed at the terminal stage of the disease. Hamsters injected with leaves and roots treated with healthy brain homogenates did not show any clinical signs up to 550 days post-inoculation. The differences in the survival curves of animals infected with 263K brain homogenate versus those infected with prion-contaminated leaves or roots were statistically significant ($p = 0.0136$ and 0.047, respectively) as analyzed by the log-rank (Mantel-Cox) test.

(B) Brains from hamsters orally infected with roots and leaves exposed to prions displayed neuropathological alterations typical of prion disease, including characteristic synaptic and diffuse patterns of PK-resistant PrP^{Sc} deposition (antibody 6H4, left panels), astrogliosis (middle panels), and spongiosis (right panels). These alterations were not observed in animals fed with plant tissue exposed to normal brain homogenate. Magnification 20× in all panels.

(C) Biochemical analysis confirmed the presence of PrP^{Sc} accumulation in the brain of all animals showing signs of prion disease. The figure shows a western blot of different brain dilutions from a representative animal per group. All samples were digested with PK, except the normal brain homogenate (PrP^{C}) used as a migration control.

exposed to normal brain homogenate (Figure 1C). However, comparing PMCA amplification in the presence (Figure 1B) or in the absence (Figure S1A) of plant tissue, it is possible to appreciate that plants (both leaves and roots) partially inhibits the PMCA reaction. This explains why in most of the experiments with plants, protease-resistant PrP^{Sc} is only observed after two rounds of PMCA. In our current PMCA settings, no false-positive PrP^{Sc} signals were ever detectable when samples did not contain PrP^{Sc} inoculum (Figure S1B). These results indicate that leaves and roots can efficiently bind PrP^{Sc}, which remains able to catalyze PrP^{C} to PrP^{Sc} conversion, leading to prion replication. In these experiments, plant tissues were incubated with prions for 16 hr, but a similar experiment in which roots and leaves were exposed to a 10^{-5} dilution of 263K brain homogenate for different times, we found that as little as 2 min of incubation was sufficient for the efficient contamination of plants (Figure S2).

Animals Can Be Infected by Oral Administration of Prion-Contaminated Plants

To investigate whether prion-contaminated plants were able to infect animals by ingestion, leaves and roots previously incubated with either 263K-infected or control hamster brain homogenates were orally administered into naive hamsters. After exposure, plants were extensively washed five times with water and animals were fed with dried material. As positive controls, we orally administered 750 μl of 5% 263K brain homogenate (same material used to contaminate plant tissue). All animals that ingested prion contaminated leaves and roots developed typical prion disease. Although the incubation times were significantly longer in animals ingesting prions attached to leaves and roots as compared with those fed directly with the brain material, the differences were not as high as one could have expected (Figure 2A). Indeed, incubation periods were 147 ± 10,

159 ± 10, and 164 ± 13 days (mean ± SEM) for the groups inoculated with brain homogenate, and prion contaminated roots and leaves, respectively. Prion disease was confirmed by histological study of PrP^{Sc} deposition, astrogliosis, and brain vacuolation (Figure 2B), as well as by biochemical detection of protease-resistant PrP^{Sc} by western blot (Figure 2C). None of the animals inoculated with leaves and roots exposed to normal brain homogenate developed disease up to 550 days post-inoculation. Histological analysis did not show any PrP^{Sc} staining or disease specific alteration in control animals.

Plants Bind Prions from Different Strains and Species

To analyze prion-plant interaction with other species and strains of the prion agent, we performed similar studies as described in Figure 1, by incubating leaves and roots with a preparation containing hamster, murine, cervid, and human prions corresponding to the Hyper, 301C, CWD, and vCJD prion strains, respectively. PrP^{Sc} from these strains and species showed good amplification by PMCA, using homologous substrates (Figure S3A). In all cases, leaves and roots bound prions from these species and retained the ability to replicate in vitro (Figure S3B), indicating that the interaction of PrP^{Sc} with plants is a general feature of infectious prions.

Contamination of Plants with Prions Excreted in Urine and Feces

Under natural conditions, it is likely that the main source of prions in the environment comes from secretory and excretory fluids, such as saliva, urine, and feces. We and others have shown that PrP^{Sc} is released in these fluids and excretions in various animal species (Gonzalez-Romero et al., 2008; Haley et al., 2009, 2011; Maddison et al., 2010; Terry et al., 2011; Moda et al., 2014). It has been estimated that the amount of infectious prions spread by excreta during the animals' lifespan could match or even surpass the quantity present in the brain of a symptomatic individual (Tamgüney et al., 2009). To study whether plant tissue can be contaminated by waste products excreted from prion-infected hamsters and deer, leaves and roots were incubated with samples of urine and feces and the presence of PrP^{Sc} analyzed by serial rounds of PMCA. For these experiments, plant tissues were incubated for 1 hr with urine or feces homogenates obtained either from 263K-infected hamsters or CWD-affected cervids. This time was chosen because longer incubation with these biological fluids affected the integrity of the plant tissue. After being thoroughly washed and dried, PrP^{Sc} attached to leaves and roots was detected by PMCA. The results clearly show that PrP^{Sc} was readily detectable after three or four rounds of PMCA in samples of wheat grass leaves and roots exposed to both urine and feces from 263K sick hamsters (Figure 3A) and CWD-affected cervids (Figure 3B). Comparing these results with studies of the direct detection of PrP^{Sc} in urine and feces (Figures 3A and 3B), it seems that the majority of PrP^{Sc} present in these waste products was effectively attached to leaves and roots. No signal was observed in plant tissue exposed to urine or feces coming from non-infected hamsters.

Prions Bind to Living Plants

To investigate a more natural scenario for prion contamination of living plants, we sprayed the leaves of wheat grass with a preparation containing 1% 263K hamster brain homogenate. Plants were let to grow for different times after exposure, and PrP^{Sc} was detected in the leaves by PMCA in duplicates for each time point. The results show that PrP^{Sc} was able to bind to leaves and remained attached to the living plants for at least 49 days after exposure (Figure 4). Considering that PrP^{Sc} signal was detectable normally in the second or third round of PMCA without obvious trend in relation to time, we conclude that the relative amount of PrP^{Sc} present in leaves did not appear to change substantially over time. These data indicate that PrP^{Sc} can be retained in living plants for at least several weeks after a simple contact with prion contaminated materials, and PrP^{Sc} remains competent to drive prion replication.

Plants Uptake Prions from Contaminated Soil

The experiments described above were done by exposure of the surface of leaves and roots with different solutions containing prions. To evaluate whether living plants can uptake PrP^{Sc} from contaminated soil, we grew barley grass plants on soil that was contaminated by addition of 263K brain homogenate. Plants were grown for 1 or 3 weeks under conditions that carefully prevented any direct contact of the aerial part of the plant with the soil. After this time, pieces of stem and leaves were collected and analyzed for the presence of PrP^{Sc} by PMCA. As shown in Figure 5A, all plants grown for 3 weeks in contaminated soil contained PrP^{Sc} in their stem, albeit in small quantities that required four serial rounds of PMCA for detection. One of the four plants analyzed contained a detectable amount of PrP^{Sc} in the leaves (Figure 5B), indicating that prions were uptaken from the soil and transported into the aerial parts of the plants, far from the soil. These results differ from a recent article reporting that infectious prions were not detectable in above the ground tissues of wheat plants exposed to CWD prions (Rasmussen et al., 2014). The lack of detection in this article is most likely due to the low sensitive techniques (western blots or ELISA) employed to analyze the presence of PrP^{Sc}. Indeed, as we reported previously, PMCA has a power of detection, which is several millions times higher than western blots or ELISA (Saá et al., 2006). In order to estimate the amount of PrP^{Sc} present in stem and leaves coming from contaminated soil, we performed a quantitative PMCA study, as previously described (Chen et al., 2010). Unfortunately, by comparing the PMCA amplification in the absence or the presence of plant tissue, it is possible to conclude that stems and leaves substantially interfered with the PMCA procedure, and thus the calculation cannot be very precise (Figure S4). Indeed, after two rounds of PMCA we cannot detect any protease-resistant PrP^{Sc}, but on the third round we observed the maximum amplification (10^{-9}), presumably because at this round the concentration of PMCA inhibitors has been reduced enough to permit good amplification. At this point, we can estimate that the amount of PrP^{Sc} that reaches the stem and leaves from contaminated soil is equivalent to the PrP^{Sc} concentration present in a 10^{-6} to 10^{-9} dilution of sick brain homogenate. Nevertheless, this result is interesting, because it indicates that the amount of prions uptaken from

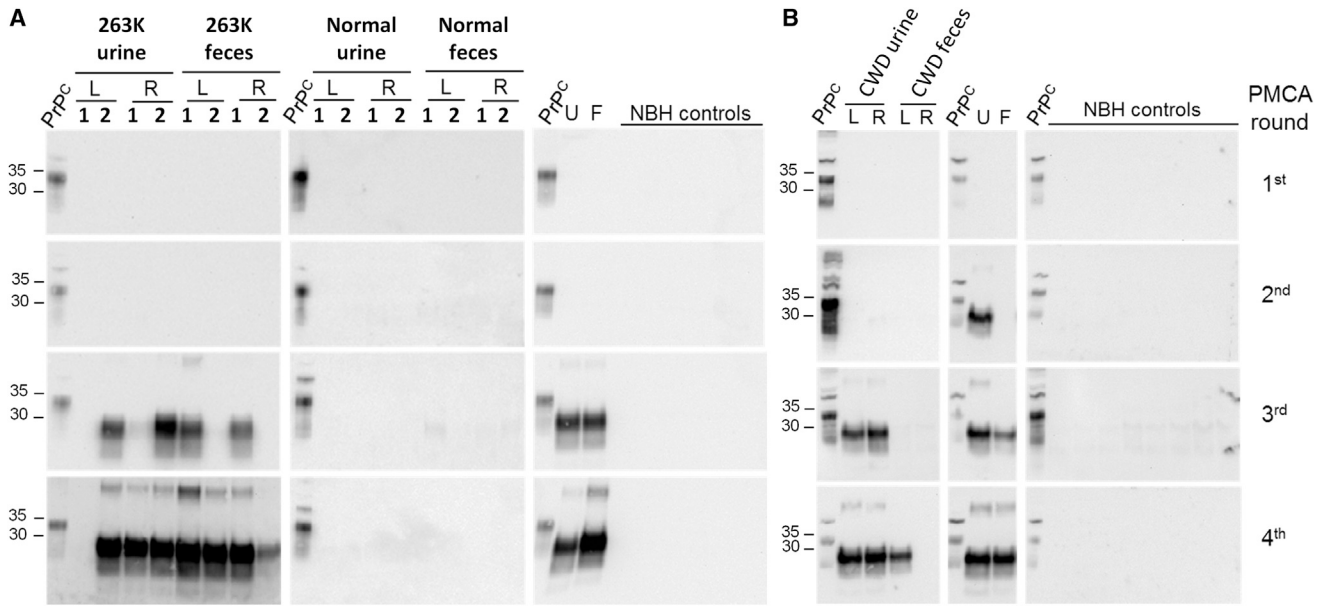

Figure 3. PrP^{Sc} Contained in Urine and Feces of Prion-Infected Animals Binds to Leaves and Roots

(A) Wheat grass roots (R) and leaves (L) were incubated for 1 hr with 1 ml of urine or 1 ml of 20% feces homogenate from sick hamsters experimentally infected with 263K prions. Controls included similar experiments using urine and feces from healthy animals. After exposure, roots and leaves were thoroughly washed five times with water and dried, and the presence of plant-attached PrP^{Sc} was detected by serial rounds of PMCA. The figure shows the results of two replicated experiments (1 and 2). In the right blot of this panel, we show the results of the positive control experiment aiming to directly detect PrP^{Sc} in urine (U) and feces (F) from 263K-infected animals. We also include several negative controls for the PMCA reaction, containing only the normal brain homogenate (NBH) used as substrate, to rule out cross-contamination or de novo formation of PrP^{Sc}.

(B) A similar experiment as described in (A) was done using urine and feces from white-tailed deer clinically affected by CWD. In this case, leaves (L) and roots (R) were incubated in 1:2.5 diluted urine or with 5% feces homogenates. The middle blot shows the positive control experiment in which PrP^{Sc} was detected directly in urine and feces from CWD-affected deer. No PrP^{Sc} signal was detected for various negative controls in which the PMCA reaction was carried out in the absence of infectious samples (right panel). Both (A) and (B) show the results obtained in the first, second, third, and fourth round of PMCA. Each round consisted of 96 PMCA cycles (2 days). All samples were digested with PK, except the normal brain homogenate (PrP^{C}) used as a migration control.

soil and transported to aerial parts of the plant is within the infectious range. Indeed, titration studies showed that the last infectious dilution of a 263K brain homogenate is $\sim 10^{-9}$ (Gregori et al., 2006).

DISCUSSION

This study shows that plants can efficiently bind prions contained in brain extracts from diverse prion infected animals, including CWD-affected cervids. PrP^{Sc} attached to leaves and roots from wheat grass plants remains capable of seeding prion replication in vitro. Surprisingly, the small quantity of PrP^{Sc} naturally excreted in urine and feces from sick hamster or cervids was enough to efficiently contaminate plant tissue. Indeed, our results suggest that the majority of excreted PrP^{Sc} is efficiently captured by plants' leaves and roots. Moreover, leaves can be contaminated by spraying them with a prion-containing extract, and PrP^{Sc} remains detectable in living plants for as long as the study was performed (several weeks). Remarkably, prion contaminated plants transmit prion disease to animals upon ingestion, producing a 100% attack rate and incubation periods not substantially longer than direct oral administration of sick brain homogenates. Finally, an unexpected but exciting result was that plants were able to uptake prions from contaminated soil and transport them to aerial parts of the plant tissue. Although it may seem farfetched that plants can uptake proteins from the soil and transport it to the parts above the ground, there are already published reports of this phenomenon (McLaren et al., 1960; Jensen and McLaren, 1960; Paungfoo-Lonhienne et al., 2008). The high resistance of prions to degradation and their ability to efficiently cross biological barriers may play a role in this process. The mechanism by which plants bind, retain, uptake, and transport prions is unknown. We are currently studying the way in which prions interact with plants using purified, radioactively labeled PrP^{Sc} to determine specificity of the interaction, association constant, reversibility, saturation, movement, etc.

Epidemiological studies have shown numerous instances of scrapie or CWD recurrence upon reintroduction of animals on pastures previously exposed to prion-infected animals. Indeed, reappearance of scrapie has been documented following fallow periods of up to 16 years (Georgsson et al., 2006), and pastures were shown to retain infectious CWD prions for at least 2 years after exposure (Miller et al., 2004). It is likely that the environmentally mediated transmission of prion diseases depends upon the interaction of prions with diverse elements, including soil, water, environmental surfaces, various invertebrate animals, and plants. However, since plants are such an important component of the environment and also a major source of food for many animal

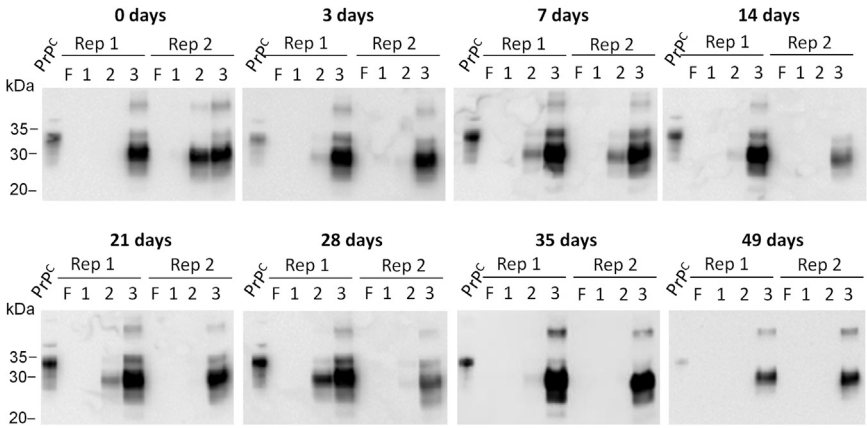

Figure 4. PrP^{Sc} Binds to Living Plants

The leaves of living wheat grass plants were sprayed three times with 10^{-2} diluted 263K brain homogenate. Plants were left to grow for a period of 0, 3, 7, 14, 21, 28, 35, and 49 days. Thereafter, leaves were collected washed five times with water, dried, and used to detect PrP^{Sc} signal by serial rounds of PMCA. The experiment was done in two independent replicates (Rep 1 and 2) for each time point. F, non-amplified control. 1, 2, and 3, number of PMCA rounds performed. Each round consisted of 96 PMCA cycles (2 days). All samples were digested with PK, except the normal brain homogenate (PrP^{C}) used as a migration control.

species, including humans, our results may have far-reaching implications for animal and human health. Currently, the perception of the risk for animal-to-human prion transmission has been mostly limited to consumption or exposure to contaminated meat; our results indicate that plants might also be an important vector of transmission that needs to be considered in risk assessment.

EXPERIMENTAL PROCEDURES

Biological Samples

This study used brain samples from animals and humans infected with various prion strains. Rodents (Syrian golden hamsters and 129S mice) were experimentally infected by intra-peritoneal route with various prion strains (263K and Hyper for hamster and 301C for mouse). The onset of the disease was monitored by the appearance of the clinical signs, using our previously described procedures (Castilla et al., 2008). Animals were sacrificed when they reached a severe stage of the disease, and the brain was collected and stored at −80°C. For deer material, a piece of brain from a white-tailed deer experimentally infected by CWD was used. For human prions, a piece of brain from a patient affected by variant Creutzfeldt-Jakob disease (vCJD) was used. For all these samples, 10% (w/v) brain homogenates (BHs) were prepared in PBS plus complete protease inhibitor cocktail (Roche). When used in protein misfolding cyclic amplification (PMCA), the BH was clarified by a short, low-speed centrifugation at 800 × *g* for 1 min. The BH was stored at −80°C until use.

For our studies, we also used urine and feces from hamsters infected by 263K prions and deer affected by CWD. Urine and feces from terminally sick hamsters was collected using metabolic cages, as described (Gonzalez-Romero et al., 2008). For cervids, urine and feces were collected as previously described from a CWD-affected white-tailed deer (Haley et al., 2009).

All animal experimentation was performed following NIH guidelines and approved by the Animal Welfare Committees of the University of Texas Medical School at Houston and the Colorado State University.

Exposure of Plant Tissue to Infectious Prions

Leaves and roots, grown from organic wheatgrass seeds (*Triticum aestivum*), were used for inoculation experiments. A 2-cm^2 piece (4 cm^2 total surface considering back and front) of wheat grass leave and a 15-mg piece of a pre-washed root were placed in a 2-ml reaction tube and incubated with 300 μl of prion-infected BH at the indicated dilution in PBS by gently rotating for 16 hr at room temperature. Afterward, the plant tissue was washed carefully five times with 1 ml tap water to remove unbound prion protein. A short spin (3 s) was included to remove remaining liquids. The presence of PrP^{Sc} attached to the plant tissue was measured by serial PMCA.

For contamination of plant tissue with prions present in urine and feces, wheat grass leaves and roots were incubated with 1 ml of whole urine (or 1:2.5 diluted urine for CWD samples) or 1 ml of 20% feces homogenate (5% for CWD samples) for 1 hr gently rotating and processed as described for the BH incubation.

For the experiments aimed to determine the survival of prions attached to living plants, we sprayed the leaves of wheat grass plants three times with a 10^{-2} dilution of 263K BH. Pieces of leaves (3.2 cm^2) from living plants were taken after 0, 3, 7, 14, 21, 28, 32, and 49 days post-treatment, washed five times with 1 ml tap water, and analyzed by PMCA.

Growing of Plants in Prion-Contaminated Soil

Barley grass (*Hordeum vulgare*) plants were grown from seeds placed in 350 g of soil until they reached a height of around 12 cm. Subsequently, the surface of the soil was contaminated with 20 ml of 5% 263K or normal brain homogenate taking especial precaution not to contaminate the plant directly. Plants were grown in this soil for 1 or 3 weeks, and samples of stem and leaves were collected. Figure S5 shows a scheme of the region of stem and leaves used for the experiments. The plant tissue was allowed to dry, and 4 cm of the stem or leaves were grinded and analyzed for PrP^{Sc} by PMCA. To prevent cross-contamination, each sample was minced with separate disposable blades in disposable Petri dishes.

Serial Replication of Prions In Vitro by PMCA

10% normal brain homogenates (NBHs) from healthy animals, perfused with PBS plus 5 mM EDTA, were prepared as described before and used as a substrate for PMCA (Morales et al., 2012). NBH prepared from Golden Syrian hamster and 129S mice were used as substrates for prions replication of hamster and mouse PrP^{Sc}, respectively. Transgenic mice overexpressing human PrP with MM at position 129 or transgenic mice overexpressing cervid PrP were used to amplify vCJD and CWD, respectively.

For the positive control reaction, 10% BH from prion-infected animals was serially diluted into NBH and loaded onto 0.2-ml PCR tubes. To determine the presence of PrP^{Sc} in urine and feces, 1 ml of whole urine or 1 ml 20% feces homogenate from 263K-infected hamsters was ultracentrifuged for 1 hr at 45,000 rpm, and, after washing in 1 ml PBS and centrifuging again, the pellet was directly added to the PMCA tube containing NBH substrate.

In order to amplify PrP^{Sc} bound to plant tissue, the contaminated tissue was placed in a reaction tube with 120 μl NBH. NBH alone was used as a negative control. Each PMCA tube, supplemented with three Teflon beads (Hoover Precision Products) was placed in a microsonicator (Qsonica Model Q700) and submitted to PMCA cycles consisting of incubation at 37°C and brief sonication. Hamster and mouse prions were amplified using cycles of 29 min 40-s incubation followed by 20-s sonication at ~260 W. For human and cervid prions, the substrate was supplemented with 0.05% Digitonin and 5 mM EDTA, and the sonication time was increased to 40 s at 260–280 W. After a round of 96 cycles, 10 μl of the amplified sample was transferred into 90 μl NBH, and another PMCA round was performed until detection limit was reached.

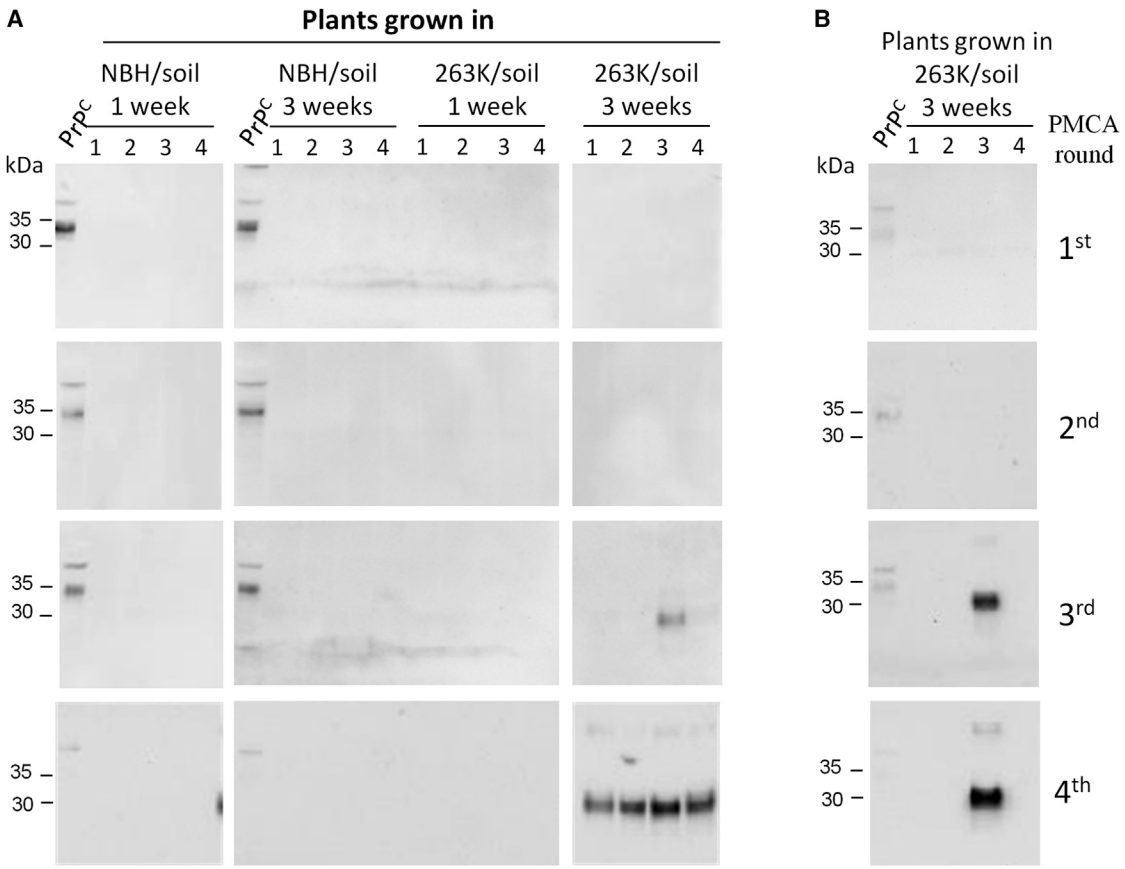

Figure 5. Uptake of Prions by Plants Grown in PrP^{Sc}-Contaminated Soil

The soil of barley grass plants, grown from seeds, was carefully contaminated on day 5 with 20 ml of 5% 263K brain homogenate and as control with the same amount of normal brain homogenate (NBH). One or 3 weeks after infection, plant samples were taken, dried, and minced. The grinded tissue corresponding to either the stem (A) or leaves (B) was analyzed for the presence of PrP^{Sc} by PMCA. Western blots of four different samples (1, 2, 3, or 4) of stems or leaves taken from plants grown for 1 or 3 weeks in 263K BH (or NBH as control) are shown. The results of four consecutive serial rounds of PMCA are depicted. Each round consisted of 96 PMCA cycles (2 days). All samples, except the normal brain homogenate used as a migration control (PrP^{C}), were digested with PK, as indicated in Experimental Procedures.

PK Digestion Assay and Western Blotting

To detect PrP^{Sc}, the samples were incubated in the presence of PK (50 μg/ml) for 1 hr at 37°C with shaking (450 rpm) in a thermomixer. When digesting samples resulting from human and cervid PMCA, 0.2% SDS was added to the PK reaction (100 μg/ml PK). The PK digestion was stopped by adding SDS sample buffer, 33 mM DTT, and boiling the samples for 10 min.

The proteinase resistant PrP was fractionated by SDS-PAGE, electroblotted into Hybond-ECL nitrocellulose membrane (Amersham GE Healthcare), and probed with 6D11 (1:5,000) for hamster, mouse, and cervid PrP^{Sc} or 3F4 (1:10,000) for human samples. The immunoreactive bands were visualized by enhanced chemiluminescence assay ECL Prime Western Blotting Detection system (GE Healthcare) using a Bio-Rad image analysis system.

Bioassay

Groups of five golden Syrian hamsters (females 6–10 weeks old) purchased from Harlan laboratories were orally inoculated with 3 U (3 × 2 cm^2 leaves or 3 × 15 mg roots) of leaves or roots previously exposed to 263K BH as indicated above. Hamsters orally injected with three similar units of leaves or roots treated with 10% NBH were used as control. The onset of clinical disease was measured by scoring the animals twice a week using our previously described scale (Castilla et al., 2008). Stage 1: normal animal; stage 2: mild behavioral abnormalities, including hyperactivity and hypersensitivity to noise; stage 3: moderate behavioral problems, including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness; stage 4: severe behavioral abnormalities, including all of the above plus jerks of the head and body and spontaneous backrolls. Animals scoring level 4 during 2 consecutive weeks were considered sick and were sacrificed. Brains were extracted and disease was confirmed by biochemical and histological analysis. The right cerebral hemisphere was frozen and stored at −70°C for biochemical studies of PrP^{Sc}, and the left hemisphere was used for histology analysis.

Neuropathology

Brains were harvested and left hemisphere fixed in Carnoy fixative (Giaccone et al., 2000), dehydrated, and embedded in paraplast. 10-μm serial sections were stained with H&E or immunostained with monoclonal antibodies to PrP (6H4, 1:1,000; Prionics) and to reactive astrocytes (GFAP, 1:2,000; Abcam). Before PrP immunostaining, the sections were treated with proteinase K (10 μg/ml, 5 min, room temperature) and guanidine isothiocyanate (3 M, 20 min, room temperature). To prevent unspecific bindings, Animal Research Kit (ARK, Dako) was used. Immunoreactions were visualized using 3-3′-diaminobenzidine (DAB, Dako) as chromogen.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.036.

AUTHOR CONTRIBUTIONS

S.P. designed the studies, carried out the majority of the experiments, analyzed the results, and prepared the final version of the figures. R.M. participated in the in vivo infectivity studies and collaborated with the histological analysis. F.M. performed most of the histological studies. U.K. performed the studies of quantitative PMCA. G.C.T. provided colonies of transgenic mice expressing human and cervid PrP. E.H. provided CWD-infected urine, feces, and brains from white-tailed deer. C.S. is the principal investigator on the project and was responsible for coordinating research activity, analyzing the data, funding, writing the manuscript, and producing the final version of the article.

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