The PRT6 N-degron pathway restricts VERNALIZATION 2 to endogenous hypoxic niches to modulate plant development

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Summary

- VERNALIZATION2 (VRN2), an angiosperm-specific subunit of the polycomb repressive complex 2 (PRC2), is an oxygen (O2)-regulated target of the PCO branch of the PRT6 N-degron pathway of ubiquitin-mediated proteolysis. How this post-translational regulation coordinates VRN2 activity remains to be fully established.
- Here we use Arabidopsis thaliana ecotypes, mutants and transgenic lines to determine how control of VRN2 stability contributes to its functions during plant development.
- VRN2 localizes to endogenous hypoxic regions in aerial and root tissues. In the shoot apex, VRN2 differentially modulates flowering time dependent on photoperiod, whilst its presence in lateral root primordia and the root apical meristem negatively regulates root system architecture. Ectopic accumulation of VRN2 does not enhance its effects on flowering, but does potentiate its repressive effects on root growth. In late-flowering vernalization-dependent ecotypes, VRN2 is only active outside meristems when its proteolysis is inhibited in response to cold exposure, as its function requires concomitant cold-triggered increases in other PRC2 subunits and cofactors.
- We conclude that the O2-sensitive N-degron of VRN2 has a dual function, confining VRN2 to meristems and primordia, where it has specific developmental roles, whilst also permitting broad accumulation outside of meristems in response to environmental cues, leading to other functions.

Introduction

Plants need to accurately regulate gene expression to appropriately coordinate their development in response to changing environmental conditions. Such regulation occurs at the direct transcriptional level, through the action of transcription factors, but also epigenetically through a combination of DNA methylation and modification of histone tails in the nucleosomes that make up chromatin. One of the best characterized histone modifying enzymes in eukaryotes is the polycomb repressive complex 2 (PRC2), a multisubunit holoenzyme that catalyses the deposition of the Histone H3 lysine 27 trimethylation (H3K27me3) mark on chromatin (Simon & Kingston, 2009; Margueron & Reinberg, 2011). H3K27me3 promotes compaction of nucleosomes, and therefore acts as a repressor of transcription through switching off gene expression by limiting transcription factor occupancy. The canonical PRC2 comprises four core subunits, and several of these have expanded in number in plants, indicating enhanced flexibility in the composition and activity of individual PRC2s (Hennig & Derkacheva, 2009; Mozgova et al., 2015). PRC2 complexes in Arabidopsis thaliana (hereafter Arabidopsis) are named according to which of three homologues of the Drosophila melanogaster SUPPRESSOR OF ZESTE 12 (Su(z)12) subunit they recruit: FERTILIZATION INDEPENDENT 2 (FIS2), EMBRYONIC FLOWERING 2 (EMF2) and VERNALIZATION 2 (VRN2). Both EMF2 and VRN2 are expressed in sporophytic tissues, where they form PRC2 complexes in association with the methyltransferases CURLY LEAF (CLF) or SWINGER (SWN). CLF and SWN can act interchangeably with EMF2 and VRN2 to regulate overlapping and distinct functions, particularly in the control of shoot development and flowering (Gendall et al., 2001; Yoshida et al., 2001). By contrast, the expression and activity of FIS2 and its cognate methyltransferase MEDEA (MED) are exclusive to the gametophyte, where FIS2-PRC2 prevents fertilization in the absence of pollination (Yadegari et al., 2000). Common to all three PRC2 complexes is FERTILIZATION INDEPENDENT ENDOSPERM (FIE), a homologue of Drosophila EXTRA SEX COMBS, and the only component in Arabidopsis that is not encoded by multiple family members (Ohad et al., 1999).

VRN2 was first identified as a major regulator of the vernalization response in Arabidopsis ecotypes that require prolonged winter to initiate flowering in spring (Chandler et al., 1996; Sheldon et al., 2000; Gendall et al., 2001). During cold exposure, VRN2-PRC2 accumulates, and through associations with other cold-specific accessory proteins – including the PHD protein...
VERNALIZATION INSENSITIVE3 (VIN3) and its homologue VRN5 – it contributes to histone methylation and epigenetic repression of the floral inhibitor gene FLOWERING LOCUS C (FLC) (Sung & Amasino, 2004; Wood et al., 2006; Greb et al., 2007; Costa & Dean, 2019). PRC2-mediated FLC silencing occurs in distinct phases; initial methylation at a nucleation site in the cold requires VRN2-PRC2 in association with SWN, whilst subsequent H3K27me3 spreading throughout the FLC gene body occurs in the warm and is dependent on PRC2-CLF (Yang et al., 2017; Costa & Dean, 2019). Mutations of VRN2 and VRN2-like proteins in Arabidopsis, rice and Medicago truncatula (Medicago) also lead to nonvernalization-associated flowering phenotypes, indicating additional roles for VRN2 in autonomous flowering pathways (Gendall et al., 2001; Yang et al., 2013; Jaudal et al., 2016). Further to its role in vernalization, VRN2 has been implicated in the control of seed dormancy (Auge et al., 2017), seed development (Roszk & Kohler, 2011), vascular patterning and root cell proliferation (de Lucas et al., 2016), somatic cell de-differentiation (Ikeuchi et al., 2015), and hypoxia and submergence tolerance (Gibbs et al., 2018).

Although many functions and targets of plant PRC2s are known, direct mechanisms controlling their activity, composition, and specificity are less well established. Spatiotemporal differences in the expression of SWN, CLF, EMF2 and VRN2 have been demonstrated in specific root cell types, indicating that the patterning of their expression contributes to their diverse functions (de Lucas et al., 2016). However, discrepancies in promoter activity, mRNA abundance, and protein accumulation indicate that PRC2 subunits are also under post-translational control (Wood et al., 2006; de Lucas et al., 2016). We previously identified VRN2 as an oxygen (O2)-regulated target of the PCO branch of the PRT6 N-degron pathway of proteolysis via its conserved Met-Cys-initiating N-terminus, and showed that it positively regulates hypoxia tolerance (Gibbs et al., 2018). VRN2 was coopted to this regulatory pathway in flowering plants, following duplication and N-terminal truncation of an EMF2-like ancestor that contained a latent internal N-degron (Chen et al., 2009; Gibbs et al., 2018). Post-translational control of VRN2 limits its accumulation in the absence of environmental stimuli that inhibit its proteolysis, including low-O2 (hypoxia) and long-term exposure to cold temperatures (Gibbs et al., 2018). How this regulation of VRN2 stability contributes to its known and undescribed functions during development remains to be determined.

Hypoxia in plants occurs frequently as a result of O2 diffusion limitation, rapid consumption rates in tissues with high energy demands, and in response to flooding stress (Bailey-Serres et al., 2012; van Dongen & Licausi, 2015; Considine et al., 2017). In plants, the transcriptional response to hypoxia is coordinated by ERFVII transcription factors, which are O2 and nitric oxide (NO) labile targets of the PRT6 N-degron pathway (Gibbs et al., 2011, 2014a, 2015; Licausi et al., 2011). In O2-replete conditions, ERFVIIIs undergo a series of N-terminal modifications, including methionine excision, cysteine oxidation and N-terminal arginylation, which promotes their degradation by the N-recognin E3 ligase PROTEOLYSIS6 (PRT6) (Gibbs et al., 2014b, 2016; Weits et al., 2014; White et al., 2017). Enhanced stabilization of ERFVIIIs before and during hypoxia is critical for survival in low O2 (Gibbs et al., 2011; Licausi et al., 2011; Schmidt et al., 2018; Dismeyer, 2019; Hartman et al., 2019; Holdsworth et al., 2019; Lin et al., 2019). Furthermore, control of ERFVII stability also regulates responses to other abiotic stresses and pathogen attack (de Marchi et al., 2016; Vicente et al., 2017, 2019).

In addition to flooding-induced O2 deprivation, endogenous hypoxic niches occur naturally in certain plant tissues, where they play a necessary and positive role in regulating development (Borisjuk & Rolletschek, 2009; Kelliber & Walbot, 2012; Meitha et al., 2015, 2018; Considine et al., 2017). For example, a conserved hypoxic niche in the shoot apical meristem (SAM) coordinates leaf development by constraining the accumulation of the locally expressed transcriptional regulator LITTLE ZIPPER 2 (ZPR2), a Cys-initiating N-degron pathway target that controls primordia formation through repressing the activity of HD-ZIP III regulators (Weits et al., 2019). A hypoxic niche is also established in lateral root primordia (LRP), triggering ERFVII stabilization to attenuate auxin signalling by inhibiting LRP developmental genes (Shukla et al., 2019). Furthermore, ERFVIIIs coordinate early seedling establishment during the skoto- to photomorphogenetic transition, sensing O2 availability to regulate apical hook opening and limit the production of harmful Chl precursors before light is perceived (Abbas et al., 2015; Zhang et al., 2018). Thus, in addition to having a general role in coordinating hypoxia stress survival, ERFVIIIs also have separate context- and tissue-specific developmental functions.

Here we investigate how regulation of VRN2 through its O2-sensitive N-degron controls its spatiotemporal accumulation and function during development in Arabidopsis. Under nonstressed and ambient growth conditions, VRN2 protein is largely confined to regions of the plant that are characterized by hypoxic niches, namely the SAM and young leaf primordia (hereafter, the shoot apex) and LRP developmental zones and ambient growth conditions, VRN2 protein is largely confined to regions of the plant that are characterized by hypoxic niches, namely the SAM and young leaf primordia (hereafter, the shoot apex) and LRP developmental zones and by limiting root branching and primary root growth. Moreover, we show that ectopic stabilization of VRN2 through genetic manipulation is insufficient to trigger the vernalization response in the absence of cold exposure, as increases in other PRC2 components and cold-specific factors are also required for appropriate silencing of FLC to induce flowering in response to winter. We conclude that the N-degron of VRN2 is necessary for preventing ectopic accumulation outside of meristems and primordia, where it has specific roles in regulating growth and development. By contrast, under certain environmental conditions, including cold exposure and hypoxia, proteolysis is inhibited and VRN2 accumulates throughout the plant where, along with other context-specific factors, it adopts a different set of developmental functions.
Materials and Methods

Plant growth and materials

Arabidopsis thaliana (L.) Heynh lines were obtained from the Arabidopsis Stock Centre (NASC), except for: Col-0 FRI-Sf2 (Lee et al., 1994), from Dr Jie Song, Imperial College London, UK; pVRN2::VRN2-FLAG and vrn2-1 fca-1 (Wood et al., 2006), from Dr Chris Helliwell, CSIRO, Australia; and pFLC::FLEC-GUS (Sheldon et al., 2008), from Dr Candice Sheldon, CSIRO, Australia. The wild-type (WT) and Cys2Ala pVRN2::VRN2-GUS transgenics in Col-0 and pri6-1, as well as vrn2-5, pri6-1, and pri6-1 vrn2-5 lines were described previously (Gibbs et al., 2018). Mutant combinations were generated by crossing, and full knockouts confirmed by PCR and reverse transcription polymerase chain reaction (RT-PCR; primers in Supporting Information Table S1). Typically, seeds were surfaced-sterilized in 20% Parazone, plated on half-strength Murashige & Skoog (½MS) medium (1% agar, pH 5.7), and stratified at 4°C for a minimum of 2 d, before being transferred to long-day (LD; 16 h : 8 h, light : dark) conditions under white fluorescent light (90–100 μmol m⁻² s⁻¹) at 22°C, and transferred to soil after 2 wk.

Plant phenotypic analyses

For flowering time assessment, seedlings were grown on vertical ½MS plates for 7 d, before being transferred to soil under LD or short-day (SD; 8 h : 16 h, light : dark, 22°C) conditions. Flowering time was determined by counting the number of rosette leaves and day number at bolting. For vernalization experiments, following an initial 7 d growth at 22°C, plates were transferred to SD at 5°C for the appropriate number of weeks. For nonvernalized controls, 1 wk at 5°C was correlated with 1 d growth at 22°C. Following these treatments, seedlings were harvested for protein/RNA extraction, or transferred to soil and grown under LD at 22°C until bolting, at which point rosette leaf number and day number were counted. For root assays, seedlings were grown vertically on ½MS for 10 d at 22°C, photographed, and primary root lengths and lateral root densities (number emerged lateral roots mm⁻¹ primary root) were calculated using ImageJ software (http://rsb.info.nih.gov/ij/). All phenotypic assays were performed at least three times.

Construction of transgenic plants

The pVRN2::VRN2-GUS and pVRN2::Ala2-VRN2-GUS constructs, used here to generate transgenics in the vrn2-2 fca-1 background, were as described previously (Gibbs et al., 2018). To generate the pER8::VIN3 construct, VIN3 was PCR-amplified from 2 wk vernalized Arabidopsis seedling cDNA using attB-flanked primers, recombined into pDONOR211 using Gateway BP clonase (11789020; Invitrogen), then transferred into the destination binary vector pER8GW (Coego et al., 2014) using LR clonase (11791100; Invitrogen). Constructs were transformed into Agrobacterium tumefaciens (strain GV3101 pMP90), then transformed into relevant Arabidopsis lines using established floral dip method. At least 10 independent transgenic plants were selected for each construct; data from two independent T3 homozygous lines are shown.

In vivo and in vitro protein stability analyses

Total protein was extracted from 7-d-old nonvernalized or vernalized seedlings as previously described (Gibbs et al., 2011). To test the effect of hypoxia on in vivo protein stability, 7-d-old seedlings were exposed to 1% hypoxia for 6 or 24 h in a Heracell VIOS 160i incubator (Thermo Scientific, Waltham, MA, USA), and ADH1 expression was used as a marker gene for hypoxia efficacy. For the β-oestradiol induction assays, 7-d-old seedlings were transferred to liquid ½MS in six-well microtitre plates supplemented with 50 μM β-oestradiol (or equivalent volume dimethylsulphoxide control), and incubated at 22°C in the light with gentle shaking for 24 h, before harvesting in liquid nitrogen.

The VRN2-HA in vitro expression construct was as previously described (Gibbs et al., 2018). To generate the VIN3-HA fusion driven by the T7 promoter, VIN3 cDNA was PCR-amplified from 2 wk vernalized Arabidopsis seedling cDNA and directionally cloned into a modified version of the pTNT (Invitrogen) expression vector (pTNTxHA; Gibbs et al., 2011). Cycloheximide-chase assays were then performed using the TNT T7 Coupled Reticulocyte Lysate system (L4610; Promega) using 250 ng of each construct per 25 μl reaction as described previously (Gibbs et al., 2018).

Immunoblotting

Equal total protein amounts were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis before transferring to polyvinylidene difluoride membrane via a MiniTrans-Blot electrophoretic transfer cell (Bio-Rad). Primary antibodies were then used to probe membranes at the following dilutions: anti-HA (H3663; Sigma-Aldrich), 1 : 2000; anti-GUS (G5420; Sigma-Aldrich), 1 : 1000; anti-FIE (AS12 2616; Agrisera, Västerås, Sweden), 1 : 2000; anti-GUS (G5420; Sigma-Aldrich), 1 : 2000; anti-GUS (G5420; Sigma-Aldrich), 1 : 2000; anti-FLAG (F1804; Sigma-Aldrich), 1 : 1000; anti-HA (H3663; Sigma-Aldrich), 1 : 2000; anti-FLAG (F1804; Sigma-Aldrich), 1 : 1000; anti-FIE (AS12 2616; Agrisera, Vännäs, Sweden), 1 : 1000. Horseradish peroxidase-conjugated anti-mouse or rabbit secondary antibodies (sc-358914 and sc-2004; Santa Cruz, Dallas, TX, USA) were used at a titre of 1 : 10 000, before developing to film using ECL Western blotting substrate (Thermo Fisher Scientific).

Reverse transcriptase PCR and qPCR

For semiquantitative RT-PCR, RNA was extracted from seedlings using the RNEasy plant mini kit (74904; Qiagen). cDNA was then synthesized with Superscript II Reverse transcriptase (18064-014; Invitrogen) using the RNEasy plant mini kit (74904; Qiagen). cDNA was then amplified by reverse transcriptase PCR and qPCR with gentle shaking for 24 h, before harvesting in liquid nitrogen.

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on an AriaMx Real-Time PCR system (Agilent, Santa Clara, CA, USA) according to the manufacturer’s instructions. Relative transcript abundance was determined by normalization to ACTIN and relative fold changes calculated. Data shown are means of three biological repeats. Error bars indicate standard deviation. For primer sequences see Table S1.

Meristem measurements

Arabidopsis seedlings (6 d old) were stained with propidium iodide (10 µg ml⁻¹) for 15 min before rinsing in water. Root meristems were visualized using a Nikon A1R Eclipse Ti inverted confocal microscope (Nikon, Tokyo, Japan). To determine meristem size, distance from the quiescent centre to the first elongated cortex cell was measured using ImageJ.

Histochemical staining

To stain for β-glucuronidase (GUS) enzyme activity, 7-d-old transgenic Arabidopsis seedlings were incubated in GUS buffer (phosphate buffer (100 mM), pH 7.0; potassium ferricyanide (2 mM); Triton X-100 (0.1% v/v); 1 mM X-Gluc solution (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt, X-GLUC Direct)). Samples were then incubated for 4 h at 37°C, cleared and fixed in 3:1 ethanol:acetic acid, 200 g chloral hydrate, 20 g glycerol, 50 ml water) before imaging on a light microscope.

Results

VRN2 accumulates at the shoot apex and modulates flowering dependent on photoperiod

We investigated VRN2 protein accumulation in the aerial tissues of WT Col-0 plants using a pVRN2::VRN2-GUS reporter line, which consists of full-length VRN2 fused to GUS, driven by c. 2 kb of endogenous promoter (Gibbs et al., 2018). Despite ubiquitous expression of VRN2 mRNA across tissues (de Lucas et al., 2016; Gibbs et al., 2018), VRN2-GUS protein was only detected in the SAM, leaf primordia, young expanding leaves, and parts of the vasculature (Fig. 1a), which correlates with a previous study that showed enrichment of VRN2-FLAG at the shoot tip (Wood et al., 2006). This localization is remarkably similar to that observed for a pHRPEx5::GFP-GUS hypoxia reporter construct expressed in Col-0 seedlings of a similar age (Weits et al., 2019). By contrast, pVRN2::VRN2-GUS in the pprt6-1 mutant accumulated throughout all tissues of the seedling, resembling pHRPEx5::GFP-GUS expression in lines grown under 5% O₂ (Weits et al., 2019). Quantitative PCR analysis confirmed that VRN2 expression was not significantly increased in pprt6-1 relative to WT (Fig. 1b). We therefore conclude that the N-degron pathway restricts VRN2 to the hypoxic shoot apex in the aerial tissues of Arabidopsis seedlings.

To investigate how control of VRN2 stability by the N-degron pathway regulates its functions in the shoot, we took advantage of a range of genetic mutants in the Col-0 background: vrn2-5 (which lacks full-length VRN2), pprt6-1 (which ectopically accumulates VRN2, as well as other N-degron targets including ERFVIIs and presumably ZPR2), and pprt6-1 vrn2-5 (which accumulates all PRT6 N-degron substrates except for VRN2). We designed VRN2-specific primers upstream and downstream of the vrn2-5 insertion site, and confirmed that the T-DNA completely abolished expression of full-length VRN2 mRNA, although a C-terminally truncated mRNA could still be detected at reduced levels compared with the WT (Figs 1b, 2d). When these lines were grown under LD conditions at 22°C, no obvious effects on phyllotaxis or morphology were observed, except for some small variations in rosette size (Fig. 1c). However, both vrn2-5 and pprt6-1 vrn2-5 flowered earlier than Col-0 and pprt6-1 when leaf number at bolting and time to flowering were assessed (Fig. 1d,e). This indicates a repressing function for VRN2 in flowering, similar to reported roles for VRN2-like proteins in Medicago (Jaudal et al., 2016). Whilst loss of VRN2 reduced time to flowering, pprt6-1 did not exhibit delayed flowering relative to WT, suggesting that ectopic accumulation of VRN2 is insufficient to enhance its effects on this process.

Col-0 is a facultative LD plant, with short photoperiods having a strong repressive effect on reproductive transition. Therefore, we also assessed flowering phenotypes under SD conditions at 22°C. Here, all three mutants had reduced developmental synchronicity relative to WT when leaf number at bolting was scored (i.e. broader variation in leaf numbers) (Fig. 1f). When chronological timing of flowering was determined, both vrn2-5 and pprt6-1 vrn2-5 flowered significantly later than Col-0, with the double mutant displaying an even stronger delay (Fig. 1g). This indicates additive positive roles in flowering for VRN2 and PRT6 under SD conditions, although a more general pleiotropic effect on quiescence cannot be ruled out. For vrn2-5 this is opposite to what was observed under LD conditions, revealing distinct photoperiod-dependent roles for VRN2 in the shoot (Fig. 1d,e). The ERFVIIs have previously been shown to positively regulate flowering (Vicente et al., 2017), which probably explains the pprt6-1 phenotype observed here. Our data reveal that VRN2 has multiple roles in regulating flowering that are separate from its role in vernalization, dependent on photoperiod, and not enhanced when its levels are ectopically increased through genetic manipulation.

VRN2 function in vernalization-dependent lines

VRN2 was initially identified as a positive regulator of the vernalization response in late-flowering Arabidopsis mutants (Chandler et al., 1996). Col-0 is an early-flowering ecotype that does not require vernalization as a result of an inactive allele of FRI (Johnson et al., 2000). ERFVIIs have a positive regulator of FLC expression. To investigate how post-translational control of VRN2 stability contributes to its functions in the vernalization response, we crossed the double pprt6-1 vrn2-5 mutant to the late-flowering Col-0 FRI-Sf2 introgression line (hereafter FRI-Sf2), which is almost identical to Col-0 except that it contains a dominant
FRIGIDA allele derived from the Sf2 ecotype (Johanson et al., 2000). Single and double mutants in the FRI-Sf2 background were then identified in the F2 generation (Fig. S1). We confirmed increased levels of FLC expression in FRI-Sf2 relative to Col-0 using qPCR, and observed a concomitant delay in flowering under LD conditions (c. 40 leaves vs 14 in Col-0) (Fig. 2a,b). Both vrn2-5 FRI-Sf2 and prt6-1 FRI-Sf2 had slightly higher levels of FLC expression relative to WT FRI-Sf2, with the double
mutant having the greatest increase (Fig. 2a). This corroborates previous work showing elevated FLC expression in late-flowering mutants carrying the vrn-2-1 mutation (Sheldon et al., 2000), and indicated that single vrn-2-5 and double prt6-1 vrn-2-5 mutants in the FRI-Sf2 background would flower later than the WT, which we observed when we grew them under LD conditions (Fig. 2b). Remarkably, the prt6-1 vrn-2-5 FRI-Sf2 line flowered extremely late relative to FRI-Sf2 (>100 leaves vs. c. 40), suggesting a similar additive role for VRN2 and PRT6 in promoting flowering to that observed in the Col-0 ecotype under SD conditions (Fig. 1d,e).

Increased FLC expression and delayed flowering in prt6-1 FRI-Sf2 indicate that ectopic accumulation of VRN2 in the absence of cold exposure is insufficient to repress FLC and abolish a requirement for vernalization. Further supporting this, activity of pFLC::FLC-GUS was not reduced in prt6-1 relative to a vernalization-dependent C24 parental line (Fig. 2c).

We exposed all FRI-Sf2 lines to 4 wk vernalization (4V) treatment at the seedling stage (8 h:16 h, light:dark, 5°C), before returning to LD conditions at 22°C and assessing leaf number at bolting. Remarkably, all four lines flowered significantly earlier in response to this treatment (Fig. 2b). This was particularly striking for the prt6-1 vrn-2-5 FRI-Sf2 mutant (from >100 leaves to <30). Thus, the repressive effects induced by loss of VRN2 and PRT6 activity can be overridden by exposure to low temperatures. This was unexpected for vrn-2-5, as VRN2 is required for vernalization, and the previously isolated vrn-2-1 mutant allele is insensitive to cold exposure (Sheldon et al., 2000; Gendall et al., 2001). The vrn-2-1 mutant was isolated from an EMS screen for plants that do not respond to vernalization (Chandler et al., 1996), and contains a codon substitution that leads to premature truncation of VRN2 at amino acid 322 (Gendall et al., 2001). By contrast, vrn-2-5 has a T-DNA insertion that is predicted to disrupt the gene downstream of the vrn-2-1 mutation site, at a residue encoding amino acid 331, and qPCR analysis confirmed that a truncated VRN2 mRNA is expressed (Fig. 1b). Both mutations occur in the VEFS-box domain of VRN2, a critical region of the protein that is conserved in Su(z)12 homologues and required for facilitating binding and catalytic function of PRC2 (Fig. 2d) (Cao & Zhang, 2004; Ketel et al., 2005). Given that vrn-2-5 has clear developmental defects related to flowering and root growth (see later), our data suggest that the vrn-2-5 allele disrupts some VRN2 functions, but, in contrast to vrn-2-1, does not abolish vernalization capacity.

Ectopic stabilization of VRN2 does not abolish the requirement for vernalization

We next investigated how regulation of VRN2 stability influences vernalization using the vrn-2-1 allele, which is in the late-flowering fca-1 mutant in Landsberg erecta (Ler) (Chandler et al., 1996). FCA is a component of the autonomous flowering pathway that regulates RNA-mediated chromatin silencing (Baurle et al., 2007); the fca-1 mutant has high levels of FLC expression, leading to a late-flowering phenotype that can be overcome by vernalization. Whilst vrn-2-1 fca-1 is in the Ler ecotype, prt6-1 is in Col-0. Owing to a lack of prt6 mutants in Ler, and to avoid mixing ecotypes, we instead ectopically stabilized VRN2 by introducing WT (Cys2) or mutant (Cys2Ala) variants of pVRN2::VRN2-GUS into vrn-2-1 fca-1. Western blotting and histochimical staining confirmed that the Cys2Ala mutation in VRN2 is sufficient to enhance its abundance and expand its domain of accumulation throughout the seedling, similar to prt6-1, whilst WT VRN2 showed characteristic localization to the hypoxic shoot apex (Fig. 3a,b; two independent lines for each transgene).
We investigated flowering phenotypes in these transgenic lines relative to the untransformed vrn2-1 fca-1 parent line. WT pVRN2::VRN2-GUS and mutant pVRN2::Ala2-VRN2-GUS plants all flowered late under LD conditions, similar to the vrn2-1 fca-1, with all lines showing low synchrony in leaf number at bolting (Fig. 3c). We exposed these lines to increasing lengths of time at 5°C (2, 3 and 4 wk). As expected, the vrn2-1 fca-1 mutant was insensitive to vernalization treatment. However, all four transgenics flowered earlier in a dose-dependent manner, signifying that the WT and mutant pVRN2::VRN2-GUS constructs can functionally compensate for the vrn2-1 mutation. Extended exposure to cold also led to greater synchronicity of flowering for all lines tested. The mutant pVRN2::Ala2-VRN2-GUS plants had a slightly enhanced response to shorter cold exposure times, which was most pronounced following 3 wk of vernalization. However, by 4 wk all transgenic lines flowered at a similar time. We therefore conclude that ectopic stabilization of VRN2 by mutating its N-degron is insufficient to significantly enhance its function during vernalization relative to WT.

**VRN2 stability in relation to other PRC2 components and the VIN3 co-factor**

Our data reveal that VRN2 accumulation in prt6-1 or through N-terminal mutagenesis does not lead to increased VRN2 function with regard to photoperiod- or vernalization-associated flowering. The former could be explained by the fact that VRN2 is already stabilized in regions of the plant (i.e. hypoxic niches) where this function is established, with enhanced abundance outside these domains having no further influence. The latter is probably a result of a lack of other cold-specific factors that are required for efficient silencing of FLC (Costa & Dean, 2019). Nonetheless, these findings prompted us to investigate the relationship between VRN2 stability and the abundance of other core PRC2 components and accessory proteins during cold exposure, hypoxia, and in the prt6-1 mutant.

The Arabidopsis PRC2 complex consists of four core subunits; three of these are encoded by multiple family members, but one of these (FIE) is not (Fig. 4a). We therefore used FIE protein as a proxy for relative PRC2 abundance in relation to VRN2 accumulation. Western blot analysis showed that whilst VRN2 accumulated to high levels in prt6-1 relative to WT, the amount of total FIE protein was unaltered (Fig. 4b). During cold exposure, however, FIE abundance increased in a dose-dependent manner, and depleted again upon return to warm temperatures, similar to VRN2 (Fig. 4c). It was previously also shown that CLF and SWN methyltransferases accumulate during cold exposure (Wood et al., 2006). We also saw similar increases in both VRN2-GUS and FIE abundance in response to hypoxia (Fig. 4d). This suggests that genetic enhancement of VRN2 levels (i.e. in prt6-1) might be insufficient to stimulate enhanced VRN2-PRC2 activity because of a lack of a concomitant increase of other complex subunits or interaction partners, which only occurs when VRN2 accumulates in environmental contexts (i.e. cold exposure or hypoxia).

We also examined the dynamics of the VRN2-PRC2 cofactor VIN3. VIN3 is a key player in the vernalization response (Sung & Amasino, 2004), which is transcriptionally induced by cold temperatures and binds specifically to VRN2-PRC2 (Sung & Amasino, 2004), which is transcriptionally induced by cold temperatures and binds specifically to VRN2-PRC2 (Sung & Amasino, 2004). We therefore used FIE protein as a proxy for relative PRC2 abundance in relation to VRN2 accumu-lation. Western blot analysis showed that whilst VRN2 accumu-lated to high levels in prt6-1 relative to WT, the amount of total FIE protein was unaltered (Fig. 4b). During cold exposure, however, FIE abundance increased in a dose-dependent manner, and depleted again upon return to warm temperatures, similar to VRN2 (Fig. 4c). It was previously also shown that CLF and SWN methyltransferases accumulate during cold exposure (Wood et al., 2006). We also saw similar increases in both VRN2-GUS and FIE abundance in response to hypoxia (Fig. 4d). This suggests that genetic enhancement of VRN2 levels (i.e. in prt6-1) might be insufficient to stimulate enhanced VRN2-PRC2 activity because of a lack of a concomitant increase of other complex subunits or interaction partners, which only occurs when VRN2 accumulates in environmental contexts (i.e. cold exposure or hypoxia).

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published hypoxia microarray datasets (Gibbs et al., 2011), indicating that transcriptional control of *VIN3* is not linked to the canonical mechanism for hypoxia-responsive gene induction through ERFVIIIs. As such, a mechanistic connection between VRN2 and VIN3 under hypoxia is still unclear.

It had previously been proposed that cold-triggered induction of *VIN3* (Fig. 4e) might enhance the abundance of PRC2 subunits during vernalization through binding and stabilizing the complex (Wood et al., 2006). We tested the possibility that VIN3 promotes VRN2 stability (e.g. through steric shielding of
the VRN2 N-degron) by coexpressing VIN3-HA and VRN2-HA in a cell free rabbit reticulocyte system that contains a functional Arg N-degron pathway (Gibbs et al., 2011), and monitoring protein abundance over time following treatment with the translational inhibitor cycloheximide (CHX) (Fig. 4b). Here, VRN2-HA was unstable even in the presence of VIN3-HA. By contrast, when VRN2-HA was co- incubated with the proteasome inhibitor bortezomib without VIN3-HA, its turnover was inhibited. Thus, VIN3 alone is not sufficient to stabilize VRN2. We also introduced β-oestradiol-inducible VIN3 constructs into the previously described pVRN2::VRN2-FLAG Arabidopsis line, to test if ectopic induction of VIN3 in planta affects VRN2 abundance. However, despite clear induction of VIN3 expression in the presence of β-oestradiol, no obvious increase in VRN2-FLAG stability was observed (Fig. 4i). Together, these data suggest that whilst VIN3 is required for vernalization through its association with VRN2-PRC2, it does not promote increased stability of VRN2.

The PRT6 N-degron pathway confines VRN2 to discrete root tissues and negatively regulates root growth

In addition to assessing the spatiotemporal pattern of VRN2 localization in aerial tissues, we investigated VRN2 abundance in the root system of seedlings. WT VRN2-GUS was detected in the primary root (PR) meristem zone, in LRP s and emerged lateral roots (LRs), and parts of the vasculature (Fig. 5a). By contrast, mutant Ala2-VRN2-GUS, and WT VRN2-GUS in prt6-1, had expanded domains of accumulation, localizing throughout the root (Fig. 5a). Quantitative RT-PCR analysis confirmed that VRN2 expression levels are not enhanced in WT root tips relative to the main root, or in prt6-1 root tips compared with the WT (Fig. 5b). Thus, similar to aerial tissues, the N-degron pathway post-translationally restricts VRN2 protein to discrete regions of the root.

Mid-to-late stage LRP s have recently been shown to be hypoxic (Shukla et al., 2019). Interestingly, in contrast to the SAM, the root meristem of young, establishing seedlings may not be hypoxic, as anaerobic gene expression is not enriched in this region and the ERFVII RAP2.12 does not accumulate there (Hartman et al., 2019; Weits et al., 2019). However, in the PR tip of older seedlings, ERFVII s are stable (Holdsworth et al., 2019). Thus, as VRN2 mRNAs are not enriched in this region relative to other parts of the root (Fig. 5b), the accumulation of VRN2 protein may be a result of other factors blocking VRN2 proteolysis, differential sensitivities to O2 availability in this tissue, or age-dependent variability in N-degron pathway activity (Giuntoli et al., 2017).

Given the localization of VRN2 to LRP s and the PR meristem, we investigated root architecture in vrn2-5, prt6-1 and prt6-1 vrn2-5 relative to Col-0. When grown on vertical agar plates, seedlings of the vrn2-5 mutant had significantly longer PRs than those of the WT, whilst prt6-1 mutant roots were shorter (Fig. 5c,d). The prt6-1 vrn2-5 mutant had PRs of a similar length to the WT, indicating that stable VRN2 contributes to the reduced root length phenotype of prt6-1. We also observed enhanced meristem size in vrn2-5 relative to the WT, which correlates with the increased PR lengths observed in this line (Fig. 5f,g). A similar pattern across the mutants was observed when emerged LR densities were scored: vrn2-5 had increased LR density, prt6-1 reduced density, and the double mutant had an intermediate phenotype (Fig. 5e). prt6-1 was recently shown to have reduced LR density as a result of an accumulation of ERFVII s, which repress LR production (Shukla et al., 2019). Our data suggest that repression of LRs in prt6-1 is controlled by stable VRN2 as well as ERFVII s.

To further investigate the role of VRN2 in regulating root system architecture, we also examined root growth in the vrn2-1 fca-1 mutant, as well as vrn2-1 fca-1 complemented with WT pVRN2::VRN2-GUS or mutant stable pVRN2::Ala2-VRN2-GUS (Fig. 3a,b). Here, both transgenes led to a reduction in PR length and emerged LR density relative to vrn2-1 fca-1, but this was most pronounced in the pVRN2::Ala2-VRN2-GUS line (Fig. 6a–c). This therefore corroborates our findings in Col-0, identifying VRN2 as a negative regulator of root growth, and indicates that, in contrast to flowering, ectopic stabilization of VRN2 is sufficient to enhance its function in roots.

Discussion

Here we investigated how control of VRN2 by the PCO-dependent branch of the PRT6 N-degron pathway relates to its localization and functions in plant development. Our data indicate that post-translational control of VRN2 plays a key role in restricting its accumulation to specific regions of the shoot and root that are hypoxic, where it contributes to the regulation of flowering time and repression of root growth. These roles are separate from its function in vernalization, which is potentiated in response to cold temperatures that inhibit proteolysis to enhance VRN2 abundance throughout the plant. Thus, our findings suggest that the N-degron of VRN2 has a distinct role in limiting VRN2 abundance to discrete tissues, whilst also permitting accumulation in response to environmental inputs where it carries out a different set of context-specific functions.

In the early-flowering Col-0 ecotype, VRN2 has opposing roles in modulating flowering, dependent on photoperiod: under LD conditions VRN2 is repressive, whilst under SD conditions it has a positive function (Fig. 1). In late-flowering FRI-Sf2, the positive function of VRN2 also manifests under LD conditions (Fig. 2). We found that ectopic stabilization of VRN2 did not enhance the photoperiod-dependent functions of VRN2 (Fig. 3). This is probably because such functions are linked to VRN2 activity in the shoot apex, where it is already stable as a result of maintenance of a hypoxic niche in this region. How VRN2 differentially influences flowering remains to be determined. Photoperiod-dependent flowering is regulated by a complex network of floral regulators, which includes components of the circadian clock and light receptors, which converge on the zinc finger transcription factor CONSTANS that, in turn, modulates levels of the Florigen gene FLOWERING LOCUS T (Song et al., 2015). VRN2 may contribute to the epigenetic regulation of any or several components in this pathway, or alternatively it could have
pleiotropic effects on this developmental process related to growth and quiescence. Future analysis of genome-wide methylation targets of VRN2-PRC2 may shed light on this.

VRN2 also had a restricted pattern of accumulation in root tissues, where the PCO branch of the PRT6 N-degron pathway limits its abundance to the root meristem zone and LRPs to repress root development (Figs 5, 6). Plants with mutations in VRN2 (vrn2-5 and vrn2-1) had increased PR lengths and emerged LR densities, whilst the prt6-1 mutant and plants expressing stable Ala2-VRN2-GUS had opposite root phenotypes (i.e. shorter PRs and reduced LR density). This effect was partially reverted in the prt6-1 vrn2-5 double mutant, indicating that ectopic accumulation of VRN2 in roots does lead to enhanced function, in contrast to the situation in aerial tissues. It was previously reported that different PRC2 subunits have distinct and opposing roles in the control of root development: mutations in SWN and MSI1 cause smaller PRs with reduced meristem size, whilst a CLF mutant (clf29) had longer roots and significantly increased numbers of cells in the meristem (de Lucas et al., 2016). We observed enhanced meristem size in vrn2-5 relative to WT, which is similar to the previous observation in clf29. Thus, it is possible that the repressive role of VRN2 in root system architecture is linked to the CLF methyltransferase.

Hypoxic niches have recently been identified in pre-emerged LRPs, which probably explains why VRN2 accumulates in these regions (Shukla et al., 2019). However, in contrast to the shoot meristem, root meristems are yet to be defined as hypoxic when assayed in normoxia (Weits et al., 2019), and so it is possible that VRN2 accumulation in the root tip is linked to alternative mechanisms inhibiting its proteolysis (e.g. perhaps steric shielding of the N-degron by a tissue-specific binding partner). Alternatively, root meristems may show different sensitivity to O2, as root tip growth occurs in the soil, which is likely to be a hypoxic environment (Abbas et al., 2015). Hypoxic niches in LRPs were recently

Fig. 5 The PRT6 N-degron pathway confines VERNALIZATION2 (VRN2) to discrete root tissues and negatively regulates root growth. (a) Histochemical β-glucuronidase (GUS) staining of 7-d-old roots in Col-0 and prt6-1 Arabidopsis thaliana seedlings expressing wild-type (WT) or mutant (Ala2) pVRN2::VRN2-GUS: (i) primary root (PR) tip; bar, 1 mm; (ii) lateral root primordium (LRP); bar, 500 μm; (iii) emerged LR. (b) Quantitative reverse transcription polymerase chain reaction (qPCR) of VRN2 in different regions of WT or prt6-1 primary roots. The tip and main regions are shown in (a). Data are means of three biological replicates. Error bars represent ±SE. (c) Representative images of 10-d-old WT and mutant seedling roots in the Col-0 background. Bar, 1 cm. (d, e) Quantified PR lengths (d) and emerged LR densities (e) of 10-d-old Col-0 WT and mutant lines (n = 13–24). (f) Representative images of 6-d-old Col-0 and vrn2-5 primary root meristems stained with propidium iodide. Asterisk represents the quiescent centre; arrowhead indicates the end of the meristem. Bar, 100 μm. (g) Quantification of meristem size in Col-0 and vrn2-5. Data calculated from confocal root images (n = 10). Box and whisker plots show maximum and minimum, 25th to 75th percentiles, median and mean (+). Letters indicate one-way ANOVA; Tukey’s test (P < 0.05–0.01).
shown to enhance ERFVIIs, which inhibit LR development through repressing the expression of the auxin-associated genes IAA29 and PUCHI (Shukla et al., 2019). It will be important to determine if any of these same genes are also repressed at the epigenetic level through the action of locally stabilized VRN2, or whether separate targets are involved.

VRN2 is well characterized as a positive regulator of the vernalization response, accumulating during cold exposure to facilitate methylation and silencing of FLC. However, ectopic accumulation of VRN2 (in either prt6-1 or through N-terminal mutagenesis) did not repress FLC or abolish the requirement for vernalization (Figs 2, 3). This was perhaps not unexpected, as other cold-specific proteins and regulatory lncRNAs are required for the epigenetic repression of FLC (Costa & Dean, 2019). However, this led us to investigate in further detail the relationship between VRN2 abundance and the presence of other PRC2 subunits that are necessary for VRN2 to carry out PRC2-associated functions. When VRN2 is ectopically enhanced, levels of the core PRC2 component FIE do not change (Fig. 4), which indicates an overaccumulation of ‘free’ VRN2 protein. By contrast, cold- and hypoxia-triggered increases in VRN2 abundance were accompanied by higher levels of FIE. Thus, when VRN2 is stabilized outside of meristems in response to environmental signals, there is the capacity for a similar overall increase in VRN2-PRC2 that cannot take place when VRN2 accumulates out of context. The PHD protein VIN3 was previously shown to be transcriptionally induced by both cold and hypoxia, two environmental conditions that also inhibit VRN2 proteolysis. Whilst we also observed cold induction of VIN3 transcripts, we did not see an increase in VIN3 expression in response to hypoxia treatment (Sung & Amasino, 2004; Bond et al., 2009; Gibbs et al., 2018). However, it should be noted that here we used 1% O2, whereas Bond et al. (2009) used 0.1%, suggesting that perhaps VIN3 induction requires extremely low O2 availability, or even anoxia. VIN3 expression was not increased in prt6-1 relative to Col-0, further highlighting that VRN2-PRC2 binding partners required for vernalization are not available when VRN2 artificially accumulates. It was previously postulated that VIN3 might promote VRN2-PRC2 increases during long-term cold exposure, through binding and enhancing stability of the complex. We explored this possibility by investigating the effect of VIN3 on VRN2 stability in vitro and in planta. VIN3 did not stabilize VRN2, which suggests alternative mechanisms promoting observed increases in PRC2 components in response to cold temperatures (Fig. 4; Wood et al., 2006). Collectively, our data indicate that functions for VRN2 outside of meristems are only activated when it accumulates in appropriate environmental contexts as a result of a requirement for other specific binding factors.

The vrn2-5 mutant used in this study displayed several phenotypes related to both flowering and root development. This mutant was also previously shown to influence hypoxia tolerance and maternal effects on seed dormancy (Auge et al., 2017; Gibbs et al., 2018). However, in contrast to the vrn2-1, vrn2-5 was still able to fully respond to vernalization treatment, indicating that vrn2-5 disrupts some but not all VRN2 functions. The VEFS domain in Su(z)12 is required for binding to PRC2 and stimulating methyltransferase activity (Cao & Zhang, 2004). Full depletion of the VEFS domain abolishes the capacity for Su(z)12 to associate with catalytic Ez subunit in Drosophila. By contrast, a series of point mutations at different positions in the VEFS box of Su(z)12 affected PRC2 activity to different degrees (Ketel et al., 2005). A D593A mutation in the latter half of the VEFS-box had no effect on PRC2 assembly or enzymatic function, whilst a D550A mutation earlier in the sequence had a modest effect on methyltransferase activity (Ketel et al., 2005). However, an E546A mutation just four residues upstream of D550 almost completely abolished PRC2 function. Thus it is plausible that the different positions of mutation in vrn2-1 and vrn2-5 could differentially affect VRN2 activity, perhaps through modulating
binding stoichiometries, as has been observed previously for other Su(12x) mutations (Birve et al., 2001). It will now be important to develop further knockouts of VRN2 in different ecotypes (e.g. through the use of CRISPR) to help dissect its different functions in development and environmental response.

In conclusion, we show that VRN2 has multiple functions in plant development that are linked to the control of its abundance through the PCO branch of the PRT6 N-degron pathway. Cooption of Su(12x) to this proteolytic system allows plants to control spatial abundance and function of VRN2 by limiting it to endogenous hypoxic niches, whilst also coupling its accumulation to the perception of specific environmental cues where it adopts a separate set of functions. In this way, regulation of VRN2 by the PRT6 N-degron pathway is similar to that for ERFVIs, which also have dual functionality in tissue-specific coordination of development, and broader environment-triggered regulation of stress responses.

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Author contributions

DJG, A-ML and MJH designed and conceived the research. DJG, A-ML and MJH conducted experiments. DJG, A-ML and MJH analysed data. DJG wrote the manuscript with input from all authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Genotyping PCR confirming homozygosity of mutants in the Col-0 FRI-Sf2 background.
Table S1 Primer sequences used in this study.

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