VERNALIZATION INSENSITIVE 3 (VIN3) is required for the response of *Arabidopsis thaliana* seedlings exposed to low oxygen conditions

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SUMMARY

VERNALIZATION INSENSITIVE 3 (VIN3), which is required for the vernalization-mediated epigenetic repression of **FLOWERING LOCUS C (FLC)** in *Arabidopsis thaliana*, is quantitatively induced in response to low temperatures. We found that hypoxic conditions also induce **VIN3** in a quantitative manner but high salt, high temperatures and osmotic stress do not. Inhibition of mitochondrial respiration did not induce **VIN3** expression, consistent with the lack of **VIN3** induction in response to other stresses that affect the rate of mitochondrial respiration. *De novo* protein synthesis is required for **VIN3** induction during hypoxic conditions; this situation is not the case for **VIN3** induction by low temperatures, indicating that different mechanisms act to induce **VIN3** expression in response to cold and hypoxic conditions. Without **VIN3** activity, fewer seedlings survived following a 72-h period of hypoxic treatment, indicating that **VIN3** is required for the survival of *Arabidopsis thaliana* in response to hypoxic stress. Complementation of the *vin3* mutant with a **VIN3** transgene restored the wild-type response to low oxygen and confirmed the role of **VIN3** in protecting both shoots and roots during low oxygen conditions. Loss of **VIN3** protein did not affect the transcriptional regulation of genes known to be important in the response to low oxygen stress, which suggests that there is a novel mechanism to combat hypoxia that involves **VIN3**. This mechanism is likely to involve chromatin remodelling and may be similar to the role of **VIN3** in the epigenetic repression of **FLC** during the vernalization response.

Keywords: *Arabidopsis*, hypoxia, chromatin remodelling, gene expression, **FLOWERING LOCUS C**, **VERNALIZATION INSENSITIVE LIKE**.

INTRODUCTION

Vernalization, the acquisition of the competence to flower after exposure to prolonged low temperatures, is associated with the repression of the *Arabidopsis thaliana* floral repressor, **FLOWERING LOCUS C (FLC)** (Michaelis and Amasino, 1999; Sheldon *et al.*, 1999). **VERNALIZATION INSENSITIVE 3 (VIN3)**, a chromatin remodelling protein that is induced by low temperatures, is required for the vernalization response in *Arabidopsis* (Sung and Amasino, 2004). Induction of **VIN3** is transient and when plants are returned to warmer temperatures **VIN3** is rapidly repressed (Sung and Amasino, 2004). **VIN3** binds to the chromatin of the **FLC** locus (Sung and Amasino, 2004), and interacts with members of the conserved polycomb-group repressive complex 2 (PRC2) (Wood *et al.*, 2006; De Lucia *et al.*, 2008). The PRC2

complex trimethylates histone H3 lysine 27 (H3K27me3) (Schubert *et al.*, 2006; De Lucia *et al.*, 2008; Jiang *et al.*, 2008), a repressive chromatin mark that increases at the **FLC** locus as a result of vernalization (Schubert *et al.*, 2006; Sung *et al.*, 2006; Finnegan and Dennis, 2007; Greb *et al.*, 2007; De Lucia *et al.*, 2008). *Arabidopsis* plants with a mutation in **VIN3** fail to respond to vernalization; **FLC** expression remains high resulting in a late-flowering phenotype (Sung and Amasino, 2004). In contrast, constitutive expression of **VIN3** is not sufficient to substitute for low temperatures (cited in Sung and Amasino, 2004).

VIN3 is also required for the repression of **MADS AFFECTING FLOWERING 1 (MAF1)** in response to vernalization (Sung *et al.*, 2006). **VIN3** is also required for the cold

induction of PRC2-regulated *AGAMOUS-LIKE 19* (*AGL19*), a MADS box transcription factor that acts as a floral promoter (Schonrock *et al.*, 2006). Therefore, VIN3 has a strong association with PRC2 complex(es) to positively and negatively regulate gene transcription during vernalization.

The Arabidopsis genome encodes four genes that are related to VIN3 (*VERNALIZATION INSENSITIVE LIKE 1-4* or *VIL1-4* as named by Sung *et al.*, 2006; Greb *et al.*, 2007). With the exception of VIL4, all members of the VIN3 family contain a plant homeodomain (PHD), a domain that can bind either tri-methylated lysine 4 residues on histone H3 (H3K4me3) (Shi *et al.*, 2006; Wysocka *et al.*, 2006) and/or phosphoinositides (Gozani *et al.*, 2003). Adjacent to the PHD domain is a fibronectin type III domain that can mediate protein–protein interactions (Potts and Campbell, 1994). The C-terminal VIN3-Interacting domain (VID), which is unique to the plant kingdom, is well conserved amongst the VIN3 family (Sung *et al.*, 2006; Greb *et al.*, 2007).

VIN3 and VIL1 interact via their VID domains in yeast two-hybrid assays (Sung *et al.*, 2006; Greb *et al.*, 2007); this interaction also occurs *in vivo* in tobacco leaves that express epitope-tagged versions of VIN3 and VIL1 (Greb *et al.*, 2007). In addition, VIN3 and VIL2 form homo-dimers and hetero-dimers in yeast two-hybrid assays (Sung *et al.*, 2006; Greb *et al.*, 2007). Biochemical purification of the PRC2 complex in vernalized plants indicates that VIN3, VIL1 and VIL2 are all part of this complex (De Lucia *et al.*, 2008).

VIN3 is also induced in response to nicotinamide treatment (Bond *et al.*, 2009). We proposed that alterations in cellular redox state, induced by cold or nicotinamide treatment, could result in the induction of VIN3. Here we report that VIN3 is induced in response to hypoxic conditions, but is not induced in response to the other stresses that affect redox homeostasis that we tested. We have found that the mechanisms that control VIN3 induction in response to cold and low oxygen conditions are different. VIN3 is important for the survival of Arabidopsis seedlings during hypoxic stress; fewer *vin3* mutant seedlings survived low oxygen conditions than wild-type seedlings, while complementation of the *vin3* mutant restored the wild-type response. The role of VIN3 in Arabidopsis during the response to the low oxygen conditions is discussed.

RESULTS

VIN3 is induced in response to hypoxic conditions

Hypoxic conditions (0.1% O₂) induced VIN3 expression; the longer the hypoxic treatment, the higher the expression of VIN3, which returned to pre-hypoxia levels when seedlings were re-exposed to atmospheric oxygen (Figures 1a and S1). The expression profile of VIN3 in response to hypoxia is similar to that seen in response to low temperatures (Figure 1a; Sung and Amasino, 2004). VIN3 induction was not observed in seedlings exposed to

high salt concentrations, high temperature or osmotic stress conditions (Figure 1a).

Arabidopsis seedlings have an improved response to anoxia if they are exposed to 5% O₂ prior to anoxic stress (Ellis *et al.*, 1999). A 5% O₂ treatment did not induce VIN3 (data not shown), but VIN3 induction was observed in the aerial tissue of soil-grown Arabidopsis seedlings that experienced waterlogging for 7 days (Figure 1b). Waterlogging conditions reduce the level of oxygen in the soil to approximately 0.2–0.3% O₂ (I. Wilson and J. Christianson, unpublished data). In contrast to VIN3 induction, VIL1 was repressed two-fold within 12 h of low oxygen conditions (Figure 1c) and this result was independent of VIN3 (data not shown), while VIL2 and VIL3 were not affected by low oxygen conditions (Figure 1d, e).

VIN3 expression is not regulated by alterations in mitochondrial respiration

Low oxygen and cold treatment are associated with alterations in mitochondrial respiration (Atkin and Tjoelker, 2003; Igamberdiev and Hill, 2009). Inhibitors of mitochondrial respiration pathways were used to determine whether alterations in the rate of mitochondrial respiration lead to the induction of VIN3. Antimycin A (AA) inhibits complex III of the cytochrome pathway (Ikuma and Bonner, 1967), while salicylhydroxamic acid (SHAM) inhibits the alternative oxidase (AOX) pathway (Schonbaum *et al.*, 1971). VIN3 was not induced when either AA or SHAM were sprayed onto Arabidopsis seedlings, alone or in combination (Figure 2a). In contrast, *ADH1*, whose expression is also induced by cold and low oxygen conditions (Dolferus *et al.*, 1994), was induced by AA and SHAM and a greater induction was observed when both inhibitors were applied (Figure 2b).

VIN3 induction in response to low oxygen conditions requires *de novo* protein synthesis

We measured the expression of VIN3 in response to low oxygen and cold, with or without cycloheximide, an inhibitor of protein synthesis. VIN3 expression was induced by a 24-h low oxygen treatment; induction was inhibited by cycloheximide (Figure 3a). Cycloheximide treatment also blocked *ADH1* induction by hypoxia (Figure S2a) (Hoeren *et al.*, 1998). In contrast, VIN3 was super-induced by 24 h of cold exposure in the presence of cycloheximide (Figure 3b), whereas cycloheximide did not alter *ADH1* induction under cold conditions (Figure S2b) (Hoeren *et al.*, 1998).

VIN3 is required for the low oxygen response in Arabidopsis

The *vin3-4* T-DNA insertion mutant was used to assess whether VIN3 is required for survival of Arabidopsis under hypoxic conditions. The survival of the *vin3-4* mutant was not different from wild-type after short periods (12 and 24 h) of exposure to hypoxia. As the duration of the hypoxic treatment increased, the survival of Col:*FRI*, the parental line of the

Figure 1. Expression of the *VIN3* gene family in response to a low oxygen environment.

(a) Expression of *VIN3* relative to *FDH* in C24 seedlings in response to exposure to 28°C (X), 150 mM NaCl (○), 0.6 mannitol (▲), 4°C (◇) and 0.1% O₂ (■) (grey area), and in samples removed from the stresses (white area), compared with 14-day-old non-treated seedlings (0 h). Error bars represent S.E.D. *F* test *P*-values indicated significant effects for cold ($P < 0.001$) and 0.1% O₂ ($P = 0.007$), but not the other treatments.

(b) Expression of *VIN3* relative to *FDH* in aerial tissue of C24 seedlings in response to waterlogging (solid line; grey area) followed by aeration (white area), compared with 21-day-old non-treated seedlings (0 days) and control seedlings grown under the same photoperiod conditions (dashed line). Error bars represent S.E.D. *F* test *P*-values for the terms in the ANOVA analysis indicated significant effects for waterlogging ($P < 0.001$).

(c–e) Expression of (c) *VIL1* (d) *VIL2* and (e) *VIL3* relative to *FDH* in Col:FRI seedlings in response to 0.1% O₂ (solid line) or control conditions (dashed line). Error bars represent S.E.D. *F* test *P*-values indicated significant effects for *VIL1* only, for the treatment type ($P = 0.007$), but not duration of treatment or their interaction.

Figure 2. *VIN3* is not induced by alterations to mitochondrial respiration.

Expression of (a) *VIN3* and (b) *ADH1* relative to *FDH* in C24 seedlings in response to treatment with 25 μ M antimycin A (AA), 5 mM salihydroxamic Acid (S), control treatments [solvents used; Tween-20 (T) and DMSO (D)], or combinations of both inhibitors (A + S) and controls (T + D). Seedlings exposed to cold (4°C) for 24 h were used as a comparison. All samples are compared with 14-day-old non-treated control seedlings (Con). Error bars represent S.E.D. For *ADH1*, *F* test *P*-values indicated significant effects for the different inhibitors and cold compared with the control conditions ($P < 0.001$); for *VIN3* cold treatment ($P < 0.001$).

vin3-4 mutant, declined to 50–60% (by 72 h) compared with the aerobic controls (Figure 4a, b). Fewer *vin3-4* seedlings survived 36 h of 0.1% O₂ compared with the wild-type seedlings and this difference became more pronounced after a 72 h treatment when approximately 30% of *vin3-4* seedlings survived (Figure 4a, b). However, exposure to hypoxia for longer time periods (120 h) resulted in death of the majority of the wild-type and mutant seedlings (Figure 4b). Even after 120 h at low oxygen, all plants remained green although some leaf tissue was limp; plants became chlorotic and died only during the 10-day recovery period after the treatment.

The expression of three genes, *FDH* (At5g43940), *At4g26410* and *At1g13320* (Czechowski *et al.*, 2005), used as controls for qPCR was comparable between control and treated plants, confirming that the plants were still alive after 120 h under low oxygen conditions. These results indicate that, without *VIN3*, *Arabidopsis* seedlings are more susceptible to hypoxia.

The survival of another allele of *VIN3* (*vin3-8*) was similar to the *vin3-4* mutant. After 72 h of hypoxic treatment 29% of the *vin3-8* mutants survived, this rate was approximately 50% less than the survival rate of wild-type seedlings (Figure 4c).

Figure 3. Cycloheximide blocks *VIN3* induction under low oxygen conditions.

(a) Expression of *VIN3* relative to *FDH* in C24 seedlings exposed to 0.1% O₂ (light grey) or control conditions (dark grey) for 24 h. A subset of seedlings were treated with 10 µM cycloheximide (right) and compared with seedlings without cycloheximide (left). All samples are compared with non-treated 14-day-old control seedlings (control, no cycloheximide).

(b) Same as (a) but measuring *VIN3* expression in C24 seedlings exposed to 4°C (light grey) or control conditions (22°C; dark grey) for 24 h. For (a) and (b), error bars represent S.E.D. *F* test *P*-values indicated significant effects for the low oxygen and cold treatment ($P < 0.001$), and the effects of cycloheximide on these treatments ($P < 0.001$).

Complementation of the *vin3-4* mutant with a genomic version of *VIN3* (*VIN3pro::VIN3::HA*) (Wood *et al.*, 2006) restored the vernalization response and seedlings flowered with a similar number of leaves as wild-type Col:*FRI* seedlings (Figure S4). After 72 h of 0.1% O₂ exposure, the survival of transgenic lines (line #2 and #4) expressing the *VIN3pro::VIN3::HA* transgene was comparable with that of wild-type seedlings (approximately 50% survival) (Figure 4c). In contrast, a line carrying an inactive transgene (line #11) was affected by the hypoxic treatment to the same extent as the *vin3-4* mutant (Figure 4d).

The phenotype we observed with the *vin3-4* mutant suggests that *VIN3* is important for survival of both roots and aerial tissues (Figure 4a). After a 10-day recovery period, the shoots and roots of Col:*FRI* seedlings that were exposed to hypoxia weighed 35 and 47% less, respectively, than the corresponding tissues of the wild-type controls grown in normal atmospheric conditions (Figure 4e, f). The weights of the shoots and roots of *vin3-4* seedlings that had been exposed to hypoxic conditions was even less, approximately 73 and 81%, respectively, than the mutant seedlings grown under control conditions (Figure 4e, f).

***VIN3* does not regulate its known targets of the vernalization pathway in response to low oxygen conditions**

VIN3 is required for the repression of *FLC* and *MAF1* and the activation of *AGL19* in response to vernalization (Sung and Amasino, 2004; Schonrock *et al.*, 2006; Sung *et al.*, 2006). There was no change in the expression of either *FLC* or *MAF1* in response to hypoxic conditions in the *vin3-4* mutant or Col:*FRI* seedlings (Figure 5a, b). *AGL19* expression trended upwards during hypoxia, but there was no difference between wild-type and the *vin3-4* mutant (Figure 5c). In agreement with the expression data, the hypoxic treatment did not induce flowering (data not shown), suggesting low oxygen stress cannot replace vernalization.

***VIN3* does not play a role in the regulation of genes of the known low oxygen pathways in Arabidopsis**

We measured expression of genes encoding the enzymes of the ethanol fermentation pathway (*ADH1*, *PDC1* and *PDC2*) and the non-symbiotic haemoglobin, *GLB1* that are required for survival under low oxygen conditions (Ellis *et al.*, 1999; Hunt *et al.*, 2002; Ismond *et al.*, 2003; Kursteiner *et al.*, 2003). The pattern and extent of induction of *ADH1*, *PDC1*, *PDC2*, and *GLB1* was almost identical in the wild-type and *vin3-4* mutant in response to hypoxia (Figure 6a–d).

Numerous other metabolic and signalling pathways are altered at the transcriptional level under low oxygen environments (Klok *et al.*, 2002; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005). As a result, multiple mechanisms play a role in altering the tolerance of Arabidopsis seedlings to low oxygen conditions. We measured the expression of a selection of genes (Table 1) from these different pathways in both Col:*FRI* and *vin3-4* seedlings to assess whether *VIN3* plays a role in the regulation of these genes during the early or late stages of low oxygen stress. The majority of these genes were induced only under hypoxic conditions (Figure 7a–k). *ANAC102* and *LDH1* were also induced in the aerobic controls compared with the 0-h time sample, which indicated that the control samples were under slight oxygen stress. In contrast, *GAD1* and *EDS1* expression was repressed two-fold over the duration of the time course.

There was no significant difference in the expression of any of these genes between Col:*FRI* and *vin3-4* in response to hypoxia (Figure 7a–k), indicating that *VIN3* does not play a role in their regulation.

Microarray analysis that compared wild-type and *vin3-4* hypoxia treated seedlings

Because *VIN3* is as a member of a complex(es) that controls the transcriptional capabilities of target genes through

Figure 4. VIN3 promotes survival of seedlings under low oxygen conditions.

(a) *Col:FRI* (left) and *vin3-4* (right) seedlings were exposed to 0.1% O₂ for 72 h (top), then aerated and left to recover for 10 days. Control samples were exposed to atmospheric oxygen for the same time period (bottom). Two independent biological replicates are shown.

(b) Survival of *Col:FRI* (solid line) and *vin3-4* (dashed line) seedlings exposed to 0.1% O₂ for various periods of time. Results are an average of four biological replicates each consisting of at least 30 seedlings. Error bars represent S.E.D. *F* test *P*-values indicated significant effects for the hours of treatment (*P* = 0.004), the genotype of seedlings (*P* < 0.001) and their interaction (*P* < 0.001).

(c) Survival of the *vin3-4* (left) and the *vin3-8* (right) T-DNA insertion mutants (light grey) exposed to 0.1% O₂ for 72 h compared with their respective wild-type parents (dark grey): *Col:FRI* (left) and *Col-0* (right). Results are an average of four biological replicates each consisting of at least 30 seedlings. Error bars represent S.E.D. *F* test *P*-values for the terms in the ANOVA analysis indicated significant effects for the hypoxic treatment on the individual mutants (*P* < 0.001).

(d) Survival of *Col:FRI*, *vin3-4* and three independent *vin3-4* + *VIN3pro::VIN3:HA* T₂ lines (#2, #4, #11) after 72 h under 0.1% O₂. The wild-type and transgenic lines containing an active transgene are shaded dark grey, while the *vin3-4* mutant and the transgenic line carrying an inactive transgene are shaded light grey. Results are an average of four biological replicates, each containing at least 30 seedlings. Error bars represent S.E.D. *F* test *P*-values indicated significant effects of the hypoxia treatment for the *vin3-4* mutant and line #11 (*P* = 0.004), compared with *Col:FRI* and lines #2 and #4.

(e, f) Dry weight (mg) of shoots (e) and roots (f) from *Col:FRI* (dark grey) and *vin3-4* (light grey) seedlings following a 72-h exposure to 0.1% O₂ (right) compared with control samples exposed to control conditions (left). Results are an average of eight biological replicates, each with at least 30 seedlings. Error bars represent S.E.D. *F* test *P*-values indicated significant effects for the hours of treatment (*P* < 0.001), the genotype of seedlings (*P* < 0.001) and their interaction (*P* < 0.001) for both shoots and roots.

altering chromatin state, we performed a gene-expression microarray with the aim of identifying genes regulated by VIN3 in low oxygen conditions. The NimbleGen *Arabidopsis*

thaliana four-plex cDNA array allowed simultaneous hybridization of four cDNA samples corresponding to RNA isolated from: *Col:FRI* 24 h control, *Col:FRI* 24 h 0.1% O₂,

Figure 5. Expression of known targets of VIN3 is not altered under a low oxygen environment. Expression of (a) *FLC* (b) *MAF1* (c) *AGL19* relative to *FDH* in Col:*FRI* (solid line) and *vin3-4* (dashed line) seedlings in response to 0.1% O₂ (◆) or control conditions (◇), compared with 14-day-old non-treated seedlings (0 h). Error bars represent S.E.D. For *FLC* and *MAF1*, the *F* test *P*-values indicated no significant effects for the hours of treatment, the genotype of the seedlings or their interaction. For *AGL19*, the *F* test *P*-values for the terms in the ANOVA analysis indicated significant effects for the hours of treatment ($P = 0.004$), but not the genotype of the seedlings or their interaction.

Figure 6. VIN3 does not regulate genes of the fermentation pathway.

Expression of (a) *ADH1* (b) *PDC1* (c) *PDC2* and (d) *GLB1* relative to *FDH* in Col:*FRI* (solid line) and *vin3-4* (dashed line) seedlings in response to 0.1% O₂ (◆) or control conditions (◇), compared with 14-day-old non-treated seedlings (0 h). Error bars represent S.E.D. For all four genes in the hypoxia treated samples, the *F* test *P*-values indicated significant effects for the hours of treatment ($P < 0.001$), but not the genotype of the seedlings, or their interaction. For expression of *PDC1* and *GLB1* in the aerobic samples, the *F* test *P*-values indicated significant effects for the hours of treatment ($P < 0.001$), but not the genotype of the seedlings, or their interaction.

vin3-4 24-h control and *vin3-4* 24 h 0.1% O₂ samples, on a single slide. A 24-h time point was used because *VIN3* was induced 10- to 15-fold at this time. We chose not to analyse later time points as fewer *vin3-4* seedlings survived after 36 h of treatment; the decreased survival of mutant seedlings could compromise the quality of RNA and bias the data set with genes associated with the cell death.

Four pair-wise comparisons of the gene expression results were made: Col:*FRI* control versus Col:*FRI* 0.1% O₂, *vin3-4* control versus *vin3-4* 0.1% O₂, Col:*FRI* control versus *vin3-4* control and Col:*FRI* 0.1% O₂ versus *vin3-4* 0.1% O₂. Firstly, we confirmed the induction of *VIN3* on the array; *VIN3* induction as measured by our array was comparable to that measured by qPCR (Table S1). We further validated our data by comparing the expression values (fold changes) of genes known to play a role in the low oxygen response from our microarray with previous low oxygen microarray results (Table S1). Control and hypoxic samples were compared within each genotype; the expression levels of known low oxygen genes in our experiment were consistent with those that have been previously published.

The comparison of wild-type and *vin3-4* seedlings under control conditions indicated that there were no statistically

significant differences ($P \leq 0.01$) in gene expression between these samples. This finding was not surprising as *VIN3* is not expressed in these conditions. The direct comparison of wild-type and *vin3-4* 0.1% O₂ treated seedlings also produced no statistically significant changes in gene expression, other than *VIN3* itself. While we have shown that the genotypes used in our study show differential survival after 72 h of hypoxia, our microarray data showed concordance of the transcriptional response after 24 h of hypoxic stress.

DISCUSSION

VIN3 expression responds to alterations in atmospheric oxygen

We have shown that *VIN3* is induced in response to a low oxygen (0.1% O₂) environment and low temperatures, but not by other stresses (Figure 1a). Consistent with cold treatment, *VIN3* transcripts became more abundant as the time under hypoxia increased; on return to atmospheric oxygen *VIN3* expression was repressed to pre-hypoxic levels (Figure 1a). *VIN3* was not induced at 5% O₂ but induction was observed in waterlogged, soil grown Arabidopsis

Table 1 Summary of low oxygen genes, and genes that have been reported to be affected by hypoxic stress, that were analysed by qPCR in this study

Locus	Short name	Function of gene	References
At5g20830	SUS1	Sucrose synthase; catalyses sucrose breakdown, acts upstream of the fermentation pathways, required for response to hypoxia	Bieniawska <i>et al.</i> (2007)
At3g43190	SUS4	Sucrose synthase; catalyses sucrose breakdown, acts upstream of the fermentation pathways, required for response to hypoxia	Bieniawska <i>et al.</i> (2007)
At2g14210	ANR1 (AGL44)	MADS-box transcription factor induced by hypoxia	Liu <i>et al.</i> (2005)
At2g47520	AtERF#71	Ethylene responsive factor (ERF); induced by hypoxia, close homologue of the <i>ERF</i> genes that make up the <i>Sub1</i> locus that determines the tolerance of rice to submergence (Xu <i>et al.</i> , 2006)	Liu <i>et al.</i> (2005)
At2g19590	ACO1	1-aminocyclopropane-1-carboxylate oxidase; catalyses the final step of ethylene biosynthetic pathway required for <i>ADH1</i> induction in later stages of hypoxia	Peng <i>et al.</i> (2001)
At5g63790	ANAC102	NAC domain transcription factor is required for full germination of Arabidopsis seeds during hypoxia	Christianson <i>et al.</i> (2009)
At1g17290 ^a	AlaAT1	Alanine aminotransferase; catalyses breakdown of alanine that accumulates during recovery from hypoxic conditions	Miyashita <i>et al.</i> (2007)
At4g17260	LDH1	Lactate dehydrogenase; enzyme of the additional fermentation pathway that is required for survival of Arabidopsis in response to hypoxia	Dolferus <i>et al.</i> (2008)
At5g17330	GAD1	Glutamate decarboxylase; the first enzyme of the GABA shunt that catalyses the breakdown of glutamate that accumulates during hypoxia	Miyashita and Good (2008)
At2g34390 ^a	NIP2;1	Transporter of lactic acid; lactic acid is exuded from the plant during hypoxia, preventing it accumulating to toxic levels in the cells (Dolferus <i>et al.</i> , 2008)	Choi and Roberts (2007)
At3g48090 ^a	EDS1	Isochorismate synthase; required for hypoxia induced lysigenous aerenchyma formation	Muhlenbock <i>et al.</i> (2007)

The reference column states the citation that reported the expression or function of the gene of interest.

^aGenes with a 'hypoxia' GO annotation.

seedlings (Figure 1b). This observation indicates that the mechanism regulating *VIN3* expression is sensitive to the degree of oxygen deprivation.

Homologues of the Arabidopsis *VIN3* gene family have been identified in the monocot plants, wheat and rice (Fu *et al.*, 2007) (Figure S5). Wheat requires vernalization to flower, while rice does not respond to vernalization. As with Arabidopsis, wheat may require *VIN3*-like proteins to respond to vernalization. However, expression of *TmVIL1-3* increases only two- to four-fold after 6 weeks at low temperatures (Fu *et al.*, 2007). This finding contrasts with the large increase in Arabidopsis *VIN3* expression in response to long-term cold exposure (Sung and Amasino, 2004).

The *VIN3* homologue, *OsVIL4*, is induced in response to low oxygen conditions (Rice Expression Database) (Yazaki *et al.*, 2002); expression of the other *OsVIL* genes did not change during this treatment (Table S2). In contrast, cold exposure did not induce any of the *OsVIL* transcripts (Table S2) (Yazaki *et al.*, 2002). Phylogenetic analysis indicates that Arabidopsis *VIN3* is more closely related to *OsVIL2* and *OsVIL4* than it is to the other rice *VIN3*-like proteins (Figure S5). The induction of *VIN3* and *OsVIL4* by low oxygen conditions could be a conserved response to low oxygen conditions. In Arabidopsis, *VIN3* may have evolved an additional role to repress *FLC* in the vernalization response.

Cold and hypoxic conditions regulate *VIN3* expression via different mechanisms

Both low oxygen conditions and low temperatures reduce the rate of mitochondrial respiration (Atkin and Tjoelker, 2003; Igamberdiev and Hill, 2009), which is associated with alterations in NAD⁺ levels and the redox state of the cell (Maciejewska and Kacperska, 1987; Szal *et al.*, 2003). However, chemical inhibition of the cytochrome and alternative oxidase respiration pathways in the mitochondria did not induce *VIN3* expression (Figure 2a), suggesting that the rate of mitochondrial respiration does not regulate *VIN3* expression. This finding is consistent with the lack of induction of *VIN3* in response to other stress conditions, which cause alterations in pyridine nucleotides and redox status (Noctor *et al.*, 2006). In contrast, treatments with AA and SHAM (Figure 2b) suggest that alterations in respiration pathways can induce *ADH1*. *VIN3* induction by hypoxia and low temperatures was observed in dark grown seedlings (Figure 1a; D.B., unpublished data), implying that photorespiration does not regulate *VIN3* expression.

Chemical inhibition of protein synthesis during hypoxia prevented the induction of *VIN3* (Figure 3a), indicating that *de novo* translation of a protein(s) that also responds to hypoxic conditions is required for *VIN3* induction during

Figure 7. VIN3 does not regulate other genes in low oxygen response pathways.

Expression of: (a) *SUS1* (b) *SUS4* (c) *ANR1* (d) *ERF#71* (e) *ACO1* (f) *ANAC102* (g) *AlaAT1* (h) *LDH1* (i) *GAD1* (j) *NIP2;1* and (k) *EDS1* relative to *FDH* in Col:*FRI* (solid line) and *vin3-4* (dashed line) seedlings in response to 0.1% O₂ (●) or control conditions (◇), compared with 14-day-old non-treated seedlings (0 h). Error bars represent S.E.D. For expression of *SUS1*, *SUS4*, *ANR1*, *ERF#71*, *ACO1*, *AlaAT1*, *ANAC102*, *AlaAT1*, *LDH1*, *NIP2;1* and *EDS1* in the hypoxia-treated samples, the *F* test *P*-values indicated significant effects for the hours of treatment ($P < 0.001$), but not the genotype of the seedlings, or their interaction. For *GAD1* in the hypoxia-treated samples, the *F* test *P*-values indicated significant effects for the hours of treatment ($P = 0.002$), but not the genotype of the seedlings, or their interaction. For expression of *ANR1*, *ERF#71*, *ACO1*, *ANAC102*, *AlaAT1*, *LDH1* and *GAD1* in the control samples, the *F* test *P*-values indicated significant effects for the hours of treatment ($P \leq 0.007$), but not the genotype of the seedlings, or their interaction.

hypoxia. In contrast, cycloheximide super-induced *VIN3* expression during cold exposure (Figure 3b) indicating that the transcriptional machinery required for *VIN3* induction is constitutively expressed. This super-induction in response to cycloheximide during cold exposure could be a result of a change in stability of *VIN3* transcripts, which is typical of mRNAs that are destabilized by constitutively synthesized proteins (Gutiérrez *et al.*, 1999; Bailey-Serres and Chang,

2005). Induction of *VIN3* during hypoxia and cold treatment is therefore directed by different mechanisms.

VIN3 is involved in the low oxygen response and has a protective role in shoots and roots

In Arabidopsis, hypoxic environments have been shown to dramatically impair protein synthesis by constraining translation of constitutive transcripts and translating selective

mRNA species induced by hypoxia (Branco-Price *et al.*, 2005, 2008). Survival assays comparing the *vin3-4* mutant and wild-type seedlings indicated that, after 72 h of hypoxic treatment, approximately half the number of mutant seedlings survived compared with wild-type (Figure 4a, b). Complementation of the *vin3-4* mutant with a *VIN3pro::VIN3::HA* transgene indicated that the level of survival of lines that carry a functional transgene was comparable with that of wild-type seedlings (Figure 4c). The phenotypic evidence confirms that the VIN3 protein is translated and has a role during hypoxic stress.

VIN3 is also induced in the aerial tissue of seedlings whose roots are waterlogged (Figure 1b), implying that a systemic signal from the roots to the shoots induces *VIN3* in aerial tissue. Root-to-shoot communication in response to low oxygen environments has been documented (Bailey-Serres and Chang, 2005). In contrast, the induction of *VIN3* in response to low temperatures is likely to be cell autonomous as there is no evidence for a transmissible signal in the vernalization response (Schwabe, 1954).

***VIN3* has a novel function in the low oxygen response of Arabidopsis**

The major metabolic response of a plant to hypoxia is conversion from aerobic respiration to anaerobic fermentation (Drew, 1997). The enzymes SUS1 and SUS4 involved in sucrose degradation, and ADH1, PDC2, PDC3, LDH1, AlaAT1, GAD1 from the different fermentation pathways are required for this switch in metabolism (Ellis *et al.*, 1999; Ismond *et al.*, 2003; Kursteiner *et al.*, 2003; Bieniawska *et al.*, 2007; Miyashita *et al.*, 2007; Dolferus *et al.*, 2008; Miyashita and Good, 2008); these pathways still proceed at the transcriptional level in the *vin3-4* mutant (Figures 6a–c and 7a, b, g–i), indicating that *VIN3* does not regulate the expression of anaerobic metabolism genes. This result is not surprising considering the slower induction of *VIN3* during hypoxia, compared with that of (for example) *ADH1*. Furthermore, loss of *VIN3* activity did not affect expression of other metabolic proteins/enzymes (*GLB1*, *ACO1*, *EDS1*), transcription factors (*ANR1*, *AtERF#71*, *ANAC102*) or structural proteins (*NIP2;1*) (Figures 6 and 7c–f, j, k) associated with the other mechanisms and pathways that are important for the response to hypoxia (Peng *et al.*, 2001; Hunt *et al.*, 2002; Liu *et al.*, 2005; Choi and Roberts, 2007; Muhlenbock *et al.*, 2007; Christianson *et al.*, 2009).

Constitutive *VIN3* expression does not replace the requirement for cold treatment for the vernalization-mediated repression of *FLC* (cited in Sung and Amasino, 2004), suggesting there is another co-factor required with *VIN3* during cold exposure, or that *VIN3* may be post-translationally modified during low temperatures. We have not been able to test the effect of constitutive *VIN3* expression on the low oxygen response due to strong silencing of the *35S::VIN3* transgene in wild-type seedlings (D. Bond and C. Helliwell, unpublished data).

In an attempt to understand the function of *VIN3* during low oxygen stress, a gene expression micro-array was conducted to identify novel chromatin targets of *VIN3* through changes in transcriptional activity. With the exception of *VIN3*, there was a high degree of similarity between the gene expression profiles of Col:*FRI* and *vin3-4* seedlings in response to a 24-h treatment of 0.1% O₂. This time point may have been too early to identify changes in transcription considering that the *vin3-4* phenotype was observed from 36–72 h of low oxygen stress. Alternatively, *VIN3* directed changes in gene expression may occur in specific tissue types and could be masked when whole seedlings are assayed. The *vin3-4* phenotype in response to hypoxia was only observed 10 days after the end of the hypoxic treatment. During reoxygenation after low oxygen stress, there are numerous changes to the transcriptional and metabolomic profiles of Arabidopsis seedlings (Branco-Price *et al.*, 2008). Perhaps *VIN3* is required to set up gene expression states for the adaptation of Arabidopsis to later stages of hypoxic stress, or the response of the plant during the recovery phase after hypoxia treatment.

The chromatin state during hypoxia: a role for *VIN3* during low oxygen conditions

There are a number of examples in Arabidopsis showing changes in histone modifications in response to a variety of abiotic stresses, including cold, drought and osmotic stress. This finding is not surprising considering that a major response to abiotic stress involves changes in gene expression. Indeed the changes recorded to date include gain or loss of histone acetylation and H3K4 methylation that has been associated with changes in gene expression (Perruc *et al.*, 2007; Sokol *et al.*, 2007; Kim *et al.*, 2008; Zhu *et al.*, 2008). Evidence from rice indicates that low oxygen conditions also induce chromatin modifications; there are dynamic, reversible changes in histone H3 acetylation and H3K4me3 on *ADH1* and *PDC1* chromatin in response to submergence (Tsuji *et al.*, 2006).

We have demonstrated a role for *VIN3* in the survival of plants under hypoxic conditions. As *VIN3* has been previously shown to associate with components of the PRC2 complex required for the addition of H3K27me3 at *FLC* chromatin during vernalization and has been implicated in deacetylation of histones at *FLC* (Sung and Amasino, 2004; Schubert *et al.*, 2006; Wood *et al.*, 2006; De Lucia *et al.*, 2008; Jiang *et al.*, 2008), it is likely that *VIN3* is also involved in histone modifications during the low oxygen response, although no target gene(s) has been identified. The finding that *VIN3* plays a role in this response suggests that, in addition to mediating the vernalization response, PRC2-like complexes (and perhaps other chromatin remodelling complexes) may also be required for response to hypoxia and other abiotic stresses.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana ecotypes C24, Col:*FRI*^{S12} (Lee *et al.*, 1994) and Col-0 were used as described. Two *vin3* T-DNA insertion mutants were also used; *vin3-4* (in the Col:*FRI* background), which contains a T-DNA in the second intron of *VIN3* and *vin3-8* (SALK_004766, in the Col-0 background), which contains a T-DNA in the third exon of *VIN3* (Alonso *et al.*, 2003). The *vin3-4* lines carrying a *VIN3pro::VIN3::HA* transgene was described by Wood *et al.* (2006).

Plant growth conditions

Arabidopsis thaliana seeds were surface sterilized with a bleach:ethanol (1:3, v/v) solution, and grown on Murashige and Skoog (MS) (1962) agar medium containing 3% sucrose (w/v). After a 2-day stratification period at 4°C, seeds were grown under a long day (LD; 16 h light:8 h dark) photoperiod at approximately 170 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ fluorescent light at 22°C. The *vin3-4* + *VIN3pro::VIN3::HA* transgenic lines were germinated and grown on agar medium containing 3% sucrose (w/v) supplemented with 30 $\mu\text{g ml}^{-1}$ Hygromycin (Roche Diagnostics, GmbH) to select plants carrying the transgene.

Hypoxia treatment

Two-week-old *Arabidopsis thaliana* seedlings were transferred into small (35 mm) Petri dishes (five seedlings per plate) containing 5 ml of liquid MS medium with 3% sucrose (v/v). Three small plates were then placed into a medium (90 mm) Petri dish, which was sealed and placed onto a gentle shaker at 22°C for 24 h, to allow the seedlings to adapt to liquid conditions. The seal was removed and plates were stacked into stainless steel racks that were placed into anaerobic tanks (Oxoid, Unipath Ltd.). The tanks were sealed and flushed with 0.1% O₂ (gases were obtained as O₂:N₂ premixes from BOC gases) at an approximate rate of 12 L min⁻¹ for 20 min. The tanks were placed onto a gentle shaker in the dark at 22°C for the specified times. The aerobic control samples were also placed into tanks with the lid on but not sealed, and kept in the dark beside the anaerobic tanks. Plants that were in liquid for 24 h, but never placed into a tank were used as the 0 h control sample. In all experiments the treatments were done in duplicate or triplicate medium Petri dishes (each containing three small plates with five seedlings each); replicates were allocated to separate treatment tanks and the positions of the Petri dishes within each tank were randomized.

Scoring of survival and growth post-hypoxia

For the analysis of survival after hypoxic treatment, the plates were removed from the tanks, sealed and placed onto a gentle shaker in LD at 22°C for a 10-day recovery period. Survival was scored by analysing the centre of the rosette containing the shoot meristem; plants showing growth of new leaves were scored as alive.

Quantitative measurements of shoot and root growth after the hypoxic treatment were performed by taking the seedlings from each small plate and aligning them on a 10 × 10 mm square Petri dish containing MS agar medium with 3% sucrose (w/v). The plates were incubated vertically in LD at 22°C for 10 days when the shoots and the roots were dissected and dried overnight at 80°C. The shoots and the roots from the five seedlings on each plate were pooled and the dry weight of the shoot and root tissue was measured.

Salt, heat, cold and osmotic treatment

Arabidopsis thaliana seedlings (14 days old) were placed onto the high salt medium (MS agar, 3% sucrose (w/v) supplemented with

NaCl, 150 mM final concentration) and grown under LD at 22°C for the specified times. For low and high temperature treatments, 14-day-old seedlings were placed on to fresh MS agar medium with 3% sucrose (w/v) and transferred to 4°C (under continuous low light) or 28°C (LD photoperiod) for the specified times. Osmotic stress was carried out by placing 10 seedlings (14 days old) into a medium Petri dish containing 15 ml liquid MS medium (3% sucrose, v/v). The plates were sealed and placed onto a gentle shaker in LD at 22°C for 24 h when mannitol (0.6 M final concentration) was added to the liquid media and the seedlings were exposed for the specified times. For all treatments non-treated plants were used as the 0 h control sample.

Waterlogging treatment

Arabidopsis thaliana seeds were sown onto soil (35% peat moss, 46% vermiculite, 19% perlite containing 1 g L⁻¹ Aboska), stratified for 2 days then grown under a short day (SD; 8 h light:16 h dark) photoperiod at approximately 120 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ metal arc light at 22°C. Pots containing 3-week-old seedlings were transferred to containers just taller than the pots. These were filled with dH₂O to simulate waterlogging for 7 days under the same light regime. Shoot samples were collected before, at the end and after the waterlogging treatment.

Mitochondrial inhibitor treatment

Antimycin A (AA, Sigma, <http://www.sigmaaldrich.com>) was prepared in a 0.01% solution of Tween-20 (Dojcinovic *et al.*, 2005). Salihydroxamic Acid (SHAM; Sigma) was dissolved in DMSO (1 M stock) then diluted in dH₂O to a final concentration of 5 mM (0.5% DMSO). Then 25 μM AA, 5 mM SHAM and the control solutions (0.01% Tween-20 and 0.5% DMSO) were sprayed onto 14 day old *Arabidopsis thaliana* seedlings on MS agar medium (3% sucrose, w/v). Plates were sealed and placed into LD growth conditions at 22°C for the specified times. Non-treated plants were used as the control samples.

Cycloheximide treatment

Ten *Arabidopsis thaliana* seedlings (14 days old) were placed into medium Petri dishes containing 15 ml liquid MS, 3% sucrose (v/v). The plates were sealed and placed onto a gentle shaker in LD at 22°C for 24 h. Cycloheximide was added (10 μM final concentration) to half the plates. The plates with and without cycloheximide were then exposed to hypoxic conditions or 4°C on a gentle shaker. The control conditions for the hypoxia treatments were atmospheric oxygen, while the control conditions for 4°C was 22°C on a gentle shaker.

RNA isolation, reverse transcription and quantitative real time RT-PCR (qPCR)

RNA was extracted from approximately 100 mg of seedlings and DNase I treated using the RNeasy Plant Mini Kit according to the manufacturer's instructions (Qiagen, <http://www.qiagen.com>). cDNA was synthesized using from 3 μg RNA using SuperScript II (Invitrogen, <http://www.invitrogen.com>) according to the manufacturer's instructions. cDNA was diluted 10-fold and then 5 μl was used in 20 μl final PCR reaction. Reaction and cycling conditions were as described in Klok *et al.* (2002). Details of the primers are provided in Table S3. RNA concentrations were normalized to *FORMALDEHYDE DEHYDROGENASE* (*FDH*; At5g43940), which is not affected by the stress conditions analysed in this study (data not shown). At least two biological replicates were measured for each qPCR result.

Microarray analysis

RNA from three biological replicates was prepared from Col:*FRI* control, Col:*FRI* 0.1% O₂, *vin3-4* control and *vin3-4* 0.1% O₂ samples. cDNA and cRNA preparation from total RNA, labelling and fragmentation of the cRNA, and hybridization to the NimbleGen *Arabidopsis thaliana* four-plex gene expression microarray was carried out by NimbleGen Systems. The four samples from each biological replicate were processed on the same slide. Quantile normalization was performed across replicate arrays (Bolstad *et al.*, 2003), and RMA (Robust Multichip Average) analysis was performed to generate gene expression values (Irizarry *et al.*, 2003). Further analysis of the data was carried out using ARRAYSTAR (DNASTAR Inc., <http://www.dnastar.com/>), including probabilistic statistical analyses (student *t*-tests, $P \leq 0.01$) that employed the FDR (Benjamini Hochberg) multiple testing correction to reduce the false discovery rate. The DNA microarray hybridization data has been submitted to ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>) with the accession number E-MEXP-2073.

Statistical analysis

For gene expression analysis the relative level of expression of each gene for each individual sample was averaged over the technical qPCR replicates, and normalized to the control gene (*FDH*). The normalized gene expression data was then Log₁₀-transformed and entered into the GenStat (11th Edition, VSN International, United Kingdom) statistical suite. A general analysis of variance (ANOVA), using a randomized block design model, was applied to examine statistical significance. For each gene, time of treatment or type of treatment was used for the treatment structure and the biological replicates as the blocks. For some analyses additional terms, such as genotype, were added to the treatment structure and are described in the respective figure legends. Significant *F* test *P*-values for the terms (treatment structure) in the ANOVA are stated in the figure legends. The resulting means were back-transformed and plotted. Error bars represent the standard errors of differences of the means (S.E.D) generated from the ANOVA. If the S.E.D error bars overlap then the data points are not statistically significant, according to an approximate Fisher's protected LSD test, at the 5% level of significance.

The survival assays and flowering time were analysed for statistical significance using the same ANOVA model as described above, but using the raw data as opposed to normalized data.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. *VIN3* expression in Col:*FRI* seedlings in response to low oxygen.

Figure S2. *ADH1* expression in cycloheximide treated seedlings.

Figure S3. Complementation of the vernalization response in *vin3-4* seedlings carrying a *VIN3pro::VIN3::HA* transgene.

Figure S4. Phylogenetic tree representing relationships between *VIN3* and related homologues.

Table S1 Comparison of microarray experiments analysing gene expression in response to hypoxic conditions.

Table S2 Expression of *OsVIL* genes. Obtained from RED database (<http://red.dna.affrc.go.jp/RED>).

Table S3 Primer sequences.

Supplementary text.

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