

RESEARCH PAPER

# Hypoxia-responsive microRNAs and *trans*-acting small interfering RNAs in *Arabidopsis*

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Received 20 July 2009; Revised 3 September 2009; Accepted 7 September 2009

## Abstract

**Low-oxygen (hypoxia) stress associated with natural phenomena such as waterlogging, results in widespread transcriptome changes and a metabolic switch from aerobic respiration to anaerobic fermentation. High-throughput sequencing of small RNA libraries obtained from hypoxia-treated and control root tissue identified a total of 65 unique microRNA (miRNA) sequences from 46 families, and 14 *trans*-acting small interfering RNA (tasiRNA) from three families. Hypoxia resulted in changes to the abundance of 46 miRNAs from 19 families, and all three tasiRNA families. Chemical inhibition of mitochondrial respiration caused similar changes in expression in a majority of the hypoxia-responsive small RNAs analysed. Our data indicate that miRNAs and tasiRNAs play a role in gene regulation and possibly developmental responses to hypoxia, and that a major signal for these responses is likely to be dependent on mitochondrial function.**

**Key words:** Abiotic stress, *Arabidopsis thaliana*, deep sequencing, hypoxia, microRNA, mitochondria, *trans*-acting small interfering RNA.

## Introduction

Waterlogging is major cause of reduced crop yields worldwide (Boyer, 1982). Initially, the foremost detrimental effect of waterlogging is the reduced access to oxygen in the rhizosphere, which is essential for mitochondrial respiration (Agarwal and Grover, 2006). Genome-wide expression studies in model plant species such as *Arabidopsis thaliana* have found that low oxygen (hypoxia) causes widespread changes in 5–10% of the transcriptome (Klok *et al.*, 2002; Branco-Price *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007; Kreuzwieser *et al.*, 2009). Co-incident with these mRNA changes are dramatic changes to protein synthesis and degradation, and a metabolic switch from aerobic respiration to anaerobic fermentation (Bailey-Serres and Voesenek, 2008).

Despite the wealth of molecular and phenotypic data on plant responses to waterlogging, very little is known about how declining oxygen levels are sensed, and how the complex and extensive expression changes are controlled

(Bailey-Serres and Chang, 2005). It is also not understood whether mitochondria play a passive role or are active in responding to decreased oxygen by retrograde regulation of nuclear genes (Rhoads and Subbaiah, 2007). To date only a few regulators of gene expression have been implicated in the hypoxia response in plants. Rice plants can increase their chance of surviving submergence in two antithetical pathways that are initiated by ethylene. Over-expression of *SUBMERGENCE 1A* (*SUB1A*) an AP2-EREBP type transcription factor aids survival under short-term flooding by conserving energy and carbohydrate consumption via the regulation of a suite of genes associated with carbohydrate metabolism, ethanolic fermentation, and cell expansion (Fukao *et al.*, 2006; Xu *et al.*, 2006), whereas, deepwater rice outgrows rising floodwaters aided by the expression of two AP2-ERFBP genes called *SNORKEL1,2* (*SK1*, *SK2*) that trigger internode elongation (Hattori *et al.*, 2009). In *Arabidopsis*, the transcription factor AtMYB2 is

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Abbreviations: smRNA, small RNA; miRNA, microRNA; tasiRNA, *trans*-acting small interfering RNA; QRT-PCR, quantitative reverse transcriptase PCR; SL-PCR, stem-loop quantitative PCR.

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induced early following hypoxia stress and trans-activates the *ALCOHOL DEHYDROGENASE (ADH)* promoter via the GT-motif (Hoeren *et al.*, 1998), aiding the metabolic shift to fermentation. Recently, VERNALIZATION INSENSITIVE 3 (VIN3) a chromatin remodelling protein was found to be required for the survival of *Arabidopsis* seedlings to hypoxic stress (Bond *et al.*, 2009), implicating histone modification as an important process in hypoxia. The *Arabidopsis* NAC domain containing gene *ANAC102* has been found to be necessary for efficient seed germination following hypoxia, but not for seedling survival (Christianson *et al.*, 2009), indicating that the response to hypoxia differs depending on developmental stage.

Small ribonucleic acids (smRNAs) have been identified as important post-transcriptional regulators of gene expression. Endogenous smRNAs in plants can be divided into two broad classes, microRNAs (miRNAs) and small interfering RNAs (siRNAs), based on their biogenesis and function. The best characterized are the miRNAs which are ~21 nucleotides (nt) in size and generated from non-coding transcripts capable of forming stable secondary structures. In plants, miRNAs often regulate groups of transcription factors, and are thought to act by targeting almost perfectly complementary mRNAs for endonucleolytic cleavage and translational inhibition (Vazquez, 2006; Brodersen *et al.*, 2008). siRNAs can be divided into a number of classes, including *cis*-acting siRNA (casiRNAs), *trans*-acting siRNA (tasiRNA), long siRNA (lsiRNA), and natural antisense transcript siRNA (natsiRNA) (for a review see Ghildiyal and Zamore, 2009). tasiRNAs and natsiRNAs are thought to regulate target transcripts in a similar manner to miRNAs, whereas lsiRNAs are thought to destabilize target mRNAs causing rapid degradation. casiRNAs promote heterochromatin formation by directing DNA methylation and histone modification.

Although the majority of characterized smRNAs have been associated with plant development (Bonnet *et al.*, 2006; Sunkar *et al.*, 2007) a growing number of smRNAs have been demonstrated to be involved in abiotic and biotic stresses such as cold (Sunkar and Zhu, 2004; Lu *et al.*, 2008; Zhou *et al.*, 2008), salt (Sunkar and Zhu, 2004; Borsani *et al.*, 2005; Lu *et al.*, 2008; Ding *et al.*, 2009), heat (Lu *et al.*, 2008), dehydration (Sunkar and Zhu, 2004; Reyes and Chua, 2007), oxidative stress (Sunkar *et al.*, 2006), mechanical stress (Lu *et al.*, 2008), pathogen infection (Katiyar-Agarwal *et al.*, 2006), and submergence in maize (Zhang *et al.*, 2008). The regulation of smRNAs appears to play an important role in transcriptome adjustments to life-threatening stresses.

In this study, high throughput sequencing technology was used to investigate the role of smRNAs in the response of *Arabidopsis* to hypoxia. Our results identified a total of 65 unique miRNA sequences (from 46 families) and 14 tasiRNAs (from three families) present in hypoxia treated and control root tissue. Of these, 25 miRNA (19 families) and 10 tasiRNAs (three families) were found to have significantly changed abundance upon hypoxia. Inhibition of mitochondrial respiration caused similar changes to the

levels of several smRNAs, indicating that these smRNAs may play a significant and specific role in plant responses to hypoxia.

## Materials and methods

*Arabidopsis* Columbia-0 seeds were surface-sterilized in 0.5% (w/v) sodium hypochlorite. Seeds were sown on 100 mm Petri plates containing a modified Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose (w/v) and 0.8% (w/v) agar and were subjected to at least 24 h of cold treatment in the dark (4 °C) to break dormancy. Each plate contained approximately 40 seeds and were grown at 21±1 °C under cool-white fluorescent lights (120 µM m<sup>-2</sup> s<sup>-1</sup>) with a 16 h photoperiod.

### Hypoxia assay

Hypoxia assays were performed as outlined previously (Christianson *et al.*, 2009). Briefly, 15 2.5-week-old plants were transferred to 100 mm Petri plates containing 15 ml of MS liquid media supplemented with 3% sucrose 1 d prior to treatment. Immediately prior to treatment, plants were transferred to liquid media which had been sparged with 0.1% O<sub>2</sub> and then placed in 3.5 l anaerobic chambers (Oxoid, Adelaide, SA, Australia) and purged with 0.1% O<sub>2</sub>, 99.9% N<sub>2</sub> at a flow rate of ~5 l min<sup>-1</sup> for 15 min. The plants were left in 0.1% O<sub>2</sub> in the dark with gentle shaking for a designated period. Control plants were subjected to the same treatment except that they were not transferred to 0.1% O<sub>2</sub> sparged media, and the chambers were not sparged with 0.1% O<sub>2</sub>. Immediately after the treatment, plants were removed from the chamber and a scalpel used to isolate root tissue, which was then placed in liquid nitrogen, and stored at -80 °C.

### Mitochondrial inhibitor treatment

The mitochondrial inhibitor assay was performed as outlined previously (Bond *et al.*, 2009) except DMSO was used as a solvent for AA (Sigma-Aldrich, St Louis, Missouri, USA). Fifteen 2-week-old plants were transferred to 100 mm Petri plates containing 15 ml of MS liquid media supplemented with 3% sucrose 1 d prior to treatment. The media were replaced with MS liquid media containing 3% sucrose, 25 µM AA, and 5 mM of SHAM (Sigma-Aldrich, St Louis, Missouri, USA) and 0.4% DMSO. The plants were gently shaken for 5 h. The control treatment consisted of MS liquid media supplemented with 3% sucrose and 0.4% DMSO. Immediately after the treatment, root tissue was collected, placed in liquid nitrogen, and stored at -80 °C.

### RNA extraction

Frozen root tissue was ground to a fine white powder using a mortar and pestle. Total RNA extraction was performed using TRI Reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) following manufacturer's instructions.

The smRNA fraction was isolated from total RNA by selectively precipitating HMW RNA in 0.5 vol. of 3 M NaCl/30% PEG (MW 8000) (Lu *et al.*, 2007). The smRNA fraction obtained represented approximately 5% of the total RNA.

### High throughput sequencing

Size-selected small RNAs (16–30 nt) was obtained from total RNA by size fractionation and sequenced by GeneWorks Pty Ltd. (Hindmarsh, SA, Australia) using high throughput pyrosequencing technology developed by Illumina, using an Illumina 1G Genome Analyzer, following the manufacturer's instruction's. All small RNA sequences have been deposited in the Gene Expression Omnibus in GenBank under the identifier GSE16971 and GPL8810.

### Bioinformatic analysis of small RNAs

Illumina sequencing data were received in FASTQ format, with all reads shown as 36 mers prior to the removal of the 3' adaptor sequence. A custom script was created to find and trim the adaptor sequence from each sequence (Zhu *et al.*, 2008). SOAP (Li *et al.*, 2008) was used to align the reads to the *Arabidopsis* genome sequence (TAIR8), allowing for one mismatch and each mapped location was annotated with genomic region and/or gene names. A summary of the origin of the small RNA fragments can be found in Supplementary Table S1 at *JXB* online. Reads perfectly matching known *Arabidopsis* miRNA or miRNA\* from the miRBase (Griffiths-Jones, 2004) and/or miRNA from the ASRP database (Gustafson *et al.*, 2005) were identified.

### Statistical analysis of miRNA frequency

To determine whether differences in miRNA frequency between control and hypoxia treated samples was significant; a  $\chi$ -squared test was performed using total sequence numbers as previously described by Qiu *et al.* (2009).

### Promoter analysis

Promoter regions from selected miRNAs were screened for the presence of common motifs using the web-based analysis tool PlantPAN (Chang *et al.*, 2008). Since mature miRNAs of the same sequence can originate from different loci, only the promoter regions from unique mature miRNAs whose frequency was altered  $\geq 1.5$ -fold in the hypoxia treatment were used for promoter analysis. Promoter sequences for miRNAs were extracted from TAIR8 (Rhee *et al.*, 2003). In each case 1000 bp upstream (minus any coding region from upstream genes) from the designated start of the miRNA stem-loop was used, and miRNA promoter regions with  $\leq 50$  bp were discarded. A total of 13 miRNA promoters were used for the analysis (miR156g, miR157d, miR158a, miR158b, miR159a, miR161, miR167d, miR169a, miR173, miR391, miR775, miR824, miR829). The default settings on PlantPan were used at all times, for both co-occurrence of transcription factor binding sites in gene group.

### Confirmation of mature miRNA expression by stem-loop quantitative PCR (SL-PCR)

The expression profiles of mature miRNAs and tasiRNAs were assayed by SL-PCR and performed as described previously (Varkonyi-Gasic *et al.*, 2007). The stem-loop reverse transcription primers were designed following the methods previously described (Chen *et al.*, 2005; Varkonyi-Gasic *et al.*, 2007) and are listed in Supplementary Table S3 at *JXB* online. The stem-loop reverse transcriptase primer for each miRNA consists of a selfed stem-loop sequence (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC) with the specificity conferred by a six nucleotide extension at the 3' end, that is complementary to the last six nucleotides at the 3' end of mature miRNA. The RT reactions were performed on 100 ng LMW RNA using Superscript III (Invitrogen Australia Pty Ltd., Vic, Australia) and carried out according to the manufacturer's instructions. For each LWM RNA sample two cDNA reactions were performed. One containing all miRNA and tasiRNA stem-loop RT primers, and one for the housekeeping control U6 (see Supplementary Table S4 at *JXB* online). The reverse transcription product was amplified using a miRNA or tasiRNA specific forward primer and a universal reverse primer on a Rotor-Gene 6000 (QIAGEN Pty Ltd, Victoria, Australia). Reactions were performed in triplicate on three independent biological replicates, and amplicons from representative samples were sequenced to confirm the specificity of the amplification. The comparative quantification procedure was used to determine relative expression levels as previously described (Wilson *et al.*, 2005).

### Quantitative reverse transcriptase PCR (QRT-PCR) of miRNA target gene expression

The expression of experimentally determined and predicted target genes were assayed by QRT-PCR as previously described by Wilson *et al.* (2005). The reverse transcription product was amplified using gene-specific primers that generated amplicons that overlapped the known or predicted cleavage site (see Supplementary Table S5 at *JXB* online). Reactions were performed in triplicate on a Rotor-Gene 6000 (Qiagen Pty Ltd, Victoria, Australia). Data were normalized to AT5G08290 (Czechowski *et al.*, 2005) and analysed using a comparative quantification procedure (Wilson *et al.*, 2005).

## Results

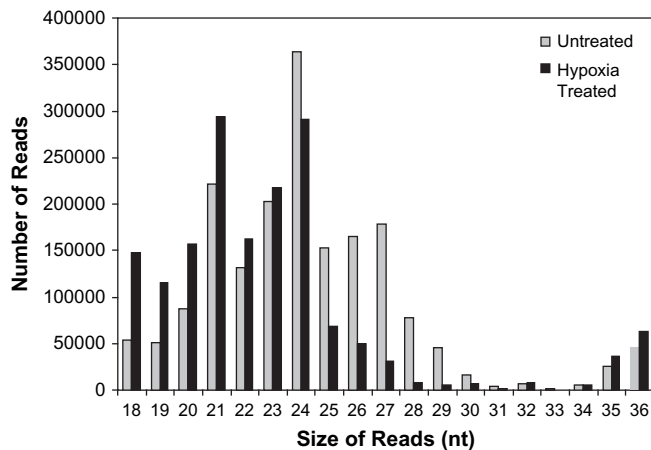
### High throughput sequencing reveals a complex small RNA population in plant roots

To investigate the role of smRNAs in hypoxia stress in *Arabidopsis*, size fractionated RNA (16–30 nucleotides) was isolated from root tissue subjected to 5 h of either 0.1% oxygen or normoxic conditions, and sequenced using Illumina high throughput sequencing. Five hours of hypoxia treatment was chosen as hypoxia responses in the roots are significant at this time as indicated by the 88-fold induction of the hypoxia responsive gene, *ADH* (see Supplementary Fig. S1 at *JXB* online), but does not result in root tip death (Ellis *et al.*, 1999). The total number of sequence reads was 2 550 664 for the normoxic sample and 2 998 711 for the hypoxia stressed sample (Table 1). After removing adaptor only sequences and sequences of sizes <18 nt there were 383 969 and 390 392 unique sequences, respectively. This totalled 698 762 unique RNA sequences across the two samples. The percentage of unique RNA sequences with only a single read (singleton) provides an indication of sequence coverage and the complexity of the sample. The unstressed sequences comprised 80% singletons, whereas hypoxia-stressed sequences contained 81% singletons, indicating that the sequencing of the RNA population had not reached saturation.

The RNA sequences were mainly within a range of 20–24 nt (61%) the size of most known smRNAs (Fig. 1). Hypoxia stress resulted in fewer 24 nt sequences and an increase in 21

**Table 1.** Summary of small RNA sequencing data from control and hypoxia treated roots

	Untreated sample	Treated sample
Total reads	2 550 664	2 998 711
Reads containing N	32 278	7 152
Reads without N	2 518 386	2 991 559
Adaptor only sequences	268 521	334 287
Low quality sequences removed	4 186	8 215
Sequences with small insert (<18 nt)	444 444	997 892
Sequences with large insert (>26 nt)	406 465	164 682
Unique sequences	383 969	390 392



**Fig. 1.** Size distribution of sequenced reads from control and hypoxia-treated roots. Small RNAs (16-30 nt) was isolated from untreated (grey bars) and hypoxia-treated (0.1% O<sub>2</sub> for 5 h (black bars) root samples and sequenced using a Illumina 1G Genome Analyzer. Sequences were trimmed of their adapter sequence.

nt sequences. Overall, the size distribution was similar to other previous studies on angiosperms (Fahlgren *et al.*, 2007; Nobuta *et al.*, 2008; Szittyta *et al.*, 2008; Zhu *et al.*, 2008).

#### Sixty-five unique sequences match known miRNAs

The small RNA sequences from the hypoxia-stressed and control samples were analysed for the presence of previously characterized miRNAs. Only sequence reads that perfectly matched known *Arabidopsis* miRNA or miRNA\* from miRBase (Griffiths-Jones, 2004) were considered. A total of 65 unique sequences from the combined RNA sequence reads matched known miRNAs (Table 2). These miRNAs belonged to 46 miRNA families and represent 45% of all known unique *Arabidopsis* miRNA sequences and 41% of all known miRNA families. Comparing normalized root miRNA frequencies (combined control and stressed RNAs) from this study to leaf and whole plant populations present in the *Arabidopsis* Small RNA Project (ASRP) database (Gustafson *et al.*, 2005) showed that most miRNA families have tissue-specific expression (see Supplementary Table S2 at *JXB* online). For example, miRNA families 156, 157, 158, 165, and 829 are observed more frequently in roots than in leaf or whole plant tissue, whereas families 167, 169, 170, 171a, 172, 319, 396, 397, and 398 are low or absent in roots, but are numerous in leaf or whole plant samples.

High throughout sequencing can identify not only miRNAs but can also accurately measure miRNA expression (t Hoen *et al.*, 2008). The abundance of miRNAs was found to vary greatly within each sample and within miRNA families (Table 2). The most abundant miRNA identified was the miR166 family ( $n=164\ 433$ ), although most miRNAs were little expressed, as almost half of the miRNAs identified had fewer than 10 reads in total.

Statistical analysis of the frequency of miRNAs (Qiu *et al.*, 2009) between the two treatments revealed that hypoxia treatment significantly changed the abundance of 25 miRNAs from 19 miRNA families ( $P$  value  $\leq 0.01$ ) with the majority of those changing by  $\geq 1.5$ -fold. In most cases, this was an increase in miRNA frequency, with only five miRNAs (from four miRNA families) in the hypoxia sample experiencing a significant decrease in expression (Table 2). Apart from miR169, multiple members of a miRNA family were regulated similarly (e.g. miR156a-f and g, and miR158a, b). These results indicate that hypoxia alters the expression of a specific subset of miRNAs.

#### Common cis-acting elements within promoter regions of miRNAs affected by hypoxia

The promoter regions of miRNAs have the same type of promoters as protein coding genes and are transcribed by RNA polymerase II (Megraw *et al.*, 2006; Zhou *et al.*, 2007). However, analysis of the promoters of miRNAs is difficult, as mature miRNAs with identical sequence can originate from different loci. In order to determine whether miRNAs with altered expression under hypoxia possessed common regulatory motifs, promoter regions from unique mature miRNAs sequences whose frequency was altered  $\geq 1.5$ -fold in the hypoxia treatment were used for motif analysis (12 miRNAs). Analysis using Plant Promoter Analysis Navigator (PlantPAN) (Chang *et al.*, 2008) indicated that two known variants of the homeobox domain leucine zipper class 1 (HDZip I) promoter motif, ATHB-5 (CAATTATTG,  $P$  value= $4E^{-6}$ ) and ATHB1 (NYNCAAT-TATTGSA,  $P$  value= $4E^{-9}$ ) were significantly overrepresented.

#### tasiRNAs change under hypoxia stress

Another major class of smRNA capable of altering the expression of genes is tasiRNA; although tasiRNA expression has not been found to alter with abiotic stress. Sequences were compared against known *Arabidopsis* tasiRNAs and 14 unique sequences (61% of total known sequences) were recognized as fully matching known tasiRNAs (Table 3). The tasiRNAs identified belonged to three of the four known tasiRNA families, TAS1, TAS2, and TAS3. All of the tasiRNAs ( $n=10$ ) that were found to change significantly under hypoxia stress showed increased abundance under hypoxia.

#### SL-PCR verifies deep sequencing results

As only one sample of stressed and unstressed RNA was sequenced, it was necessary to examine the expression of a selection of smRNAs to verify that the changes observed were biologically consistent. The SL-PCR protocol of Chen *et al.* (2005), that uses stem-loop primers in the reverse transcriptase reaction, was used to measure mature smRNA expression on at least three independent replicate samples from untreated and hypoxia-stressed roots. QRT-PCR was conducted for seven miRNAs, and one tasiRNA that

**Table 2.** Known miRNAs present in hypoxia treated and control roots

miRNA family	miRNA	Sequence	Untreated reads	Hypoxia reads	Relative change <sup>a</sup>
156	156a,b,c,d,e,f	UGACAGAAGAGAGUGAGCAC	4 382	6 958	1.35*
	156g	CGACAGAAGAGAGUGAGCAC	17	55	2.75*
	156h	UGACAGAAGAAAGAGAGCAC	2	5	2.13
157	157a,b,c	UUGACAGAAGAUAGAGAGCAC	4 119	8 409	1.74*
	157d	UGACAGAAGAUAGAGAGCAC	58	282	4.14*
158	158a	UCCCAAUUGUAGACAAAGCA	2 427	7 981	2.80*
	158b	CCCCAAUUGUAGACAAAGCA	34	69	1.73*
159	159a	UUUGGAUUGAAGGGAGCUCUA	73	162	1.89*
	159b	UUUGGAUUGAAGGGAGCUCUU	11	14	1.08
160	160a,b,c	UGCCUGGCUCCUGUAUGCCA	1	2	1.70
161	161.1	UGAAAGUGACUACAUCCGGGU	39	65	1.42*
	161.2	UCAAUUGCAUUGAAAGUGACUA	20	119	5.06*
163	163	UUGAAGAGGACUUGGAACUUCGAU	2	4	1.70
164	164a,b	UGGAGAAGCAGGGCAGUGCA	2 362	3 151	1.13*
	164c	UGGAGAAGCAGGGCAGUGCG	452	550	1.04
165	165a,b	UCGGACCAGGCUUCAUCCCC	14 977	15 750	0.89*
166	166a,b,c,d,e,f,g	UCGGACCAGGCUUCAUCCCC	79 589	84 844	0.91*
167	167a,b	UGAAGCUGCCAGCAUGAUCUA	1 429	1 709	1.02
	167d	UGAAGCUGCCAGCAUGAUCUGG	1	4	3.40*
168	168a,b	UCGCUUGGUGCAGGUCGGGAA	7 584	9 932	1.11*
169	169a	CAGCCAAGGAUGACUUGCCGA	10	3	0.26*
	169b,c	CAGCCAAGGAUGACUUGCCGG	1	8	6.80*
	169d,e,f,g	UGAGCCAAGGAUGACUUGCCG	92	61	0.56*
	169h,i,j,k,l,m,n	UAGCCAAGGAUGACUUGCCUG	3	6	1.70
171	171a	UGAUUGAGCCGCGCCAUAUUC	0	2	NA**
172	172a, b	AGAAUCUUGAUGAUGCUGCAU	15	93	5.27*
	172c,d	AGAAUCUUGAUGAUGCUGCAG	2	2	0.85
	172e	GGAAUCUUGAUGAUGCUGCAU	4	2	0.43
	173	173	UUCGCUUGCAGAGAGAAUAC	213	375
319	319a,b	UUGGACUGAAGGGAGCUCUCCU	1	0	NA
390	390a,b	AAGCUCAGGAGGGAUAGCGCC	1 652	3 124	1.61*
391	391	UUCGCAGGAGAGAUAGCGCCA	25	77	2.62*
395	395a,d,e	CUGAAGUGUUUGGGGGAACUC	1	2	1.70
	395b,c,f	CUGAAGUGUUUGGGGGGACUC	2	5	2.13
399	399a	UGCCAAAGGAGAUUUGCCUUG	1	1	0.85
	399b,c	UGCCAAAGGAGAGUUGCCUUG	4	3	0.64
	399d	UGCCAAAGGAGAUUUGCCCG	5	1	0.17
	399f	UGCCAAAGGAGAUUUGCCCGG	11	12	0.93
400	400	UAUGAGAGUAUUUAAGUCAC	2	1	0.43
403	403	UUAGAUUCACGCACAACUCG	2	4	1.70
408	408	AUGCACUGCCUCUCCUUGGC	125	138	0.94
447	447a,b	UUGGGGACGAGAUUUUUGUUG	5	3	0.51
775	775	UUCGAUGUCUAGCAGUGCCA	9	27	2.55*
780	780.1	UCUAGCAGCUGUUGAGCAGGU	0	1	NA
822	822	UGCGGAAGCAUUUGCACAUG	115	121	0.89
823	823	UGGGUGGUGAUCUAUAAGAU	6	10	1.42
824	824	UAGACCAUUUGUGAGAAGGGA	20	39	1.66*
827	827	UUAGAUGACCAUCAACAAACU	10	20	1.70
829	829.1	AGCUCUGAUACCAAUUGAUGGAU	1 564	1 228	0.67*
	829.2	CAAAUUAAAGCUUCAAGGUAG	1	0	NA
830	830	UAACUAUUUUGAGAAGAAGUG	1	0	NA
837	837-3p	AAACGAACAAAAACUGAUGG	14	27	1.64*
	837-5p	AUCAGUUUCUUGUUCGUUUA	1	1	0.85
838	838	UUUUCUUCUACUUCUUGCACA	0	1	NA
841	841	UACGAGCCACUUGAAACUGAA	1	0	NA
842	842	UCAUGGUCAGAUCCGUCAUCC	23	24	0.89
843	843	UUUAGGUCGAGCUUCAUUGGA	0	2	NA

**Table 2.** *Continued*

miRNA family	miRNA	Sequence	Untreated reads	Hypoxia reads	Relative change <sup>a</sup>
846	846	UUGAAUUGAAGUCUUGAAUU	3	3	0.85
848	848	UGACAUUGGACUGCCUAAGCUA	10	14	1.19
852	852	AAGAUAGCGCCUUGAUUCUG	1	2	1.70
860	860	UCAAUAGAUUGGACUAUGUAU	0	2	NA
861	861-5p	CCUUGGAGAAUUGCGUCAA	1	0	NA
862	862-5p	UCCAUAJAGGUCGAGCAUGUGC	0	3	NA
869	869.2	UCUGGUGUUGAGAUAGUUGAC	50	57	0.97
1888	1888	UAAGUUAAGAUUUGUGAAGAA	0	2	NA

<sup>a</sup> Relative change is the ratio of hypoxia-treated sample to the control and takes into account the differences in total read between samples. \**P* value ≤0.01. \*\*Relative change was not calculated as they contained 0 reads in one sample.

**Table 3.** Known tasiRNA present in control and hypoxia treated roots

tasiRNA	TAS locus	Sequence	Untreated reads	Hypoxia reads	Relative change <sup>a</sup>
1786	TAS1a	UGAUUUUUGUAGUAAUGGCG	1	6	5.10*
1852	TAS1a	AUGAUUUUUGUAGUAAUGGCG	430	863	1.71*
255	TAS1a,b,c	UUCUAAGUCCAACAUAGCGUA	20	61	2.59*
289	TAS1a,b,c	UUCUAAGUCCAACAUAGCAUA	3	16	4.54*
619	TAS1c	AUAUCCAGGAUUGCAAAG	0	1	NA**
850	TAS1c	UUCUAAGUCCAACAUAGCGAC	0	2	NA
1413	TAS1c	UUCUAAGUCCAACAUAGCGACG	0	1	NA
143	TAS2	AUAUCAAGUGAAUAGUUUAA	3	9	2.55*
614	TAS2	GAUGGUAGUCCAAGUAAUCCA	7	21	2.55*
1511	TAS2	UCCAAGCGAAUGAUUACU	25	90	3.06*
1767	TAS2	UUUGAACUUGUGUAAUUUGAA	10	26	2.21*
1940	TAS2	GAAUACUUGAACUACCAUCUA	6	4	0.57
2140	TAS2	AUAUCCAUUUCUACCAUCUG	2	14	5.95*
2142	TAS3a	UUCUUGACCUUGUAGACCCC	337	469	1.18*

<sup>a</sup> Relative change is the ratio of hypoxia-treated sample to the untreated control and takes into account differences in total read between samples. \**P* value ≤0.01; \*\* Relative change was not calculated as they contained 0 reads in one sample.

showed significant increases in frequency under hypoxia stress based on the deep sequencing data. The QRT-PCR results were similar in magnitude to the deep sequencing results and confirm that smRNAs identified from high throughput sequencing change in response to hypoxia treatment (Table 4).

#### *Inhibition of mitochondrial respiration induces the expression of hypoxia responsive miRNAs and tasiRNA*

Hypoxia is associated with a reduction in mitochondrial respiration (for a recent review see Igamberdiev and Hill, 2009). Inhibitors of mitochondrial respiration pathways were used to determine whether the alterations in mitochondrial respiration that resulted, also cause induction to hypoxia-induced smRNAs. 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). In combination, these inhibitors induced *ADH* expression in roots by 10-fold, similar to changes

**Table 4.** Expression levels of selected miRNAs and a tasiRNA under hypoxia stress and mitochondrial inhibitor treatment

smRNA	Hypoxia		Mitochondrial inhibitor
	Sequencing	QRT-PCR <sup>a</sup>	QRT-PCR <sup>a</sup>
miR156g	2.75	3.9 <sup>b</sup>	2.6
miR157d	4.14	3.2	2.5
miR158a	2.8	3.2	2.0
miR159a	1.89	3.8	2.0
miR172a, b	5.27	2.8	1.1
miR391	2.62	4.0	2.0
miR775	2.55	1.9	2.1
tasiR289 (Tas1a,b,c)	4.54	13	1.9

<sup>a</sup> Average of at least three biological replications performed in triplicate.

<sup>b</sup> Relative change is the ratio of hypoxia-treated sample to the control.

previously observed by Bond *et al.* (2009). Of the set of seven miRNAs and one tasiRNA verified by SL-PCR to be induced by hypoxia, only one, miR172, was not induced by

the mitochondrial inhibitors, suggesting that mitochondrial respiration may be a major factor in smRNA regulation under hypoxia (Table 4).

#### miRNA and tasiRNA target expression analysis

Genes targeted by miRNA and tasiRNAs are thought to be regulated in plants mainly via endonucleolytic cleavage of mRNAs due to their near-perfect complementarity to their targets, although recent studies indicate the existence of widespread translational inhibition (Brodersen *et al.*, 2008). A large proportion of miRNAs target transcripts that encode transcription factors required for growth, development, and stress responses. The targets (either experimentally determined or predicted) of 42 of the verified hypoxia induced smRNAs were assayed by QRT-PCR (Table 5). The high molecular weight (HMW) fraction of RNA, taken from the same plants used for miRNA and tasiRNA QRT-PCR verification, was used. Two biological replicates of hypoxia-stressed and unstressed samples were tested.

Many of the potential target genes assayed were transcription factors of the Squamosa Promoter Binding-like (SPL), MYB, and AP2-EREBP classes. Twelve of the targets had more than a 33% decrease in expression, indicating that they may be actively cleaved by smRNAs induced by hypoxia (Table 5). Overall, there was no obvious pattern in the expression of target genes under hypoxia stress, as the magnitude of the change varied within predicted targets for specific smRNAs. For example, miR172a, b had six potential targets (five experimentally determined and one predicted); three showed decreased expression, one had increased expression, and two experienced almost no change (Table 5). However, the significant number of target genes down-regulated suggests that miRNA and tasiRNAs do play a role in regulating gene expression under hypoxia stress. The genes potentially regulated by hypoxia stress-responsive smRNAs, encode five SPL proteins, three AP2-EREBP proteins, and four proteins of unknown function.

## Discussion

High throughput sequencing of smRNAs from roots subjected to hypoxia revealed a large and complex population of smRNAs. Apart from known miRNA and tasiRNA our smRNA datasets were also analysed for tasiRNAs that are implicated in gene regulation during stress (Borsani *et al.*, 2005). Many putative tasiRNA were identified (see Supplementary Table S6 at *JXB* online), however, they usually contained very few reads and were not obviously hypoxia regulated.

Analysis of publically available information on miRNA frequency in leaf tissue and whole seedlings shows that the majority of the miRNAs identified in this study have tissue preferential expression (see Supplementary Table S2 at *JXB* online). Therefore, it is likely that miRNAs play an important role in root-specific processes. Hypoxia treatment

**Table 5.** QRT-PCR relative expression of experimentally determined or predicted gene targets of selected smRNAs

Targets in bold have been verified experimentally.

smRNA	Target genes	Relative change by QRT-PCR <sup>a</sup>	Target gene details
miR156g	<b>AT1G27360</b>	<b>1.27</b>	<b>Squamosa promoter-binding-like 11</b>
&	<b>AT1G27370</b>	<b>0.47</b>	<b>Squamosa promoter-binding-like 10</b>
miR157d	<b>AT1G53160</b>	<b>0.41</b>	<b>Squamosa promoter-binding-like 4</b>
	<b>AT1G69170</b>	<b>1.29</b>	<b>Squamosa promoter-binding-like 6</b>
	<b>AT2G42200</b>	<b>0.62</b>	<b>Squamosa promoter-binding-like 9</b>
	<b>AT3G15270</b>	<b>1.64</b>	<b>Squamosa promoter-binding-like 5</b>
	<b>AT3G57920</b>	<b>0.31</b>	<b>Squamosa promoter-binding-like 15</b>
	<b>AT5G43270</b>	<b>0.55</b>	<b>Squamosa promoter-binding-like 2</b>
	<b>AT5G50570</b>	<b>1.16</b>	<b>Squamosa promoter-binding-like 13A</b>
	<b>AT5G50670</b>	<b>AS ABOVE*</b>	<b>Squamosa promoter-binding like 13B</b>
miR157d	<b>AT2G33810</b>	<b>1.85</b>	<b>Squamosa promoter-binding-like 3</b>
miR158a	AT1G64100	3.01	Pentatricopeptide repeat-containing
	AT3G03580	1.2	Pentatricopeptide repeat-containing
miR159a	AT2G26950	1.99	Myb domain 104
	<b>AT2G26960</b>	<b>4.57</b>	<b>Myb domain 81</b>
	<b>AT2G32460</b>	<b>2.56</b>	<b>Myb domain 101</b>
	<b>AT2G34010</b>	<b>0.24</b>	<b>Unknown</b>
	<b>AT3G11440</b>	<b>1.72</b>	<b>Myb domain 65</b>
	<b>AT3G60460</b>	<b>4.68</b>	<b>Duo Pollen 1 (R2R3 Myb domain)</b>
	<b>AT4G37770</b>	<b>0.85</b>	<b>1-Amino-cyclopropane-1-carboxylate synthase 8</b>
	<b>AT5G06100</b>	<b>1.31</b>	<b>Myb domain 33</b>
	<b>AT5G18100</b>	<b>1.99</b>	<b>Copper/Zinc superoxide dismutase 3</b>
	<b>AT5G55020</b>	<b>ND**</b>	<b>Myb domain 120</b>
miR172a,b	<b>AT2G28550</b>	<b>0.46</b>	<b>Target of Eat1 1</b>
	AT2G39250	1.85	Schnarchzapfen
	<b>AT3G54990</b>	<b>1.01</b>	<b>Schlafmutze</b>
	<b>AT4G36920</b>	<b>1.07</b>	<b>Apetala 2</b>
	<b>AT5G60120</b>	<b>0.32</b>	<b>Target of Eat1 2</b>
	<b>AT5G67180</b>	<b>0.57</b>	<b>Target of Eat1 3</b>
miR391a	At1g50990	6.49	Protein kinase
miR775	<b>AT1G53290</b>	<b>0.69</b>	<b>Galactosyltransferase family</b>
tasiR289 (TAS1)	At1g15940	ND**	Unknown
	At1g51670	0.23	Unknown, contains domain cysteine proteinases

Table 5. Continued

smRNA	Target genes	Relative change by QRT-PCR <sup>a</sup>	Target gene details
	At1g68840	2.57	Regulator ATPase of the vacuolar membrane (RAP2.8)
	At2g42090	6.15	Actin 9
	At3g27230	0.31	Unknown
	At3g61320	0.67	Chloroplast precursor
	At4g05360	4.65	Zinc knuckle (CCHC-type) family
	At4g29760	ND**	Unknown, contains domain cysteine proteinases
	<b>At4g29770</b>	<b>3.91</b>	<b>Unknown</b>
	<b>At5g18040</b>	<b>2.44</b>	<b>Unknown, contains domain cysteine proteinases</b>
	At5g18065	5.13	Unknown, contains domain cysteine proteinases
	At5g42670	1.09	Agenet domain-containing
	At5g52070	1.25	Agenet domain-containing

<sup>a</sup> Relative change is the ratio of hypoxia-treated sample to the control. The value is an average of at least two biological replications performed in triplicate. \*Primers amplified both these genes. \*\*Not detected (ND).

on roots resulted in changes to 25 unique miRNAs (19 families) and 10 unique tasiRNAs (three families; Table 2), the majority of these increased under hypoxia, indicating that miRNAs are mainly involved in down-regulating gene expression under hypoxia. Of the hypoxia-regulated miRNAs, only three families (miR166, miR169, and miR172) have previously been reported to be responsive to another abiotic stress in *Arabidopsis* (cold stress) (Zhou et al., 2008). Of the five miRNAs originally reported by Sunkar and Zhu (2004), as either cold, dehydration, NaCl, or ABA responsive (miR393, miR397b, miR402, miR319c, and miR389a.1), none were sequenced in either root small RNA sample. Heterologous microarray analysis on low-oxygen-stressed maize root cells identified 15 submergence-responsive miRNA families (nine maize families and six families from three other plant species), four of which were common with this study (miR159, miR166, miR167, and miR168). Of these, two families have different predicted target genes from the *Arabidopsis* miRNAs, indicating that smRNAs regulated by hypoxia may be species specific. Until now, tasiRNAs have not been found to be responsive to abiotic stresses, although few studies have tracked their abundance. The uniqueness of these results may reflect the lack of other root-specific small RNA analyses, or indicate that many of these miRNAs may target hypoxia-specific processes. The latter is supported by the replication of some of the hypoxia responses by chemical inhibition of mitochondrial respiration (Table 4).

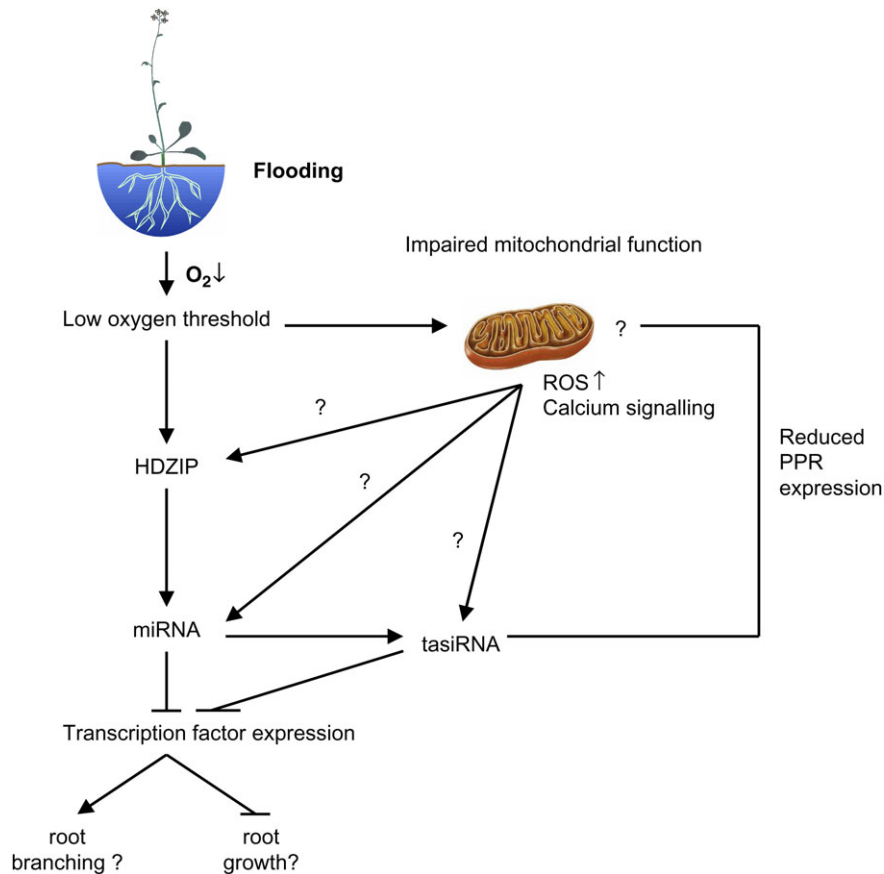
Although there was little overlap with other abiotic stresses in *Arabidopsis*, there is a surprising degree of overlap between hypoxia-responsive miRNAs and virus-induced miRNAs. *Arabidopsis* infected with tobacco mosaic virus-Cg (TMV-Cg) was found to induce 20 miRNAs families of which 15 are also hypoxia-responsive (Tagami et al., 2007); seven of these hypoxia-responsive miRNA families were also induced in tobacco infected with TMV (Bazzini et al., 2007). However, infection with TMV-Cg did not significantly affect the percentage of tasiRNAs (Tagami et al., 2007). These results indicate that either specific cross-talk exists between miRNA pathways for hypoxia and virus infection, or that the two stresses affect similar cellular processes such as energy production.

Analysis of hypoxia-responsive miRNAs with promoter sequences that could be unambiguously determined (12 miRNAs in total), revealed an over-representation of two HDZip I motifs (ATHB1 and ATHB-5). It is known that in order for HDZip I proteins to bind DNA, homo- or heterodimer formation with proteins of the same class is required, and that they can activate or repress transcription (Singh, 1998). Many *HDZip I* genes are regulated by environmental factors such as drought, extreme temperatures, osmotic stresses, and illumination conditions and are active in leaf light perception signalling, cotyledon and leaf development, and ABA-related and abiotic stress responses (Henriksson et al., 2005). Of the 16 *HDZip I* genes in *Arabidopsis* that have extensive publicly available gene expression information, 10 were found to be down-regulated and two up-regulated, early in hypoxia stress treatments (Zimmermann et al., 2004). Therefore, it is possible that members of this group may directly regulate the expression of a subset of these hypoxia-responsive miRNAs, and indicate that changes in miRNA frequencies occur after early hypoxia-responsive transcription factors (Fig. 2).

QRT-PCR verification of the putative targets for eight hypoxia-induced smRNAs confirmed a number of genes had the expected change in steady-state levels of mRNA if targeted for endonucleolytic cleavage (12 out of 42 targets). However, it has recently been shown that, in addition to RNA cleavage, miRNAs in plants have widespread translational repressor capability (Brodersen et al., 2008), so many of these targets may in fact be regulated through this translational pathway but not be detected using QRT-PCR and will require further validation. Hypoxia is known differentially to affect translational rates of specific mRNA, but few (3 out of 44) of the potential targets of hypoxia-induced smRNAs are known to possess altered translation rates based on polysome analysis (Branco-Price et al., 2008). Analysis of the spatial expression of miRNAs has shown that they often possess specific tissue patterns (Valoczi et al., 2006). For example, the hypoxia-responsive miR159a is strongly expressed in root tips and lateral roots. Putative miR159a target genes expressed throughout the root may not show a change in steady-state mRNA levels when whole roots are assayed.

The early events in a plant's response to hypoxia stress are characterized by a cascade of signalling events involving





**Fig. 2.** A hypothetical model of the regulation of hypoxia-responsive miRNAs and tasiRNAs and their potential roles on *Arabidopsis* root responses. Flooding reduces oxygen levels until it reaches a critical threshold that is sensed by an unknown mechanism either in the nucleus or mitochondria. Once sensed, a HD-ZIP is induced that regulates a suite of miRNAs. These miRNAs are potentially involved in halting root growth and developmental responses in the root that occur after hypoxia such as root branching, as well as regulating tasiRNA expression that regulates PPRs involved in mitochondrial function. Impairing mitochondrial function can also regulate a subset of hypoxia-responsive miRNAs and tasiRNAs possibly as a result of increased ROS levels and calcium signalling leading to retrograde regulation. Promotive effects are shown by '→'; repressive effects by '−'.

the differential expression of a wide range of transcription factors including the AP2-EREBP, MADS, MYB, Homeobox, WRKY, bZIP, C2H2 zinc-finger, bHLH, and NAC families (Klok *et al.*, 2002; Liu *et al.*, 2005; Christianson *et al.*, 2009). As is characteristic of miRNAs, the majority of the putative targets for the hypoxia-responsive miRNA families are transcription factors mainly from the MYB, NAC, Homeobox, SPL, ARF, AP2, MADS, and CCAAT-HAP2 families. miRNAs may therefore play a role in regulating secondary signalling events, especially the *SPL* and *AP2* genes that show inverse regulation to miR156/157 and miR172 expression (Tables 2, 4, 5). Unfortunately, the bulk of these transcription factors are poorly characterized with regard to their contribution to root development. However, *SPL* and *AP2* genes have important roles in plant growth and floral development. Loss-of-function mutants of *SPL9* and *SPL15* have demonstrated that they are active in controlling juvenile-to-adult phase transition, vegetative plastochron and branching (Schwarz *et al.*, 2008). Analysis of the AP2 proteins TOE1, and TOE2, indicates that they are involved in the transitions between developmental states of the shoot apical meristem as the *toe1-1:toe2-1* double

mutant flowers much earlier than the wild type, whereas overexpression of TOE1 leads to delayed flowering (Aukerman and Sakai, 2003). Initially, hypoxia stress results in the cessation of growth (Van Dongen *et al.*, 2009) with longer term exposure causing cell death, especially at the metabolically active root tip, and aerenchyma formation in some species. Upon restoration of normal oxygen levels in *Arabidopsis*, adventitious roots form (Ellis *et al.*, 1999). Overexpression of miR166 in *Medicago truncatula* results in decreased lateral root formation and changes in root vascular bundles, and this highly expressed family was one of the few to decrease under hypoxia stress (Boualem *et al.*, 2008). miRNAs such as miR156/157, miR166, and miR172 may be involved in controlling or stopping root growth, and redirecting root development when normoxia resumes. These miRNAs may therefore link sub-lethal exposure to hypoxia with the developmental changes that occur during and after the stress (Fig. 2).

tasiRNAs are a specialized class of siRNAs that are generated by miRNA processing of a TAS gene transcript, resulting in the production of 21 nt RNAs that are phased with respect to the miRNA cleavage site. Four families of

*TAS* genes have been identified in *Arabidopsis*, with *TAS1* and *TAS2* transcripts recognized by miR173, *TAS3* recognized by miR390, and *TAS4* targeted by miR828 (Allen *et al.*, 2005; Rajagopalan *et al.*, 2006). *TAS1*, *TAS2*, and *TAS3* tasiRNAs all showed increased expression in the hypoxia-treated samples, whereas no *TAS4* RNAs were identified (Table 3). These changes in tasiRNA levels are reflected in the changes to tasiRNA targeting miRNAs; both miR173 and miR390 showed increased expression (1.5-fold and 1.6-fold respectively), whereas miR828 was not sequenced (Table 2). The majority of the genes targeted by *TAS1* and *TAS2* (and as well as miR158) are of the pentatricopeptide repeat (PPR) family. PPRs are a large family containing 442 genes in *Arabidopsis* that are putative RNA-binding proteins, and can be divided into two subfamilies; P and PLS (Lurin *et al.*, 2004). Most of the PPRs targeted by hypoxia-responsive smRNAs are from the P subfamily that is predicted to localize to the mitochondria (Lurin *et al.*, 2004). Relatively few of these proteins have described functions but they have been implicated in different stages of plastid gene expression, including transcription, splicing, processing, editing, translation, and stability, as well as mitochondrial induced fertility restoration (Andres *et al.*, 2007). It is likely that some of the observed changes in tasiRNAs are the result of decreased mitochondrial function during hypoxia as *TAS1* is induced by chemical inhibition of the cytochrome and alternative oxidase respiration pathways in the mitochondria (Fig. 2). The down-regulation of these PPR genes may either aid mitochondrial protection during hypoxia, or simply reflect a decreased requirement for these gene transcripts.

*TAS3* targets members of the Auxin Response Factor (ARF) family that transmits auxin signals during growth and development. Specifically, *TAS3* is thought to target ARF3 and 4 that are implicated in adult-juvenile transition functions, similar to the putative SPL and AP2 targets (Hunter *et al.*, 2006). Recent evidence indicates that *TAS3* could have a specific role in regulating root architecture (Maizel *et al.*, 2008).

Oxygen-deprivation triggers an immediate and reversible rise in cytosolic Ca<sup>2+</sup> that is co-localized around the mitochondria (Subbaiah and Sachs, 2003). It is known that Ca<sup>2+</sup> signalling occurs before gene expression changes, and is required for the induction of important hypoxia-responsive genes such as *ADH* (Subbaiah *et al.*, 1994a; b) and the transduction of ethylene signalling in the formation of aerenchyma (He *et al.*, 1996). Anoxia causes increases in hydrogen peroxide formation in *Arabidopsis* seedlings (Baxter-Burrell *et al.*, 2002) and is associated with RHO-LIKE SMALL G PROTEIN OF PLANTS (ROP) that regulate intracellular Ca<sup>2+</sup> gradients. Chemical inhibition of the cytochrome pathway also results in increased mitochondrial reactive oxygen species (Maxwell *et al.*, 1999). One hypothesis for oxygen sensing that would be shared by both plant and animals, is that changes in reactive oxygen species levels within and around the mitochondria stimulate Ca<sup>2+</sup> signalling, and that this influences nuclear gene expression by retrograde communication (Semenza, 1999). Our data does

not resolve the nature of sensing, but indicates that smRNAs play a role in gene regulation and possible developmental responses to hypoxia, and that a major signal for these responses is likely to be dependent on mitochondrial function and not on oxygen *per se*.

## Supplementary data

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Relative expression of *ADH* during hypoxia stress.

**Supplementary Table S1.** Summary of the origin of small RNA sequencing data.

**Supplementary Table S2.** Comparison of read numbers of miRNAs from different tissues.

**Supplementary Table S3.** Stem-loop RT and QRT-PCR primer sequences.

**Supplementary Table S4.** Primers for U6 loading control for QRT-PCR analysis of mature smRNAs.

**Supplementary Table S5.** Target genes and primers used for QRT-PCR analysis.

**Supplementary Table S6.** Potential tasiRNA present in control and hypoxia stressed roots

## Acknowledgements

Dov Moldovan was supported by a CSIRO Office of the Chief Executive postgraduate scholarship. BJP and DM acknowledge the support of the Australian Research Council Centre of Excellence in Plant Energy Biology (CE0561495). We would like to thank Tony Millar and Ian Small for fruitful discussions. We are also grateful to Chris Helliwell, Narayana Upadhyaya, and anonymous reviewers for their critical reading of the manuscript.

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