The Activity of the Stress Modulated Arabidopsis Ubiquitin Ligases PUB46 and PUB48 is Partially Redundant

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Abstract

The Arabidopsis ubiquitin ligases PUB46, PUB47 and PUB48 are encoded by paralogus genes; *pub46* and *pub48* mutants display increased drought sensitivity compared to wild type (WT). Although the phenotype displayed in the single gene mutants, suggest that each has specific biological activity, PUB46 and PUB48 activity may be also redundant. To test functional redundancy between two gene products requires a double mutant. Unfortunately, the close proximity of the *PUB46* and *PUB48* gene loci precludes obtaining a double mutant by crossing the available single mutants. We thus applied microRNA technology to reduce the activity of all three gene products of the *PUB46-48* subfamily by constructing an artificial microRNA (aMIR) targeted to this subfamily. Expressing aMIR46-48 in WT plants resulted in increased drought-sensitivity, a phenotype resembling that of the single *pub46* and *pub48* mutants, and enhanced sensitivity to methyl viologen, similar to that observed for the *pub46* mutant. Furthermore, the WT plants expressing aMIR46-48 also revealed reduced inhibition by ABA at seed germination, a phenotype not evident in the single mutants. Expressing aMIR46-48 in *pub46* and *pub48* mutants further enhanced the drought sensitivity of each parental single mutant and of WT expressing aMIR46-48. Thus, whereas the gene-specific activity of the PUB46 and PUB48 E3s is partially redundant in that absence of either E3 leads to drought sensitivity, our ability to eliminate the activity of both *PUB46* and *PUB48* in the same plant reveals additional gene specific facets of their activity in the reaction to abiotic stress.

Introduction

Plants being sessile lack the option of relocating to escape harsh conditions and have evolved physiological, developmental, biochemical, and molecular mechanisms to endure periods of stress. Plant exposure to abiotic stress evokes a global shift in gene expression to achieve a new proteostasis in which both protein translation and degradation are specifically modulated. The Ubiquitin Proteasome System (UPS) is a central, highly regulated mechanism for protein degradation in all eukaryotes [1,2]. Proteins are ubiquitylated in a three-step cascade comprising E1 ubiquitin activating, E2 ubiquitin conjugating and E3 ubiquitin ligase enzymes. The ubiquitylated proteins are bound by the 26S proteasome; the ubiquitin tag is removed and recycled, and the target protein is denatured and transferred to the 20S catalytic chamber of the proteasome for degradation into short peptides. The E3s recruit specific substrates and bind the E2 and transfer UB molecules onto lysine residues of the substrate. Thus they determine the half-life of particular protein substrates. Plant genomes contain an exceptionally high number of genes encoding components of the UPS: over 1,600 UPS loci, almost 6% of the Arabidopsis genome encode UPS proteins several times that of other organisms from yeast to human [1]. The E3s Ub recognize the degradation substrates conferring specificity and over 1,400 Arabidopsis genes encode putative E3s suggesting that this high number is essential for maintaining functional plants.

E3s can be grouped into a number of subclasses based on their motifs and oligomeric forms [3]. One of the smaller gene families comprises Plant U-box (PUB) E3s defined by a highly conserved ~70 amino acid long U-box domain first identified in yeast Ufd2 [4]. The U-box fold is similar to the E3 RING domain [5] but is stabilized by salt-bridges rather than zinc-binding motifs [6]. Unlike yeast, which has two U-box proteins, plants have a large number of genes encoding these putative E3: the Arabidopsis genome encodes 64 PUB [7,8] in addition to ~700 putative F-box and 470 RING E3s [9,10]. Many plant genomes have been analyzed for PUBs; gene numbers range from 56 in grapevine to 213 in wheat [7,11-21]. In contrast, only 8 U-box E3 genes are

present in the human genome [22]. PUBs are divided into subclasses based on their domain composition [23,24]. The largest class of Arabidopsis and rice PUBs contains ARM-repeat protein-protein interaction domains [25-27]. U-box-ARM proteins are unique to plants [28] and PUB-ARM E3s play a role in diverse biological processes, including development, immunity and abiotic stress (reviewed by [8]).

We have studied the paralogous Arabidopsis *PUB46*, *PUB47* and *PUB48* genes [29,30], particularly *PUB46* and *PUB48* as they are more highly expressed and their mutants give a strong hypersensitivity to water stress. The three genes are located in tandem on chromosome 5 and probably resulted from a recent gene duplication [29]. Many plant genomes have undergone multiplication resulting in high numbers of gene families encoding homologous proteins [31,32]. Surprisingly, despite encoding highly homologous proteins, a single T-DNA insertion mutant of either *PUB46* or *PUB48* results in hypersensitivity to water stress, indicating that each of these genes is essential for the drought response in a gene specific manner [29]. However, the biological roles of *PUB46* and *PUB48* may also be partially redundant and these two E3s may share some substrates.

Gene multiplication often results in functional redundancy and to obtain a mutant phenotype in redundant genes it is necessary to produce double or multiple gene mutants. The physical proximity of the paralogous *PUB46, PUB47 and PUB48* genes precludes obtaining these by crossing the single mutant plants. However, due to the high homology of these genes we were able to employ RNA interference technology [33] to severely reduce the activity of all three *PUB46-48* genes in a single plant and to test whether *PUB46* and *PUB48* function is redundant in the response to abiotic stress. In RNAi, double stranded (ds) RNA is digested by a DICER-family RNase to yield 21-24 bp RNA products. One strand is then bound by an AGRONOUT (AGO) protein that is subsequently bound by the RNA-Induced Silencing Complex (RISC). In plants RISC associates with the target mRNA and either degrades it or inhibits its translation (reviewed by [33,34]. RNAi comprises two classes of small RNAs: siRNA, small interfering RNAs that have perfectly complementary sequences and are prevalent in animals, and microRNA

(miRNA), an imperfect dsRNA resulting from a genome-encoded short highly structured non-coding RNA precursor [34]. Schwab et al developed a method for constructing artificial miRNA (aMIR) by introducing the desired siRNA sequence into the backbone of a natural plant miRNA. This allows design of an aMIR that can be targeted to a single mRNA or to a group of homologous mRNAs of choice [35].

Here we report that expression of a single artificial microRNA (aMIR46-48) that targeted all three *PUB46, PUB47* and *PUB48* genes in wild type (WT) plants led to uncreased drought sensitivity. Expressing aMIR46-48 in *pub46* and *pub48* mutants enhanced their drought sensitivity which exceeded that of the WT-aMIR46-48 plants. The creation of artificial triple *pub46-48* mutants exposed facets of their role in the abiotic stress response that are not redundant. Overexpressing aMIR46-48 also resulted in a reduced inhibition of germination by ABA, a phenotype not observed in the single mutants. Germination of WT expressing aMIR46-48 was also less sensitive to methyl viologen (MV). Thus, the creation of artificial triple *pub46-48* mutants exposed facets of their role in the abiotic stress response that are both redundant and nonredundant.

Results

Design of aMIR to silence *PUB46, PUB47* and *PUB48*

The high sequence homology of the *PUB46-PUB48* gene cluster allowed us to design a single aMIR sequence termed aMIR46-48. This aMIR46-48 targets the mRNA sequences encoding amino acids close to the N-terminus of each protein (nucleotides 140-160 of *PUB46* and *PUB48* and nts 119-139 of *PUB47*), and is predicted to be a high potency candidate sequence for aMIR affecting all three genes with a hybridization energy ranging between -31.56 and -38.76 kcal/mol which should achieve stable hybridization with the target mRNAs. In addition, aMIR46-48 is not expected to have any off-targets. The designed sequence was included in the backbone of MIR319A [35] (Fig.1 and Fig. 1S) and then cloned into the plant transformation vector pCAMBIA

99-1 downstream of the constitutive 35S Cauliflower Mosaic Virus (35S CaMV) promoter. The resulting plasmid was used to transform Arabidopsis plants.

RNA interference of *PUB46 PUB47* and *PUB48* in WT plants

The observed drought hypersensitivity of the *pub46* and *pub48* single gene mutants [29] serves as an unequivocal assay for effectiveness of gene silencing by aMIR46-48 in WT plants. We focused on *PUB46* and *PUB48* in our experiments as *PUB47* is expressed at a considerably lower level and *pub47* mutants do not show a stress related phenotype [29]. We transformed WT plants with aMIR-46-48 and assayed four homozygous lines from independent transformation events each with a single T-DNA insertion. The phenotype of the aMIR46-48 transformants resembled WT plants when grown under optimal conditions on agar plates with 0.5 X MS medium or in pots. However, when rosettes of pot-grown irrigated plants were exposed to drought stress by withholding irrigation the plants expressing aMIR46-48 were hypersensitive to drought compared to the WT plants (Fig. 2). The observed drought-hypersensitivity of WT expressing aMIR46-48 resembled that of the *pub46* and *pub48* single genes [29], thus, confirming our working hypothesis that aMIR46-48 is indeed silencing the *PUB46-48* genes.

pub46 and *pub48* mutants overexpressing aMIR46-48

Here a direct comparison of single *pub46* or *pub48* mutant plants with or without aMIR46-48 expression enables us to test for an additive effect of severely downregulating the expression of all three genes, in essence creating a double/triple mutant plant. We determined sensitivity to drought stress, a phenotype observed in both *pub46* and *pub48* single gene mutants [29] and in WT plants transformed with aMIR46-48 (Fig. 2). Under optimal conditions there was no difference in the phenotypes of all the plants (Fig. S2). However, under water withholding stress *pub46* and *pub48* mutant plants expressing aMIR46-48 were far more drought sensitive than the parental

single mutant lines or the WT-aMIR46-48 plants (Fig. 3). Drought stressed plants were rewatered and recovery was followed for the next 10 days. WT plants recovered best, followed by WT-aMIR46-48 plants. The *pub46* and *pub48* mutants struggled to recover whereas the *pub46* and *pub48* mutants that expressed aMIR46-48 did not recover from this stress at all (Fig. 4).

Germination in the presence of ABA

The plant hormone abscisic acid (ABA) is the central hormone in drought and germination [36]. We previously showed that germination in the presence of 1μM ABA of WT and single *pub46*, *pub47* or *pub48* mutants seeds was inhibited equally [29]. This suggests that either the PUB46-PUB48 E3s are not involved in ABA signaling or their activity may be redundant. Here we addressed the role of functional redundancy by assaying the effects of ABA on germination of seeds of WT plants expressing aMIR46-48, in essence of the double/triple mutants. We found that these seeds were less sensitive to inhibition of germination and greening by ABA: 69-86% of seeds of the WT-aMIR46-48 line developed green seedlings 10 days after plating on medium containing 1 μM ABA as opposed to 45% of seeds of WT plants, suggesting that this activity of the PUB46-PUB48 E3s appears to be redundant (Fig. 5).

Germination in the presence of MV

Drought stress often results in oxidative stress [37]. However, although each *pub46* or *pub48* single mutant is required for resilience to drought, we previously found that germination and seedling greening of *pub46* mutant seeds was hypersensitive to MV-induced oxidative stress whereas germination of *pub47* and *pub48* mutants resembled that of WT seedlings [29]. We therefore tested the MV sensitivity of germination of WT-aMIR46-48 seeds. We found that WT-aMIR46-48 germination is hypersensitive to oxidative stress induced by MV (Fig. 6). For example, 28-43% of seeds of WT expressing aMIR46-48, developed green seedlings when germinated on 0.5 μM MV as opposed to 76% of the WT seeds.

Discussion

Our results indicate that RNA interference provides an efficient and accurate alternative means of severely reducing the activity of the entire *PUB46-48* gene family. This enabled us to address the question of the extent of redundancy in the function of these PUB E3s. The drought hypersensitivity of single *pub46* and *pub48* mutants [29] served as a reliable assay by which we now show that transformation of WT plants with aMIR46-48 recreates the drought hypersensitivity phenotype of each single mutant. This is a clear indication that the artificial sequence has indeed severely reduced the activity of the targeted *PUB46-48* genes. aMIR expression substantially reduces the expression of its target genes leaving some minor residual activity and a single aMIR targeted to multiple mRNAs may downregulate each target gene to a variable degree [35]. Based on this result we then tested aMIR46-48 in single *pub46* and *pub48* mutants to differentiate between redundant and nonredundant functions of these E3s. The enhanced hypersensitivity of single *pub46* and *pub48* mutants to water withholding stress when they expressed aMIR46-48 indicates that each E3 performs unique function(s). Redundancy could indicate that each E3 participates in a single pathway whereas the nonredundant functions suggest that each E3 targets additional unique substrates for proteasomal degradation. Alternatively, the redundancy may be the result of partially overlapping temporal expression of these two PUBs.

Functional redundancy is a common feature of plant genomes with many genes belonging to gene families as a result of whole-genome duplication events [31,32]. In the Arabidopsis *PUB* gene family there are various examples of redundancy in PUB E3 function: e.g., *pub4* mutants that have smaller rosette size show impaired stamen development resulting in lower fertility than that of WT plants [38] and increased proliferation of shoot and root meristems [39,40]. In contrast, mutants of the highly homologous PUB2 E3 do not display any detectable phenotype [41]. However, rosette size and stamen development of double *pub2 pub4* mutants were more pronounced than of pub4 mutants, suggesting partial redundancy in the function of PUB2 and

PUB4 [41]. Arabidopsis PUB12 and PUB13 ubiquitylate the brassinosteroid receptor BRI1 and the endosomal pool of BRI1 was reduced in *pub12 pub13* double mutants implying redundancy in their function [42]. In addition, the Arabidopsis PUB22 and PUB23 E3s showed redundancy in modulating the degradation of the ABA receptor PYL9 [43]. Furthermore, single mutants of homologous Arabidopsis *PUB25* and *PUB26* have weak freezing sensitivity compared to WT whereas *pub25 pub26* double mutants are hypersensitive to freezing, suggesting redundant function for these genes in ubiquitylation of the cold signaling negative regulator MYB15 [44]. Arabidopsis PUB40 reduces the cellular concentrations of BRASSINAZOLE RESISTANT1 (BZR1) and BZR1 levels and the enhanced root growth phenotype of *pub39 pub40 pub41* triple mutant could be complemented by overexpressing *PUB40*, suggesting that these genes are redundant [45].

ABA is the key hormone involved in the plant response to drought [46]. ABA also modulates germination and seedling greening (reviewed by [47]) via various genes that encode transcription factors [48-51], participate in RNA metabolism [52-55] and via interaction with the ABA coreceptor-protein phosphatases 2C (PP2C)[56-58] and their inhibitors [59]. Ubiquitylation of ABA receptors and PP2Cs, by E3s from the PUB, EING, and RBR families plays a central role in modulating ABA signaling, germination and response to stress (reviewed by [60,61]).

Functional redundancy in degradation of proteins involved in ABA signaling may occur between non-homologous E3s. For example, AFP1, DWA1/DWA2, KEG and ABD1 are all involved in the ubiquitylation of the ABI5 transcription factor [62-65]. Similarly, a number of different E3s ubiquitylate the ABA receptors and co-receptors (reviewed by [60]). Partial redundancy is also found in the signaling pathways of other plant hormones [45,66]. In our experiments WT plants expressing aMIR46-48 were less sensitive to ABA inhibition of germination and seedling greening than the parental WT plants (Fig. 2). Interestingly, ABA sensitivity of the single *pub46*, *pub47* or *pub48* mutants is similar to that of the WT plants [29], suggesting that the ABA response may be redundant in these single E3 mutants.

Additional PUBs regulate ABA signaling by ubiquitylating ABA receptors and coreceptors (reviewed by [61]: *pub 22 pub23* double mutants display enhanced tolerance to drought and target the ABA receptor PYL9 [67]. In contrast, *pub12 pub13* double mutants are ABA-insensitive: PUB12 and PUB13 regulate ABA signaling by mediating the stability of the ABA co-receptor ABI1 [68]. Interestingly, both PUB gene pairs display functional redundancy. Furthermore, PUB18 and PUB19 also showed redundancy in ABA inhibition of germination, as double *pub18 pub19* mutants were less sensitive compared to WT, whereas single mutants had similar ABA sensitivity to that of WT seeds [69]. Our results suggest that PUB46 and PUB48 affect ABA signaling, the target is yet to be determined. If PUB46 and PUB48 affect ABA perception, it is likely that they target one or more of the ABA coreceptors, given that WT-aMIR46-48 germination displayed reduced ABA sensitivity, resembling that of the *pub12 pub13* double mutant [68].

Our results suggest that whereas *PUB46* and *PUB48* possess gene-specific biological activities [29], they also share some functional redundancy, *i.e*., their redundancy is partial. The extent of redundancy differs for the different activities/stresses. For example, redundancy can be observed in a more pronounced water stress hypersensitivity of a single gene mutant (Figs. 3 and 4), or in display of a phenotype such as germination in the presence of ABA observed solely when the activity of all the genes is reduced (Fig. 5). The partial functional redundancy of PUB46 and PUB48 may result from the protein substrate preferences of these E3s and their expression pattern in different plant tissues under various conditions. PUB46, PUB47 and PUB48 each contain 3 ARM motifs involved in protein-protein interaction, in particular with their ubiquitylation substrates. Each of the corresponding ARM motifs shares high sequence identity (60-71%) [29], suggesting that these ARM motifs are both close enough to bind the same proteins substrates, resulting in functional redundancy, as well as having differences that would enable them to target additional specific targets. Similarly, promoter activity of these 3 genes shows both overlapping and gene specific expression patterns [29], suggesting that gene expression patterns of paralogous genes may also contribute to the functional redundancy of their activity.

Classical models of gene duplication maintain that to survive through evolution, at least one of the paralogs must acquire a new function or else will be lost by deletion of nonfunctional genes at duplicate loci [70-73]. The existence of truly redundant genes has been questioned [74-76], suggesting that all paralogs are likely to have some gene specific function. Our study joins other examples of partial redundancy of paralogous E3s and further comparative studies of the expression pattern of each paralog, their transcriptional, post transcriptional and post translational modifications, and their substrate preferences are needed to fully elucidate the complexity of the biological function of these E3s. Application of RNAi technology as we demonstrate here adds a new dimension to dissect the contribution of each E3 and to distinguish between overlapping redundant functions and those that are unique for each E3.

Methods

Plant material

Arabidopsis thaliana (Col). WT, *pub46* and *pub48* T-DNA insertion mutants were previously described [29].

Construct for the expression of aMIR

Artificial MicroRNA (aMIR) was designed using the Web MicroRNA Designer (http://wmd.weigelworld.org/cgi-bin/webapp.cgi) [35]. A synthetic DNA fragment with the appropriate 21 bp aMIR sequences was inserted into the MIR319a backbone [35] with flanking DNA sequences conferring *Xba*I and *Bam*HI sites for integration into the pCAMBIA 99-1 vector where expression is directed by the constitutive highly active 35S Cauliflower Mosaic Virus (35S CaMV) promoter.

Plant Transformation

WT or the indicated T-DNA insertion mutant plants were transformed by the floral dip method [77] using *Agrobacterium* strain GV3101 harboring the respective plant transformation plasmid. Transgenic plants were selected on medium containing hygromycin, and homozygous plants resulting from independent transformation events by a single copy of the respective T-DNA were isolated as described [77].

Plant growth and stress application

Plants were grown on agar plates with half-strength Murashige and Skoog (MS) nutrient solution [78] supplemented with 0.5% sucrose, or in planting mix in pots. Seed surface sterilization, imbibition, and growth conditions are detailed in [29]. Where indicated, the growth medium was supplemented with the indicated concentrations of ABA or MV. Green seedlings were scored 7 days after plating. Each experiment was carried out in three biological repeats with treatment comprising ca 40 plants.

Drought tolerance

For drought tolerance assays, seeds were planted in pots with equal amounts of potting mix. Plants were irrigated for three weeks, drought stress was applied by water withdrawal for the indicated time, and wilted plants were scored. Recovery was assayed by rewatering drought-treated plants and survival was scored ten days later.

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Author contributions

GZV performed and analyzed the experiments and participated in writing the draft of the manuscript; DR contributed to experiments analysis and writing the manuscript; DBZ conceived the project idea, analyzed the experiments, and wrote the manuscript. All authors reviewed the manuscript.

Ethics declarations

The authors declare no competing interests.

Figures and Legends

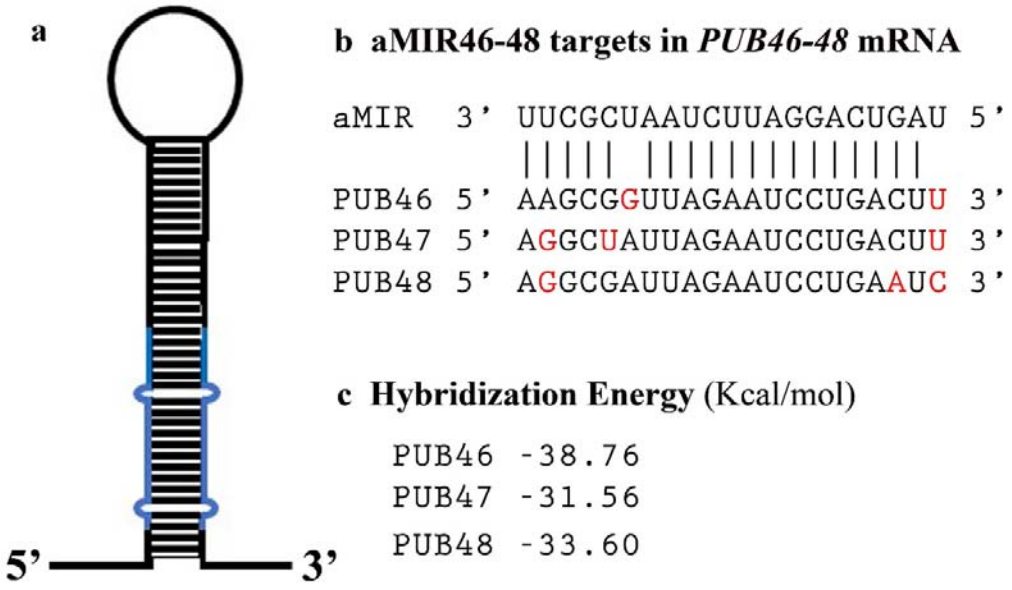

Fig. 1. Diagram of aMIR46-48 and its targets in *PUB46-48* mRNAs.

a. Diagram of aMIR46-48. Sequences in blue represent the two strands of sequence designed to specifically target the mRNA encoded by *PUB46, PUB47* and *PUB48*.

b. Proposed hybridization by aMIR46-48 and the respective mRNA target sequences. Mismatches between aMIR46-48 and each target sequence are shown in red. The top two sequence lines show the hybridization between aMIR46-48 and *PUB46* mRNA. The respective sequences in *PUB47* and *PUB48* are aligned in the bottom two lines.

c. Hybridization energy calculated for aMIR46-48 and its target mRNAs.

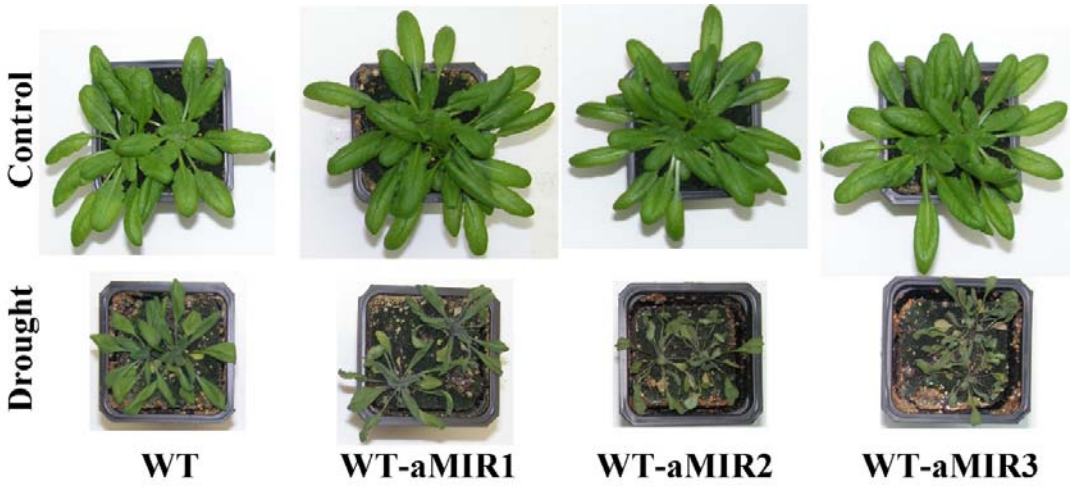

Fig. 2. Water stress performance of WT-aMIR46-48 plants. WT plants and 3 lines of WT plants expressing aMIR46-48 were grown in pots for three weeks. Water was then withheld from drought treated plants, whereas control plants was watered. Plants were pictured 10 days after water withholding.

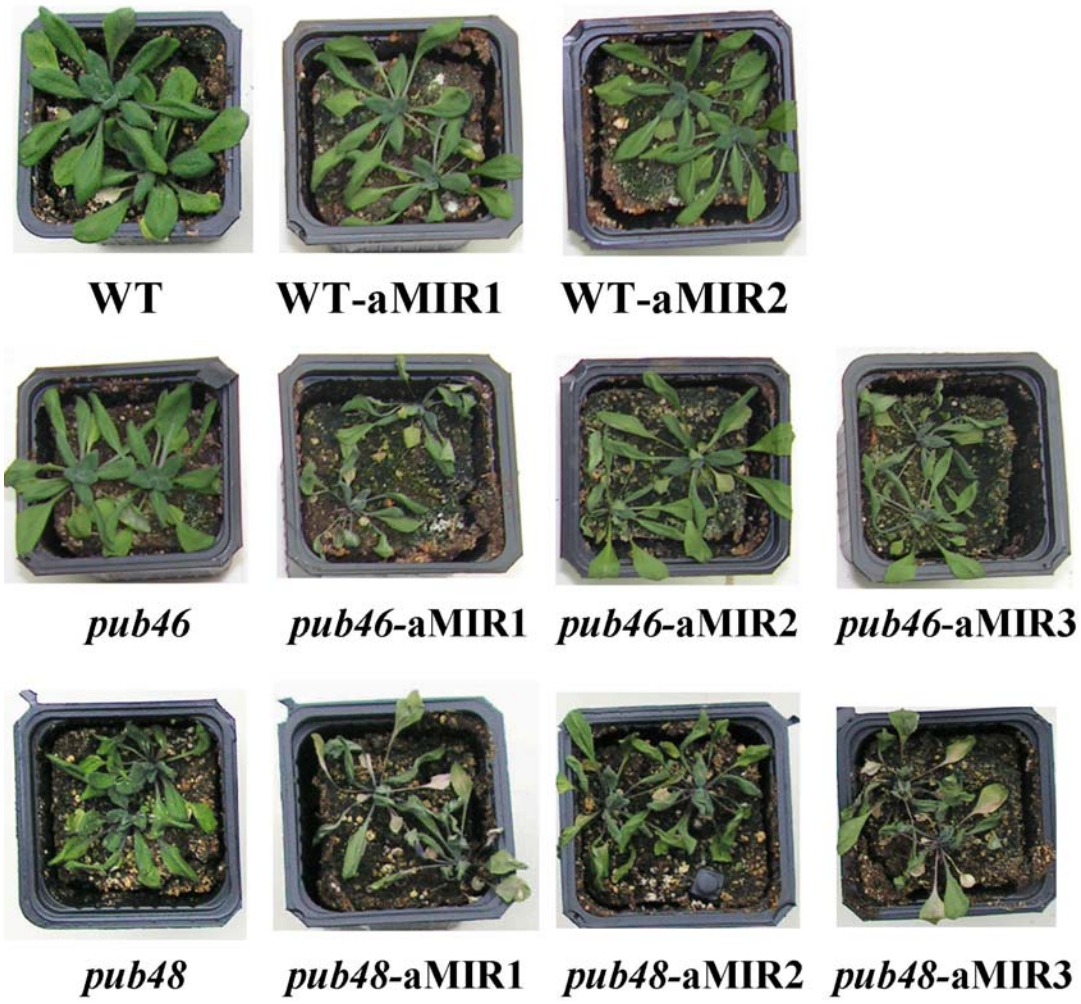

Fig. 3. Phenotype of *pub46* and *pub48* mutants expressing aMIR46-48. Plants of the indicated genotype were grown in pots for 3 weeks. Water was then withheld for eight days. Control plants are shown in Fig. S2.

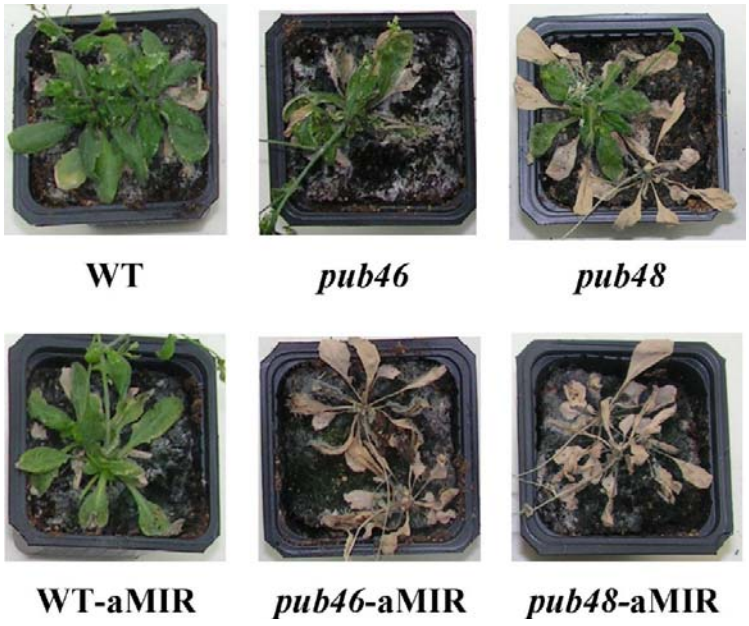

Fig. 4. Plant recovery from drought stress. Plants treated as described in Fig. 3 were re-irrigated, and pictures were taken ten days later.

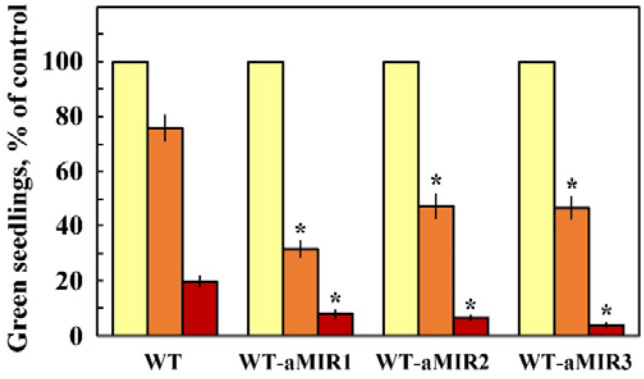

Fig. 5. Effect of ABA on seedling germination. Surface sterilized cold treated seeds of WT and WT expressing aMIR46-48 were plated on agar media containing 0.5 X MS, 0.5% sucrose (yellow bars) supplemented with 1 μM (orange bars) or 1.5 μM (brown bars) ABA. Green seedlings were scored 7 days later. Data shown are average ± SE of 5 biological repeats with 40 seedlings for each treatment. Statistically significant changes from WT plants are marked with an asterisk.

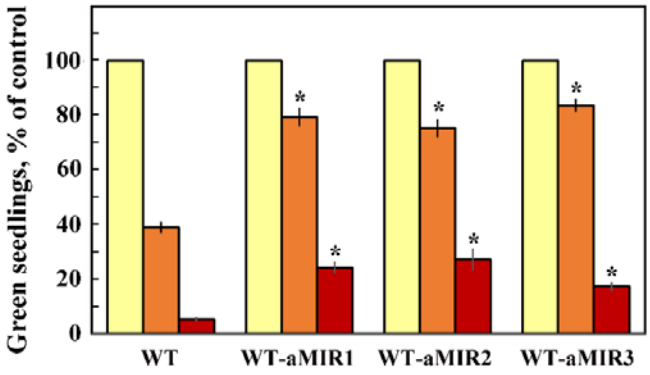

Fig. 6. Effect of methyl viologen (MV). on seedling germination. Surface sterilized cold treated seeds of WT and WT expressing aMIR46-48 were plated on agar media containing 0.5 X MS, 0.5% sucrose (yellow bars) supplemented with 1 μM (orange bars) or 1.5 μM (brown bars) MV. Green seedlings were scored 7 days later. Data shown are average ± SE of 5 biological repeats with 40 seedlings for each treatment. Statistically significant changes from WT plants are marked with asterisk.