

A

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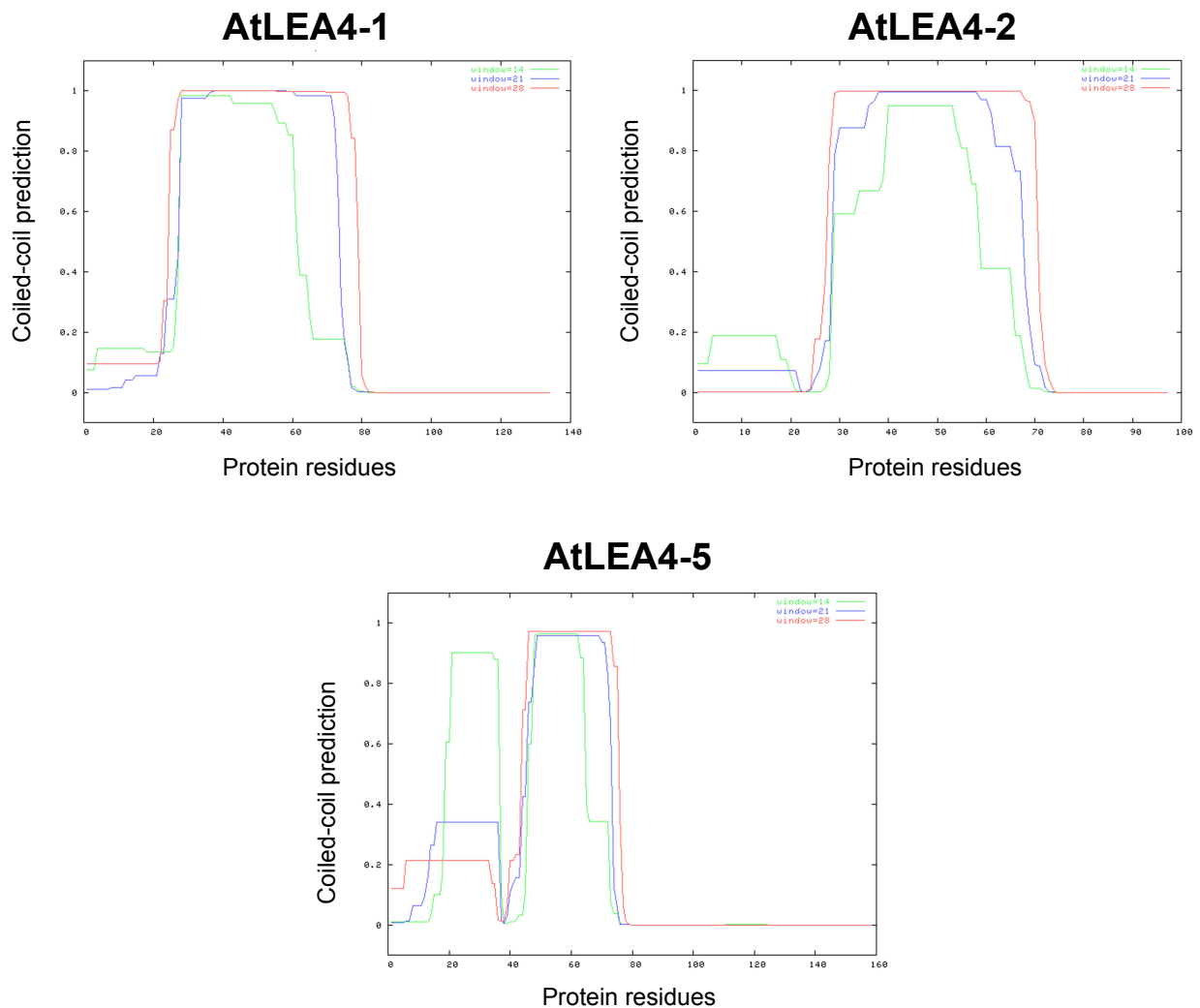
AtLEA4-1  MQSAKQKISDMASTAKEKMICQAKADEKAERAMARTKEEKEIAHQRRKAKEAEANMDMH
AtLEA4-2  MQSAKEKISDMASTAKEKLNIGGAKAQGHAEKTMARTKKEKKLAQEREKSKEAQAKADLH
AtLEA4-5  MQSMKETASNIAASAKSGMDKTATLEEKAEKMKTRDPVQKQMATQVKEDKINQAEHKKR
*** *:. *:::*. : *.:**:* :*:** :.: * :*: . :

AtLEA4-1  MAKAHAEDKDMA-----KQSHYHVTDHGPHVPQAPVPAPAPVPMGHGYGHNPT
AtLEA4-2  QSKAEHAADAQV-----HGHHLPGHSTYPTRA-----T
AtLEA4-5  ETRQHNAAAMKEAAGAGTGLGLGTGTHSTTGQVGHGTGTHQMSALP-----GHGTGQLTD
.: :* * * * * * * * * * * * * * * * * * * * * * * * * * * *

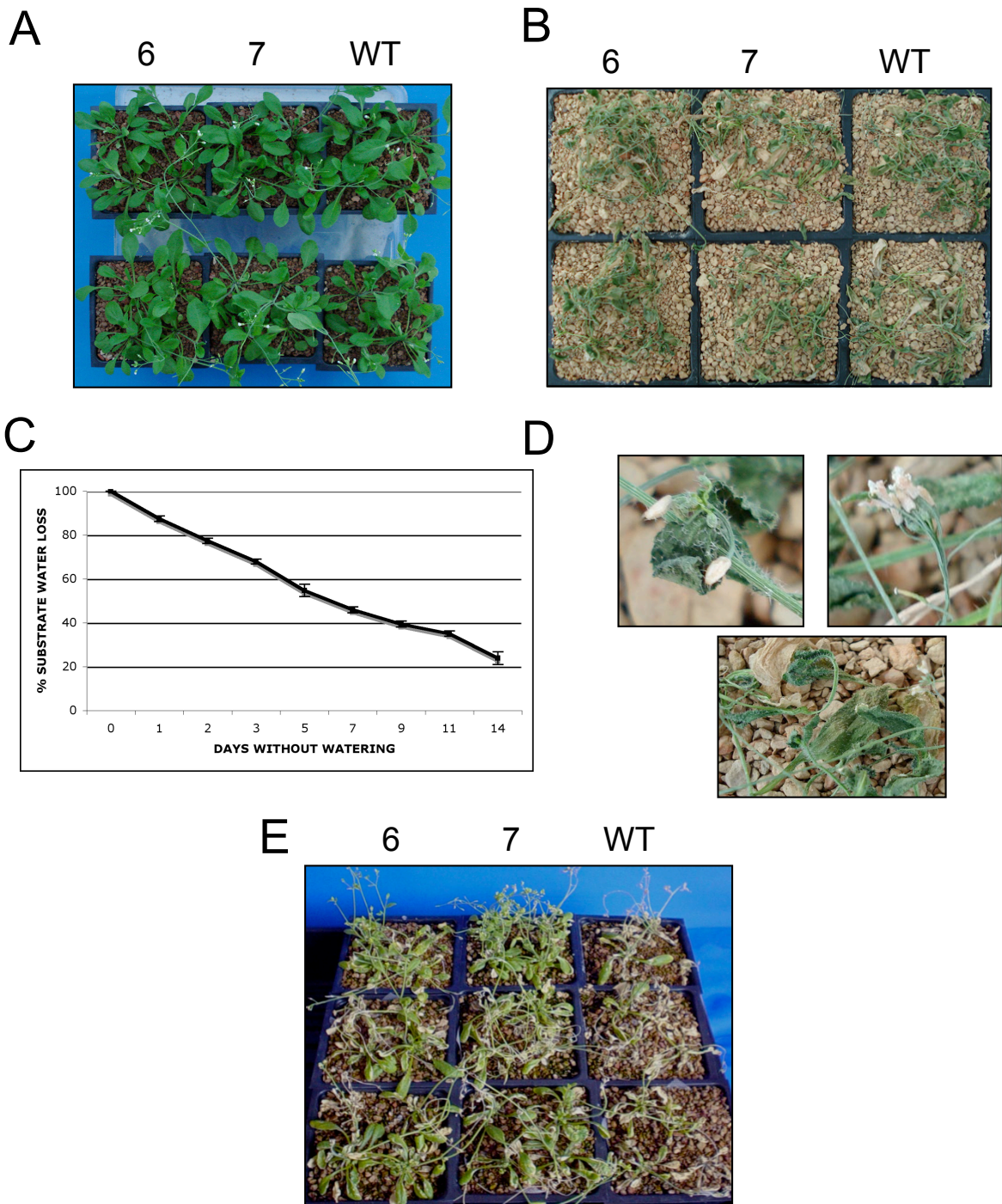
AtLEA4-1  GV-----TSVPPQTYHPTY----PPTGHHNHHHY--
AtLEA4-2  GA-----NYPPGQI-----
AtLEA4-5  RVVEGTAVTDPIGRNTGTGRRTAHNTHVGGGGATGYGTGGGYTG

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B

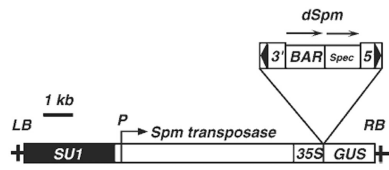


Supplemental Figure S1. Sequence similarity between group 4 LEA proteins in Arabidopsis and prediction of coiled-coil regions in these proteins. A) Sequence alignment of group 4 LEA proteins showing the high conservation among the three members at their amino portion. The highest similarity was found between AtLEA4-1 and AtLEA4-2 proteins. Identities are marked with asterisks (*) and similarities with dots (.) (:). Sequences were obtained from the Swissprot database for previously reported LEA4 proteins (listed as LEA_1, PFAM 03760) B) Predicted coiled-coil regions of group 4 LEA proteins are present in the conserved N-terminal region, while the predicted C-terminal (which varies in length 20-70 residues) is predicted to be unstructured or “random-coil” (Lupas et al., 1991). The “y” axis represents the confidence on the coiled-coil prediction, using different lengths of the coiled-coil (14, 21, 28 residues).

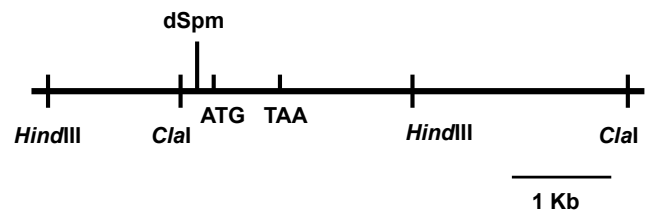


Supplemental Figure S2. Phenotypic analysis of plants overexpressing AtLEA4-5 protein. A) Wild type (WT) plants and five homozygous transgenic lines were germinated *in vitro* and transplanted to a low-water-retention substrate, kept under optimal irrigation conditions with nutrient solution until they reached the flowering stage; watering was stopped at this point. B) A representative image of Arabidopsis plants after 14 days of dehydration ($\Psi_{\text{substrate}} = -6.45 (\pm 0.57)$ MPa). C) Graph showing the water loss rate from the substrate (\pm SD) as a percentage of the initial water content in each pot during the course of the drought experiment. D) Detail of floral buds, inflorescences and leaves to denote severe dehydration in all the aerial tissues and senescence of some rosette leaves. Plants were rehydrated with nutrient solution at this point. E) Arabidopsis plants after 10 days of rehydration showing that transgenic plants were capable not only of leaf recovery, but also bud maintenance and recovery, while WT plants were only able to recover some rosette leaves but the floral axis became senescent and could not maintain their reproductive tissues.

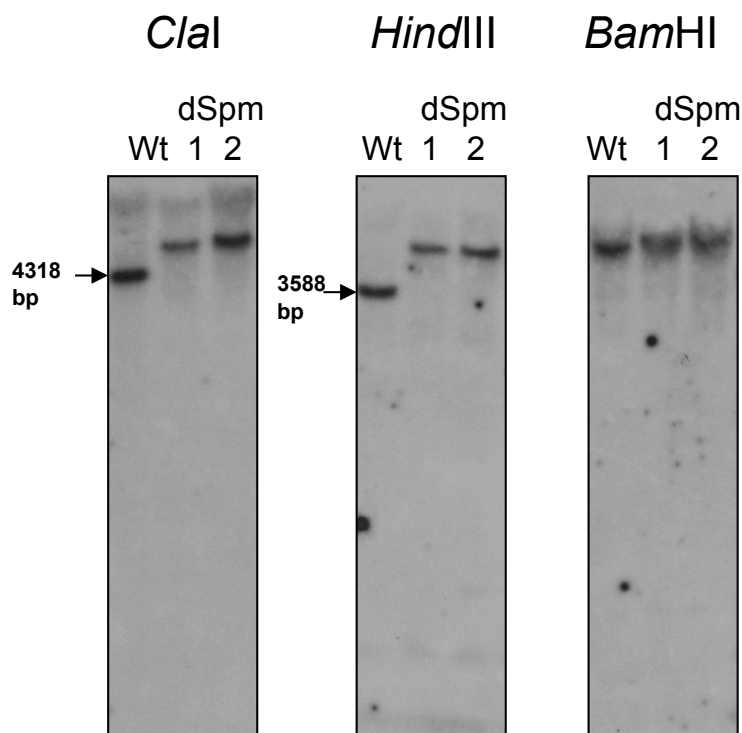
A



B



C



Supplemental Figure S3. Description of the transposon insertion mutant (dSpm 4-5) in the *AtLEA4-5* gene. A) Construct used to select stable dSpm transpositions in the SLAT mutant collection from the John Innes Centre (image reproduced from Tissier et al. *Plant Cell* **11**:1841-1852, 1999). An Enhancer/Suppressor-mutator (En/Spm) element was introduced into Arabidopsis in a single T-DNA construct, which carried a non-autonomous defective Spm (dSpm) element with a phosphinothricin herbicide resistance (BAR) gene, a transposase expression cassette, and a counter-selectable gene. B) Location of the stable dSpm insertion in the promoter region of *AtLEA4-5* gene (208 bp from the ATG codon) and restriction sites used for Southern blot analysis. Mutant seeds were obtained from the NASC European Arabidopsis Stock Centre (N122943). Plants were grown from each seed and its progeny was collected separately from each plant. C) Southern blot analysis using a specific probe to detect the insertion of dSpm in the expected region of *AtLEA4-5* gene promoter and genomic DNA (40 μ g) extracted from wild type (WT) and mutant plants from two T₄ lines (dSpm 1, 2). Genomic DNA was digested with *ClaI* or *HindIII*, which gave a restriction fragment in the expected size for WT plants and a higher fragment in the mutant lines, in agreement to the transposon size and orientation. Digestion with *BamHI* was used as negative control.

A

1) Search for a region of 22 nucleotides with maximal identity between *AtLEA4-1* and *AtLEA4-2* transcripts.

```

AtLEA4-1 AUGCAAUCGGCGAAACAGAAGAUAAAGCAUAUGGCUAGUACAGCCAAGGA
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
AtLEA4-2 AUGCAGUCGGCGAAGGAAAAGAUCAGUGACAUGGCCAGUACGGCCAAGGA
  
```

2) Search for a region with the least number of unpairings with the microRNA template (*ath-miR159a*).

```

AtLEA4-1/2 mRNA      5' GACAUGGCCAGUACAGCCAAG 3'
                   ||| ||| ||| ||| |||
ath-miR159a      3' AUCUCGAGGGAAGUUAGGUUU 5'
  
```

3) Introduce point mutations in the microRNA template (*ath-miR159a*) to eliminate unpaired sites.

```

AtLEA4-1/2 mRNA      5' GACAUGGCCAGUACAGCCAAG 3'
                   ||||| ||||| ||||| ||||| |||||
a-miRLEA4-1/2      3' CUGUACCGUCAUGUCGGUUU5'
  
```

4) From the original stem-loop precursor sequence in *ath-miR159*,

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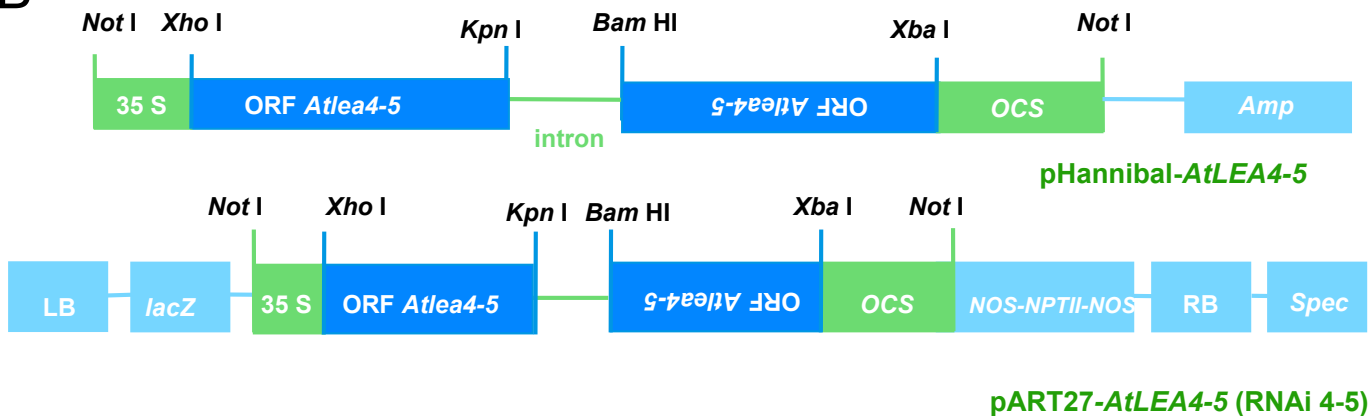
ath-miR159a* 5' -- UAGAGCUCCUUAAGUUCAAA-----
                ||||| |||||
ath-miR159a 3' -- AUCUCGAGGGAUUAGGUUU-----
                AG
                U G GCU UUA A U
ath-miR159a* 5' -- A A CC A G UCAAA-----
                | | || | |
a-miRLEA4-1/2 3' -- U U GG U U GGUUU-----
                C G ACC UCA G C
  
```

5) restore the secondary structure with the corresponding changes in the sequence of *ath-miR159**.

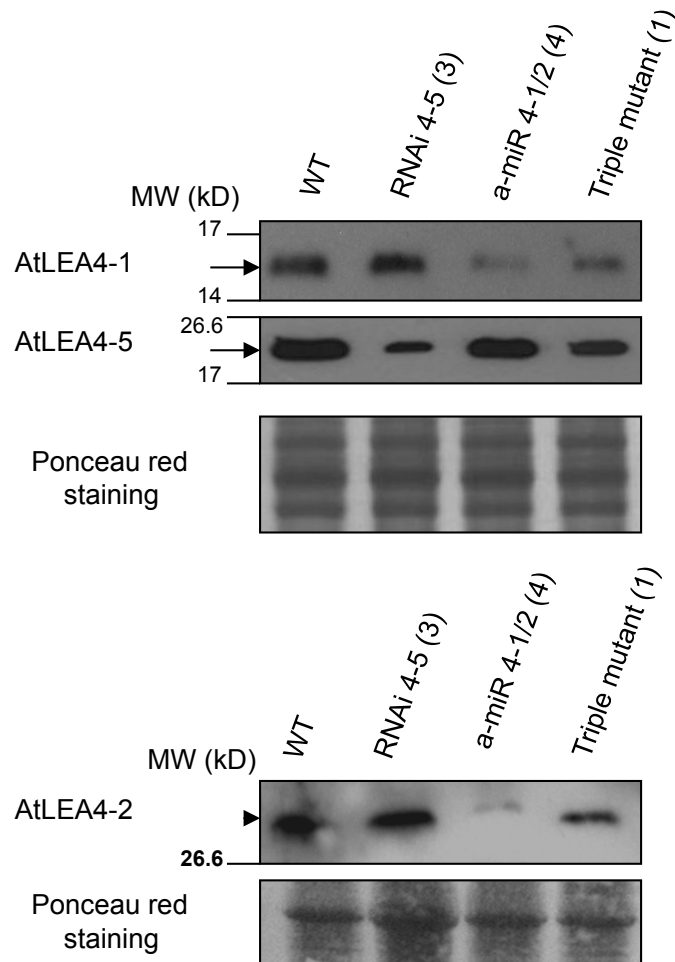
```

ath-miR159a* 5' -- GACAUGGCCAGAA CGGUCAAA-----
                ||||| |||||
a-miRLEA4-1/2 3' -- CUGUACCGUCC GUCGGUUU-----
                AU
  
```

B

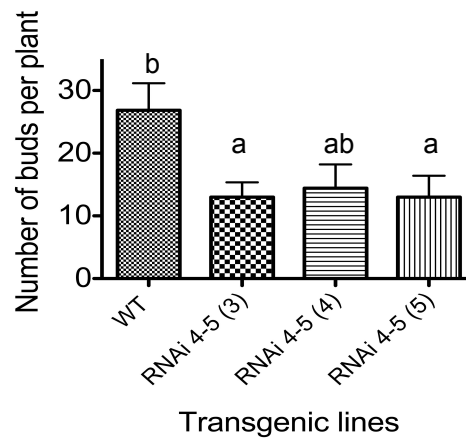


Supplemental Figure S4. Design of artificial microRNA to silence *AtLEA4-1* and *AtLEA4-2* genes and construction of RNAi-triggered silencing for *AtLEA4-5* gene. A) Point mutations were inserted into *ath-miR159a* precursor by PCR in order to target *AtLEA4-1* and *AtLEA4-2* genes as silencing targets of artificial microRNA (*a-miR 4-1/2*) as depicted in the diagram. B) RNAi silencing of *AtLEA4-5* gene was achieved by constitutively expressing the ORF as inverted repeats to trigger the specific silencing of the endogenous gene by siRNAs.

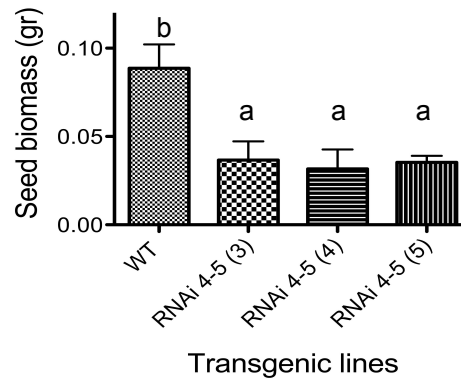


Supplemental Figure S5. Protein reduction levels in PTGS single, double and triple mutants of *AtLEA4* gene family subjected to drought. Western blot analysis using specific antibodies against each member of *AtLEA4* protein family and total protein extracts (10 μ g) from adult plants under dehydration treatments to show the reduction in the accumulation levels of silenced mutants compared to wild type plants (WT). The homozygous transgenic lines used were: RNAi-directed silencing mutant for *AtLEA4-5* gene (RNAi 4-5), artificial microRNA-directed silencing double mutant for *AtLEA4-1* and *AtLEA4-2* transcripts (a-miR 4-1/2), triple silencing mutant resulting from the cross between the single and double mutant described above (Triple mutant). Reversible stain after transfer is shown as loading reference. The arrowhead indicates the protein band (\approx 30 kD) specifically detected by the antibodies against *AtLEA4-2* protein.

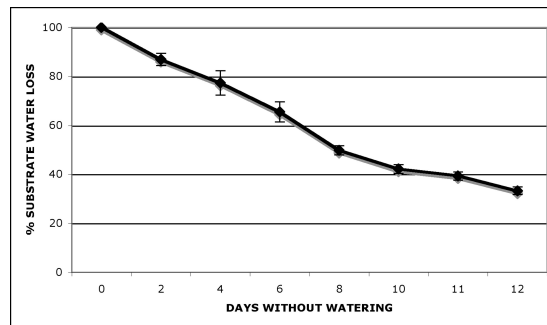
A RECOVERY AFTER DROUGHT



B SEEDS PER PLANT AFTER DROUGHT

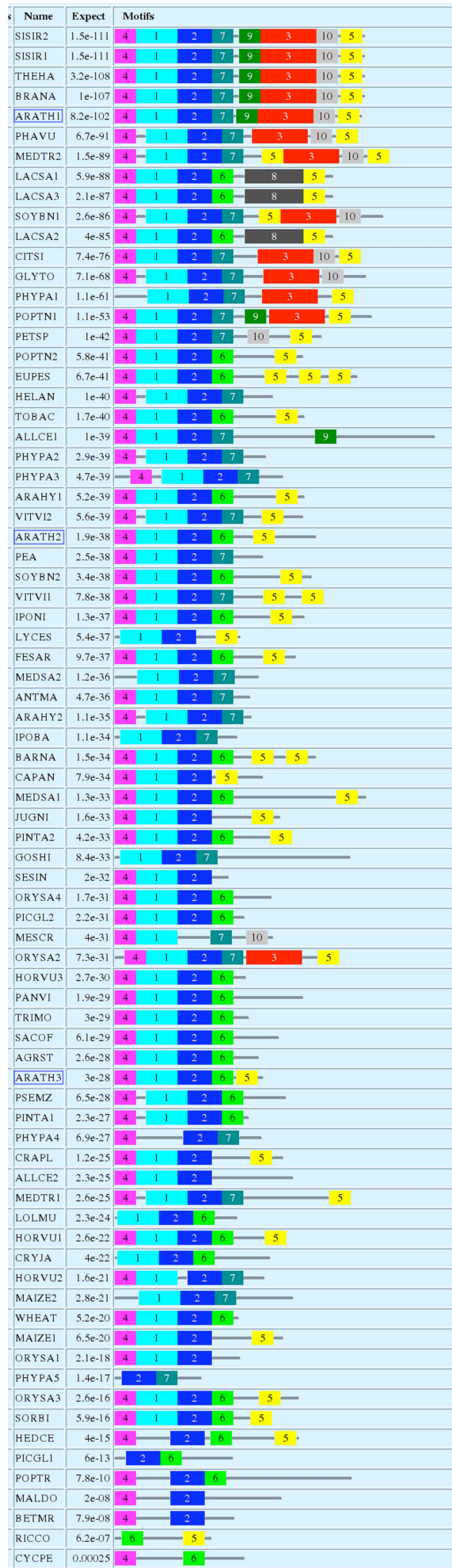


C Transgenic lines



Supplemental Figure S6. Phenotypic analysis of T_2 homozygous plants expressing an RNAi to silence *AtLEA4-5* transcript after recovery from dehydration. Wild type (WT) and three silenced lines (transgenic lines 3 - 5 as in Fig. 8), which showed significant reduction of *AtLEA4-5* protein levels under drought were used. Plants were grown in a greenhouse, watered until flowering and subjected to dehydration during a 12 d period ($\Psi_{\text{substrate}} = -4.62 (\pm 0.62)$ MPa). A) Average number of floral and axillary buds per plant were counted after 6 d of recovery. Bars indicate means (\pm SE, $n=7$), significant differences between groups were found with $P = 0.0273$, using one-way ANOVA. B) Rehydrated plants were kept under optimum irrigation with nutrient solution and seeds were harvested from each plant until senescence. Bars indicate means (\pm SE, $n=3$). Significant differences were found between groups with $P = 0.0133$, using one-way ANOVA. Different letters show significant differences between groups as indicated by Duncan's post-tests ($P < 0.05$). C) Water loss rate from the substrate (\pm SD) during dehydration for mutant lines with reduced levels of *AtLEA4* family members, shown as a percentage of the initial water content in each pot during the course of the drought experiment.

Supplemental Figure S7. Conserved motifs and their arrangement in the LEA4 protein sequences from plants. Motifs were found using MEME from 77 protein sequences obtained from protein and EST databases (Bailey and Gribskov, 1998). Based on the low e-value, the two last sequences (RICCO and CYCPE) were not used for further analysis.



Supplemental Table S1. Phenotypic analysis of T₃ homozygous adult plants overexpressing AtLEA4-5 protein (35S::AtLEA4-5::NOS) during drought and after recovery from stress. Plants were grown in greenhouse environment, watered with nutrient solution until flowering and subjected to severe dehydration ($\Psi_{\text{substrate}} = - 6.447 (\pm 0.574)$ MPa). At this point plants were rehydrated and recovered during 10-days. The number of floral and axillary buds per plant were counted after 10-days of recovery; dry weight of whole plants was also recorded. In separate experiments, the relative water content (RWC) was calculated in control (well-irrigated) plants and in dehydrated plants ($\Psi_{\text{substrate}} = - 3.851 (\pm 0.704)$ MPa).

Genotype	Dry biomass of whole plants kept under optimum irrigation (gr)[§]	Dry biomass of whole plants after recovery from severe drought^δ (gr)	Number of buds per plant under optimum irrigation[§]	Number of buds per plant after recovery from severe drought^δ	RWC of plants kept under optimum irrigation[§]	RWC of plants after 10 days of withholding irrigation[§]
WT (\pm SD) (lower 95% CI, upper 95% CI)	0.1457 (\pm 0.00153) (0.1419, 0.1495)	0.03528 (\pm 0.00435) (0.02448, 0.04608)	48.33 (\pm 3.215) (40.35, 56.32)	0	0.936 (\pm 0.01669) (0.8946, 0.9775)	0.2771 (\pm 0.01034) (0.2514, 0.3027)
OE 4-5 (2) (\pm SD) (lower 95% CI, upper 95% CI)	0.1433 (\pm 0.01901) (0.0961, 0.1906)	0.04278 (\pm 0.002999) (0.03534, 0.05021)	55.67 (\pm 3.786) (46.26, 65.07)	4.375 (\pm 4.658) (0.4808, 8.269)	0.9237 (\pm 0.02077) (0.8721, 0.9753)	0.3856 (\pm 0.02945) (0.3125, 0.4588)
OE 4-5 (3) (\pm SD) (lower 95% CI, upper 95% CI)	0.156 (\pm 0.00361) (0.147, 0.165)	0.05233 (\pm 0.00808) (0.03226, 0.0724)	37 (\pm 11.27) (9.005, 64.99)	5.875 (\pm 5.643) (1.158, 10.59)	0.948 (\pm 0.02254) (0.892, 1.004)	0.3987 (\pm 0.03116) (0.3213, 0.4761)
OE 4-5 (4) (\pm SD) (lower 95% CI, upper 95% CI)	0.1332 (\pm 0.03435) (0.04783, 0.2185)	0.05233 (\pm 0.00351) (0.04361, 0.06106)	75.33 (\pm 6.028) (60.36, 90.31)	8.375 (\pm 8.331) (1.41, 15.34)	0.9454 (\pm 0.00055) (0.9441, 0.9468)	0.3689 (\pm 0.03419) (0.284, 0.4539)
OE 4-5 (6) (\pm SD) (lower 95% CI, upper 95% CI)	0.1565 (\pm 0.00769) (0.1374, 0.1756)	0.04767 (\pm 0.00298) (0.04026, 0.05508)	66.33 (\pm 8.737) (44.63, 88.04)	6 (\pm 2.878) (3.593, 8.407)	0.9477 (\pm 0.02026) (0.8974, 0.998)	0.3629 (\pm 0.04513) (0.2508, 0.475)
OE 4-5 (7) (\pm SD) (lower 95% CI, upper 95% CI)	0.1793 (\pm 0.00635) (0.1636, 0.1951)	0.04414 (\pm 0.00768) (0.02505, 0.06323)	30 (\pm 7) (12.61, 47.39)	5.125 (\pm 5.276) (0.7139, 9.536)	0.9363 (\pm 0.01563) (0.8975, 0.9751)	0.3315 (\pm 0.03201) (0.252, 0.4111)

[§]Equivalent to $\Psi_{\text{substrate}} = - 0.451 (\pm 0.212)$ MPa

^δEquivalent to $\Psi_{\text{substrate}} = - 6.447 (\pm 0.574)$ MPa

[§]Equivalent to $\Psi_{\text{substrate}} = - 3.851 (\pm 0.704)$ MPa

Supplemental Table S2. Phenotypic analysis of T_4 homozygous transposon insertion mutant in *AtLEA4-5* gene and of homozygous T_2 PTGS mutants in *AtLEA4* gene family during drought and after recovery from stress. Plants were grown in greenhouse environment and watered until flowering, subjected to dehydration ($\Psi_{\text{substrate}} = -4.617 (\pm 0.619)$ MPa). At this point plants were rehydrated and recovered during 6-days. The number of floral and axillary buds per plant were counted after 6d of recovery; dry weight of whole plants was also recorded. In separate experiments, the relative water content (RWC) was calculated in control (well irrigated) plants and in dehydrated plants ($\Psi_{\text{substrate}} = -5.415 (\pm 0.543)$ MPa).

Genotype e	Dry biomass of whole plants kept under optimum irrigation (gr)^ε	Dry biomass of whole plants after recovery from drought (gr)^φ	Number of buds per plant after recovery from drought^φ	RWC of leaf discs from plants kept under optimum irrigation^ε	RWC of leaf discs from plants after 13 days of withholding irrigation^ε
WT 1 (\pm SD) (lower 95% CI, upper 95% CI)	0.1021 (\pm 0.02358) (0.07734, 0.1268)	0.03505 (\pm 0.008034) (0.02833, 0.04177)	13.75 (\pm 2.486) (12.42, 15.58)	0.9019 (\pm 0.02323) (0.8775, 0.9263)	0.17 (\pm 0.00674) (0.1593, 0.1808)
dSpm 4-5 (1) (\pm SD) (lower 95% CI, upper 95% CI)	0.1141 (\pm 0.01072) (0.1028, 0.1253)	0.01826 (\pm 0.00529) (0.01384, 0.02268)	7.58 (\pm 3.059) (5.64, 9.527)	0.8985 (\pm 0.03627) (0.8604, 0.9365)	0.1552 (\pm 0.01283) (0.1347, 0.1756)
WT 2 (\pm SD) (lower 95% CI, upper 95% CI)	0.1078 (\pm 0.0398) (0.06601, 0.1496)	0.03485 (\pm 0.00798) (0.02818, 0.04152)	16.25 (\pm 3.991) (12.91, 19.59)	0.9147 (\pm 0.03054) (0.8826, 0.9467)	0.2164 (\pm 0.01408) (0.194, 0.2388)
RNAi 4-5 (3) (\pm SD) (lower 95% CI, upper 95% CI)	0.1224 (\pm 0.03627) (0.08434, 0.1605)	0.01816 (\pm 0.00397) (0.01485, 0.02148)	6.5 (\pm 2.39) (4.502, 8.498)	0.9135 (\pm 0.05177) (0.8591, 0.9678)	0.1337 (\pm 0.0368) (0.07519, 0.1923)
WT 3 (\pm SD) (lower 95% CI, upper 95% CI)	0.106 (\pm 0.0199) (0.08515, 0.1269)	0.03769 (\pm 0.00398) (0.03436, 0.04102)	15.88 (\pm 5.276) (11.46, 20.29)	0.9255 (\pm 0.02639) (0.8978, 0.9532)	0.1567 (\pm 0.02432) (0.118, 0.1954)
a-miR 4-1/2 (4) (\pm SD) (lower 95% CI, upper 95% CI)	0.1088 (\pm 0.02774) (0.07968, 0.1379)	0.02015 (\pm 0.00420) (0.01664, 0.02366)	7.5 (\pm 2.507) (5.404, 9.596)	0.9138 (\pm 0.01799) (0.8949, 0.9327)	0.1478 (\pm 0.03539) (0.09152, 0.2041)
WT 4 (\pm SD) (lower 95% CI, upper 95% CI)	0.1099 (\pm 0.02415) (0.07155, 0.1484)	0.0342 (\pm 0.00649) (0.02877, 0.03963)	16.38 (\pm 4.897) (12.28, 20.47)	0.9203 (\pm 0.02924) (0.8896, 0.951)	0.1883 (\pm 0.00859) (0.1746, 0.202)
Triple mutant (1) (\pm SD) (lower 95% CI, upper 95% CI)	0.1236 (\pm 0.01129) (0.1056, 0.1415)	0.0207 (\pm 0.00538) (0.0162, 0.0252)	7.875 (\pm 3.523) (4.93, 10.82)	0.9247 (\pm 0.01299) (0.911, 0.9383)	0.1279 (\pm 0.01971) (0.0965, 0.1592)
WT 5 (\pm SD) (lower 95% CI, upper 95% CI)	0.0967 (\pm 0.02718) (0.06813, 0.1252)	0.03986 (\pm 0.00759) (0.03352, 0.04621)	12.88 (\pm 3.563) (9.896, 15.85)	0.9019 (\pm 0.02323) (0.8775, 0.9263)	0.1932 (\pm 0.01446) (0.1702, 0.2162)
Compl dSpm (1) (\pm SD) (lower 95% CI, upper 95% CI)	0.1143 (\pm 0.03298) (0.08381, 0.1448)	0.04573 (\pm 0.00547) (0.04115, 0.0503)	11.25 (\pm 5.97) (6.259, 16.24)	0.9083 (\pm 0.02954) (0.8773, 0.9393)	0.1987 (\pm 0.03705) (0.1398, 0.2577)
WT 6 (\pm SD) (lower 95% CI, upper 95% CI)	0.1244 (\pm 0.01351) (0.08965, 0.1591)	0.03721 (\pm 0.00811) (0.03044, 0.04399)	11.88 (\pm 4.824) (7.842, 15.91)	0.9338 (\pm 0.02097) (0.9117, 0.9558)	0.228 (\pm 0.007428) (0.2162, 0.2398)
OE 4-5 (4) (\pm SD) (lower 95% CI, upper 95% CI)	0.1244 (\pm 0.01947) (0.104, 0.1448)	0.04755 (\pm 0.00674) (0.04191, 0.05319)	14.38 (\pm 4.438) (10.66, 18.09)	0.9536 (\pm 0.02697) (0.9253, 0.9819)	0.1971 (\pm 0.03261) (0.1452, 0.249)

^εEquivalent to $\Psi_{\text{substrate}} = -0.451 (\pm 0.212)$ MPa

^φEquivalent to $\Psi_{\text{substrate}} = -4.617 (\pm 0.619)$ MPa

^εEquivalent to $\Psi_{\text{substrate}} = -5.415 (\pm 0.543)$ MPa

