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Supplemental information

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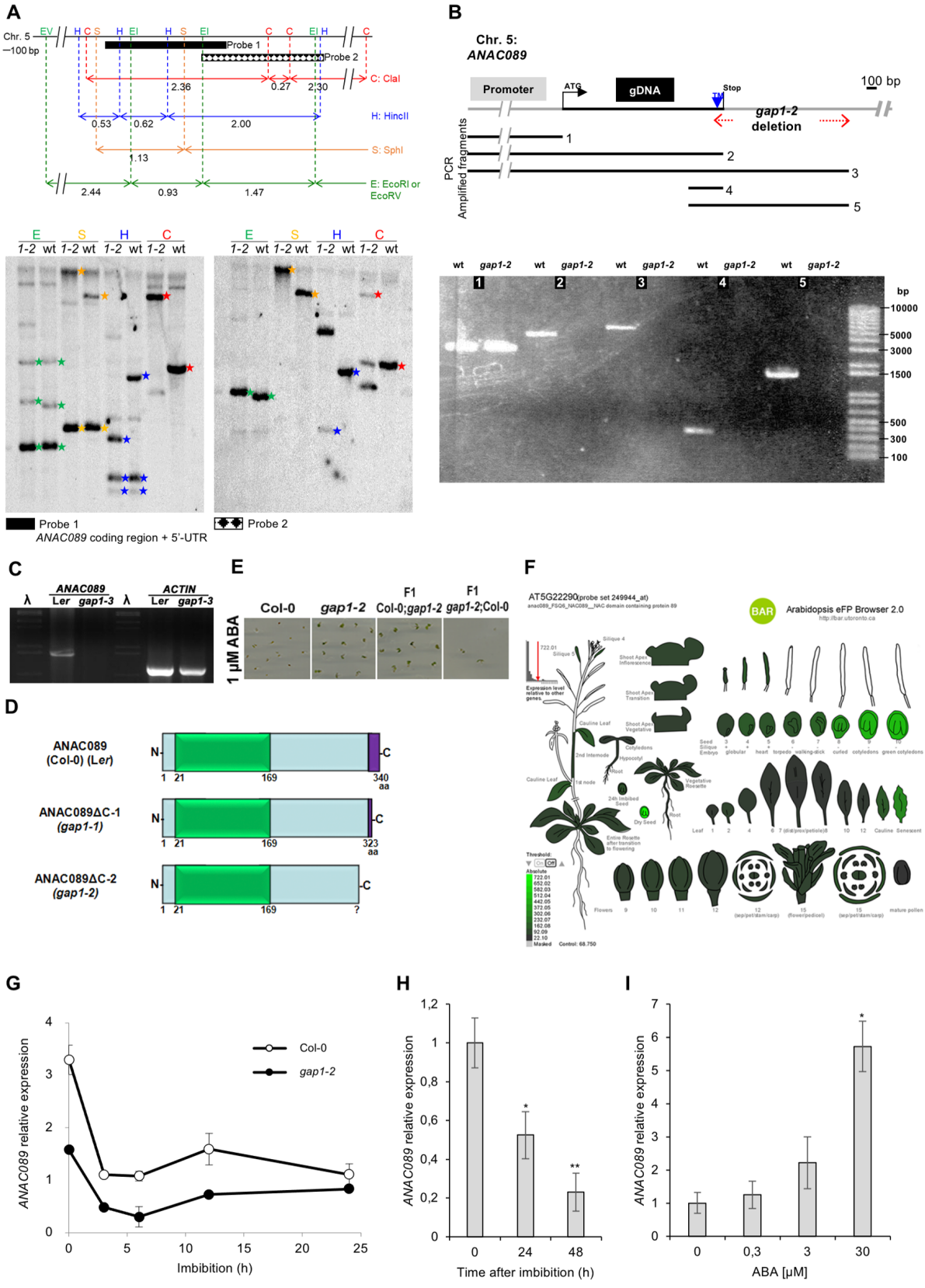


Figure S1 (related to Figure 1). *GAP1* encodes ANAC089 transcription factor expressed in seeds.

(A) Southern blot analysis of *gap1-2* mutation was a ~700 bp deletion that includes the C-terminal region.

(B) Identification of the DNA deletion in *ANAC089* locus of *gap1-2* mutant in chromosome 5. Illustration of the *ANAC089* locus to show promoter, genomic DNA, transmembrane domain (TM)

and DNA regions that were amplified by PCR to study the position of the deletion. PCR amplified fragments from wild type (Col-0) and *gap1-2* corresponding to the illustration above. Fragments from 2 to 5 were not amplified, indicating that the DNA deletion in *gap1-2* mutant compromised the 3´end of *ANAC089* locus.
(C) *gap1-3* allele is a knockout mutant from the Martienssen laboratory at CSHL. Semi-Q RT-PCR analysis of the band corresponding to *ANAC089* detected in Col-0 and not detected in *gap1-3*.
(D) ANAC089 protein scheme present in Col-0, L*er*, *gap1-1* and *gap1-2* backgrounds, respectively. NAC domain (green) and transmembrane domain (TM, purple) are indicated.
(E) ABA-insensitive phenotype of *gap1-2* and F1 progeny of Col-0 and *gap1-2* genetic crosses indicating that *gap1-2* is a dominant mutation.
(F) *ANAC089* expression patterns during different developmental stages. Transcription levels of *ANAC089* (At5g22290) in different plant tissues, based on data obtained using the eFP Browser 2.0 (http://bar.utoronto.ca). *ANAC089* expression achieves highest levels in dry seeds.
(G) *ANAC089* expression levels in dry and imbibed seeds of Col-0 and *gap1-2* mutant. Q RT-PCR analysis of *ANAC089* relative transcript abundance in Col-0 and *gap1-2* mutant seeds after 0, 3, 6, 12 and 24 hours of imbibition. Bars represent standard deviation from triplicate Q RT-PCR experiments. The expression data was normalized by the abundance of 18S *rRNA* mRNA.
(H) *ANAC089* relative expression levels in dry seeds and during seed imbibition in wild type Col-0. *ANAC089* transcript levels are highly present in dry seeds and reduced during seed imbibition in water.
(I) ABA induces the expression of *ANAC089*. Seeds were imbibed during 48 h in 0 (control), 0.3, 3 and 30μM ABA and changes in the expression levels of *ANAC089* were analysed by RT-qPCR. In all the graphs the mean ± SE (n=3) are represented. Asterisks indicate significant differences compared control vs treatments (*t*-test, $^{*}P<0.05$, $^{**}P<0.01$).

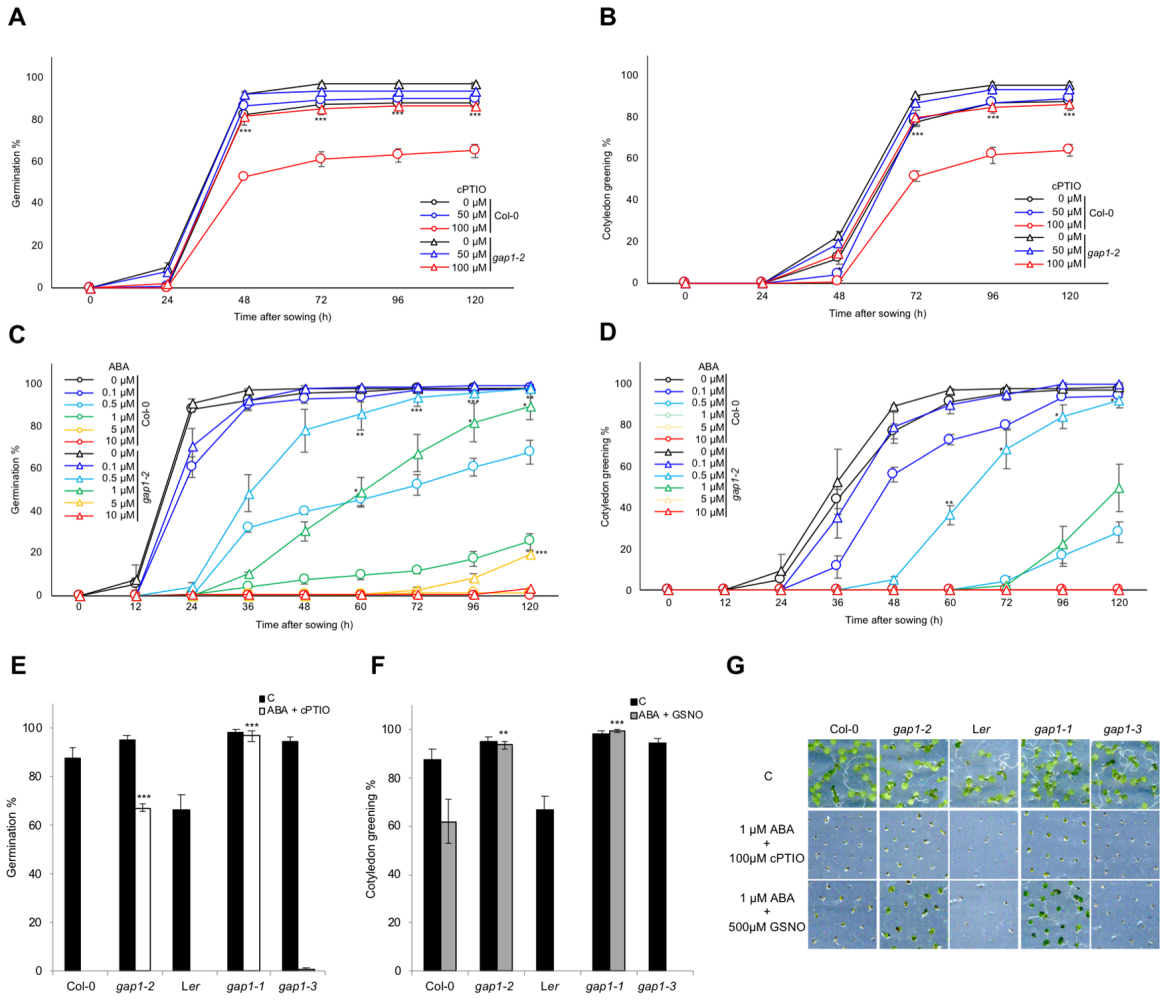


Figure S2 (related to Figure 1). Dose-response and treatment combinations during seed germination assays.

(A and B) Insensitivity of *gap1-2* mutants to cPTIO dose response concentrations compared to the wild type (Col-0) during seed germination and cotyledon greening. Wild type (Col-0) and *gap1-2* seeds were sown on control media (0µM) or media containing 50µM and 100µM cPTIO and seed germination (A) and cotyledon greening (B) are shown.

(C and D) Insensitivity of *gap1-2* mutants to ABA dose response concentrations compared to the wild type (Col-0) during seed germination and cotyledon greening. Wild type (Col-0) and *gap1-2* seeds were sown on control media (0µM) or media containing 0.1, 0.5, 1, 5 and 10µM ABA and seed germination (C) and cotyledon greening (D) are shown. In A-D graphs each value represents the average germination percentage of 50 to 100 seeds with error bars the SE of three replicates. Asterisks indicate significant differences compared wild-type vs mutant respectively for each concentration (*t*-test, $^{*}P<0.05$, $^{**}P<0.01$ $^{***}P<0.001$).

(E) Insensitivity of *gap1-1* and *gap1-2* mutants to a combined treatment with ABA and the NO scavenger cPTIO compared to the wild type (Col-0, L*er*) and *gap1-3* mutant during seed germination and seedling establishment. Seeds of the indicated genotypes were sown on control media (C) or media containing 1µM ABA plus 100µM cPTIO.

(F) Insensitivity of *gap1-1* and *gap1-2* mutants to a combined treatment with ABA and the NO donor GSNO compared to the wild type (Col-0, L*er*) and *gap1-3* mutant during seed germination and seedling establishment. Seeds of the indicated genotypes were sown on control media (C) or media containing 1µM ABA plus 500µM GSNO. In E-F graphs the mean ± SD are represented at 7 days after sowing. Each value represents the average germination and seedling establishment percentage of 50 to 100 seeds with three replicates. Asterisks indicate significant differences compared mutant vs wild type in the different treatments (*t*-test, $^{**}P<0.05$, $^{***}P<0.01$).

(G) Representative picture of *gap1* mutants and wild types sowing the insensibility of *gap1-1* and *gap1-2* to the combined treatments of ABA and cPTIO or GSNO.

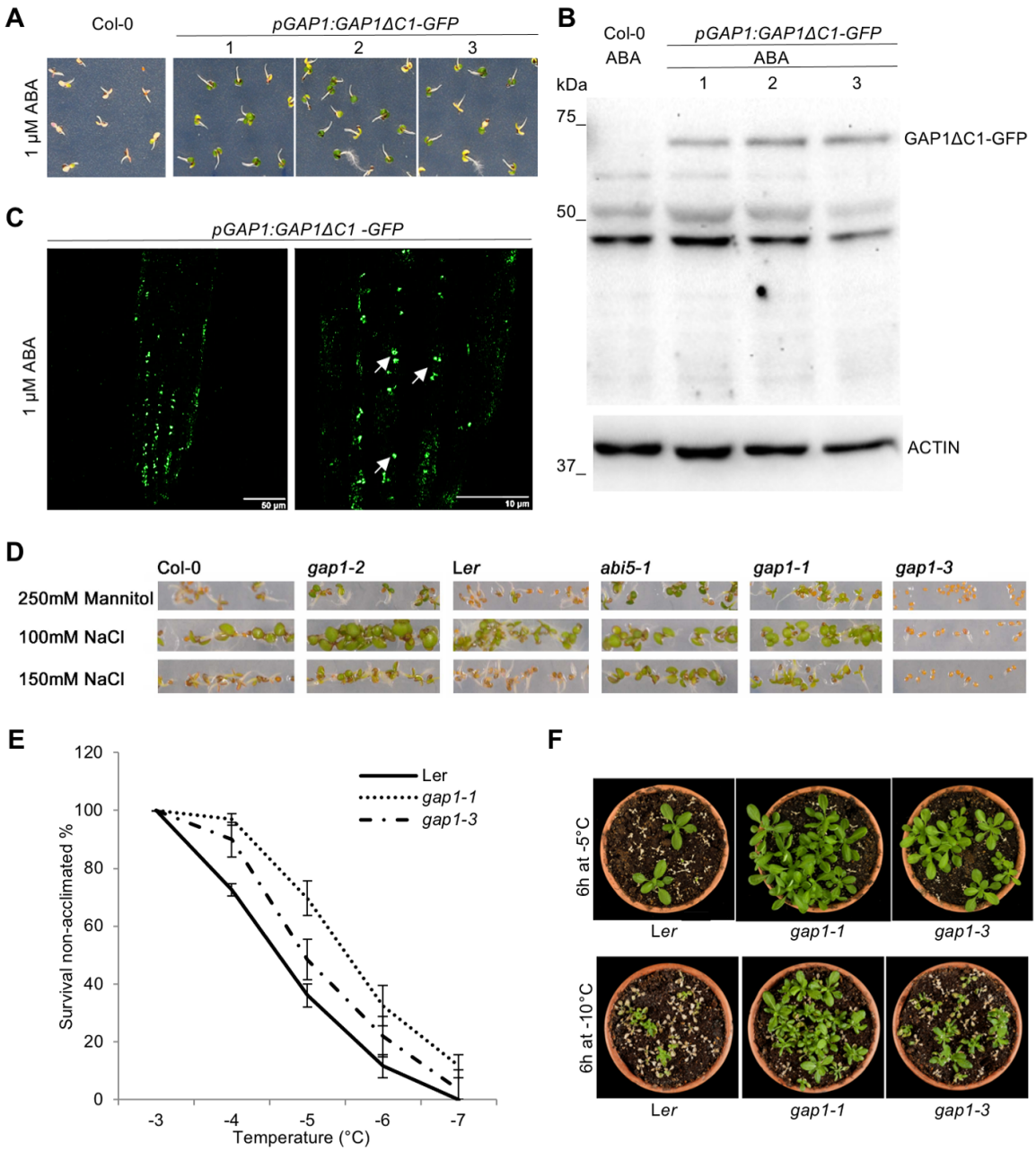


Figure S3 (related to Figure 1). Gain-of-function phenotype of *pANAC089:ANAC089ΔC-1-GFP* expression lines, abiotic stresses (NaCl and mannitol) and freezing tolerance assays of *gap1* mutants.

(A) Three independent and homozygous *pANAC089:ANAC089ΔC-1-GFP* lines were stratified for 3 days at 4ºC and sown in MS media supplemented with 1µM ABA. Photographs of ABA-insensitive seedling establishment were taken 10 days after sowing.

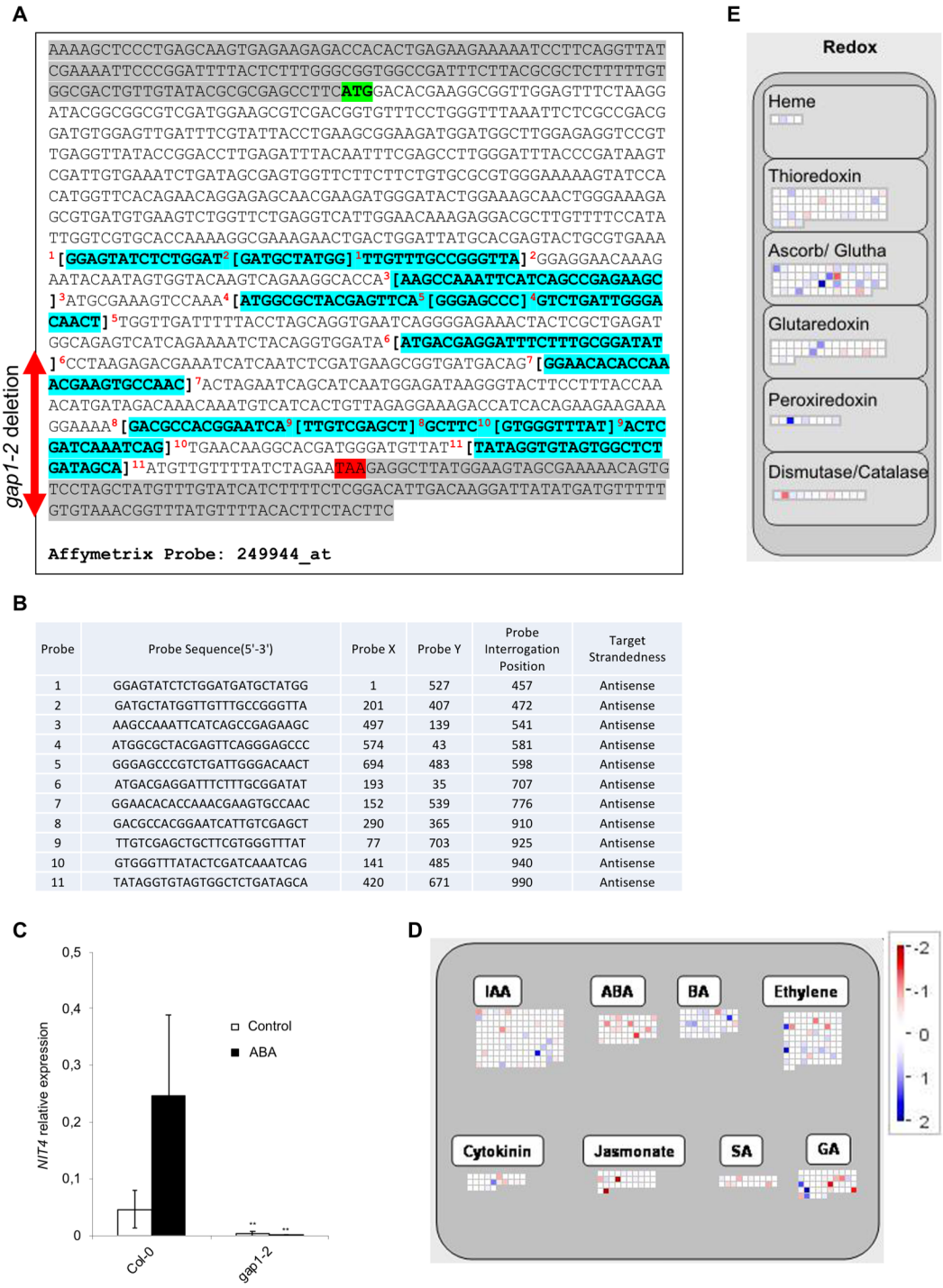
(B) Corresponding ANAC089ΔC-1-GFP protein levels in seedlings after 10 days in 1µM ABA. Actin protein levels were also determined as a loading control.

(C) Confocal microscopy of 10-day-old Arabidopsis roots of *pANAC089:ANAC089ΔC-1-GFP*-expressor line in 1µM ABA. ANAC089ΔC-1-GFP protein localized inside the cell nucleus in speckles.

(D) Stress germination assays in MS medium supplemented with either 250mM mannitol or 100 and 150mM NaCl.

(E) Freezing tolerance of two-week-old non-acclimated plants exposed to the indicated freezing temperatures for 6h. Survival percentages were evaluated after one week of recovering at 22°C under long-day conditions.

(F) Representative two-week-old non-acclimated (upper panel) and cold acclimated (7d, 4ºC) (lower panel) L*er*, *gap1-1* and *gap1-3* plants one week after being exposed 6h to -5ºC or -10ºC, respectively.



Probe	Probe Sequence(5'-3')	Probe X	Probe Y	Probe Interrogation Position	Target Strandedness
1	GGAGTATCTCTGGATGATGCTATGG	1	527	457	Antisense
2	GATGCTATGGTTGTTTGCCGGGTTA	201	407	472	Antisense
3	AAGCCAAATTCATCAGCCGAGAAGC	497	139	541	Antisense
4	ATGGCGCTACGAGTTCAGGGAGCCC	574	43	581	Antisense
5	GGGAGCCCGTCTGATTGGGACAACT	694	483	598	Antisense
6	ATGACGAGGATTTCTTTGCGGATAT	193	35	707	Antisense
7	GGAACACACCAAACGAAGTGCCAAC	152	539	776	Antisense
8	GACGCCACGGAATCATTGTCGAGCT	290	365	910	Antisense
9	TTGTCGAGCTGCTTCGTGGGTTTAT	77	703	925	Antisense
10	GTGGGTTTATACTCGATCAAATCAG	141	485	940	Antisense
11	TATAGGTGTAGTGGCTCTGATAGCA	420	671	990	Antisense

Figure S4 (related to Figure 2). Probes of *ANAC089* gene in the Affymetrix GeneChip Arabidopsis ATH1 Genome Array and the deletion in *gap1-2* allele. Expression of representative genes.

(A and B) *ANAC089* gene (Fold Change = -29,93) is the most strongly repressed gene in the microarray. To explain this, we search for the 11 probes of the *ANAC089* gene present in the Affymetrix GeneChip Arabidopsis ATH1 Genome Array and 6 of them (from 6 to 11) are included in the deletion present in *ANAC089* of *gap1-2* allele. In grey 5´ and 3´ UTRs, in green initial codon and in red stop codon.

(C) *NIT4* relative expression levels during seed imbibition and ABA treatment in wild type Col-0 and *gap1-2*. *NIT4* transcript levels are downregulated in *gap1-2* mutant under both conditions. Seeds were imbibed during 3 h in control and 5μM ABA and changes in the expression levels of *NIT4* were analysed by RT-qPCR. The mean ± SE (n=3) is represented. Asterisks indicate significant differences compared control vs treatments (*t*-test, $^{**}P<0.01$).

(D and E) Scheme representing the expression of genes involved in hormone (C) and redox metabolism (D), comparing expression levels in *gap1-2 versus* Col-0 seeds. The results were analyzed using the MapMan software (Thimm et al., 2004; Usadel et al., 2005). Those genes that do not change are displayed in white, in red range are plotted induced genes and the repressed genes are represented in blue range.

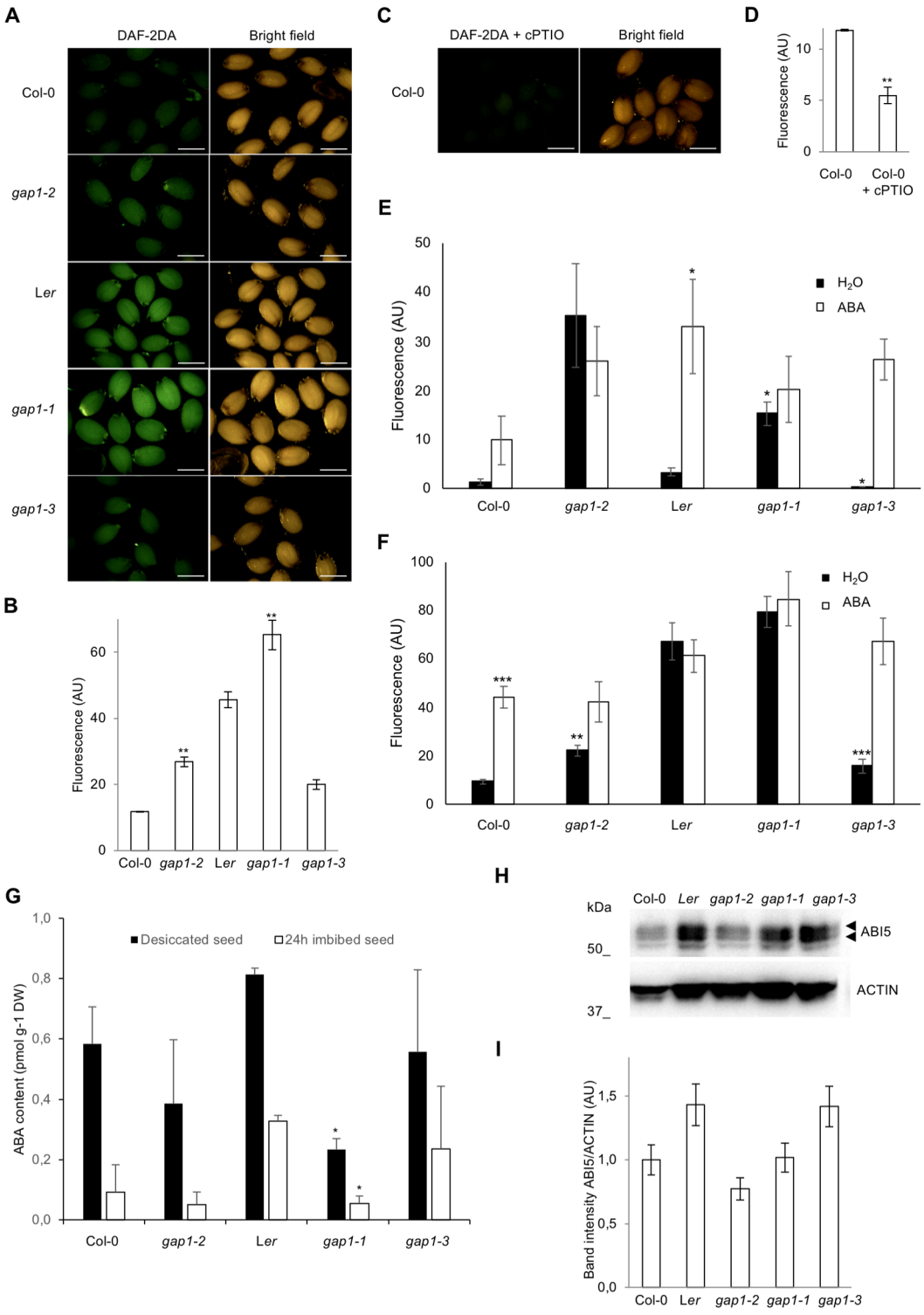


Figure S5 (related to Figure 3). Increased endogenous NO levels in *gap1-1* and *gap1-2* imbibed seeds reduce ABI5 protein accumulation.

(A) NO production detected by DAF-2DA in 24 hours imbibed seeds of the indicated genotypes, showing higher levels for *gap1-1* and *gap1-2* mutants compared with respective wild type. Scale bars 500 µm.

(B) Quantitative data of NO-dependent DAF-2DA fluorescence seed images. Values represent the mean ± SE (n=3). Asterisks indicate statistically significant difference between a: *gap1-2 vs* Col-0 (*t*-test, $^{**}P$<0,01); b: *gap1-1 vs* L*er* (*t*-test, $^{**}P$<0,01). AU, arbitrary units.

(C) Fluorescence of seeds treated with the NO scavenger cPTIO (1mM) imbibed for 24 hours and then subjected to DAF-2DA incubation. Scale bars 500 µm.

(D) Quantitative data of NO-dependent DAF-2DA fluorescence seed images. Values represent the mean ± SE (n=3). Asterisks indicate statistically significant difference with Col-0 (*t*-test, $^{**}P$<0,01). AU, arbitrary units.
(E) Quantitative data of NO-dependent DAF-2DA fluorescence in 24-hour-imbibed embryos in water and 5µM ABA. Values represent the mean ± SE (n=3). Asterisks indicate statistically significant difference between a: L*er* H_2O *vs* L*er* ABA (*t*-test, $^{*}P$<0,05); b: *gap1-1 vs* L*er* (*t*-test, $^{*}P$<0,05).
(F) Quantitative data of NO-dependent DAF-2DA fluorescence in 24-hour-imbibed seeds in water and 5µM ABA. Values represent the mean ± SE (n=3). Asterisks indicate statistically significant difference between a: Col-0 H_2O *vs* Col-0 ABA (*t*-test, $^{***}P$<0,001)*;* b: *gap1-2 vs* Col-0 (*t*-test, $^{**}P$<0,01); c: *gap1-3 vs* L*er* (*t*-test, $^{***}P$<0,001). AU, arbitrary units.
(G) Endogenous ABA levels in desiccated and 24-hour-imbibed seeds. Values represent means ± SE (n=3). Asterisks indicate statistically significant differences with L*er* ($^{*}P$<0,05) according to a basic ANOVA test.
(H) Immunoblot analysis of ABI5 protein levels in seed extracts of Col-0, L*er*, *gap1-1*, *gap1-2* and *gap1-3* after 24 hours of imbibition. Actin protein levels are shown as a loading control.
(I) Quantitative data of immunoblot analysis of ABI5 degradation in *gap1* mutant backgrounds. Values represent the mean ± SE (n=3).

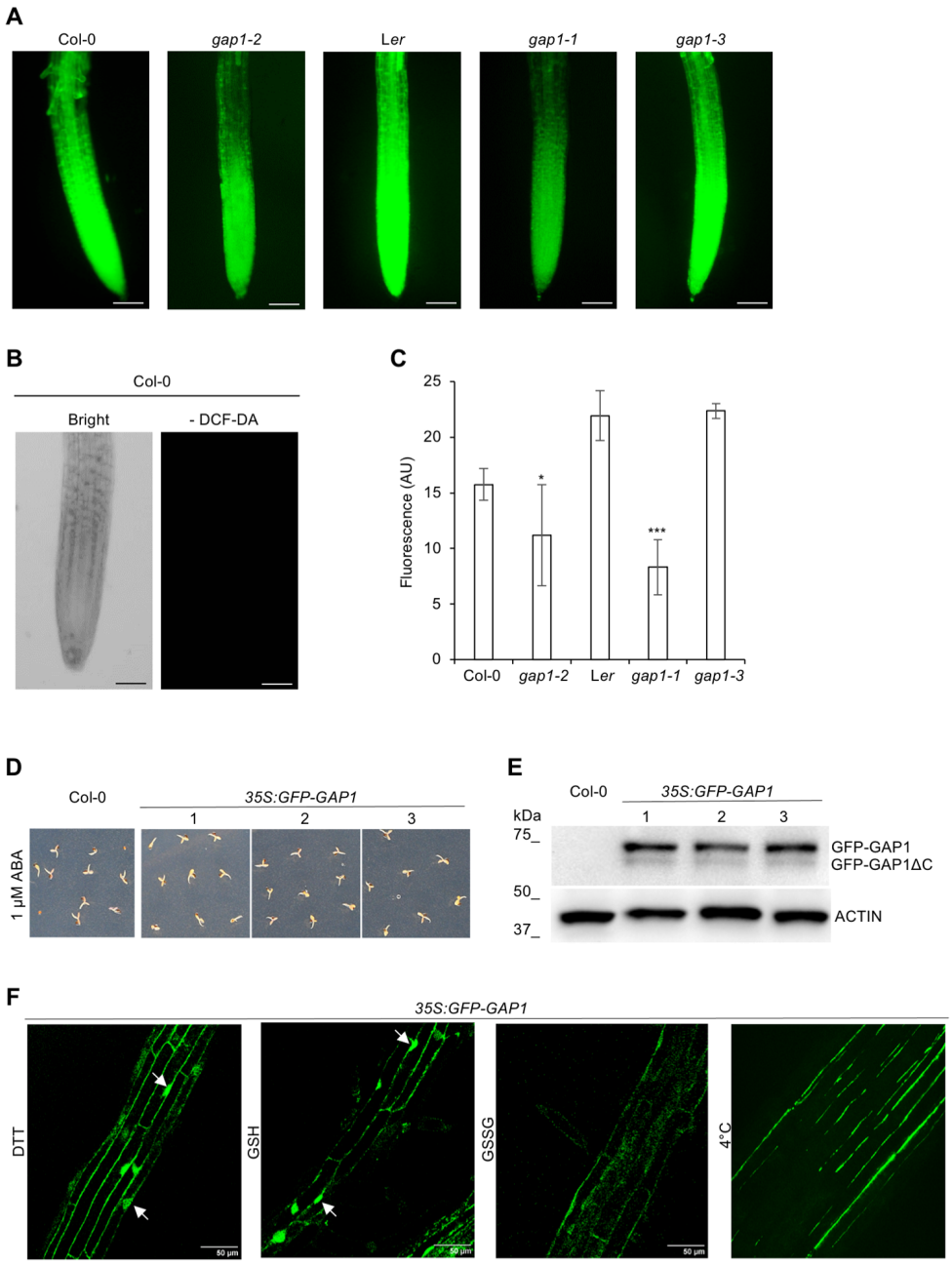


Figure S6 (related to Figure 4). Decreased endogenous ROS levels in *gap1-1* and *gap1-2* mutants. Generation and molecular analysis of *35S:GFP-ANAC089* transgenic lines.

(A) Endogenous ROS levels in *gap* mutants. Fluorescence corresponding to ROS accumulation in root tips of 6-day-old seedlings after incubation with DCF-DA. Scale bars 100 µm.

(B) 6-day-old Col-0 root tip used as a negative control without ROS detecting probe to adjust fluorescence signal to cero. Scale bars 100 µm.

(C) Quantitative data of ROS-dependent DCF-DA fluorescence root images. Values represent the mean of 10-12 roots for each genotype and error bars the SD of three replicates. Asterisks indicate statistically significant differences between a: *gap1-2 vs* Col-0 (*t*-test, $^{*}P$<0,05); b: *gap1-1 vs* L*er* (*t*-test, $^{***}P$<0,001).

(D) Wild type ABA-sensitive seedling establishment in three independent and homozygous *35S:GFP-ANAC089* lines in Col-0 background. Photographs were taken 10 days after sowing.

(E) GFP-ANAC089 protein levels in *35S:GFP-ANAC089* seedlings after 10 days. Actin protein levels were also determined as a loading control.

(F) Confocal microscopy of 7-day-old Arabidopsis roots of *35S:GFP-ANAC089*-overexpressor lines after 4 hours of 1mM DTT, 1mM GSH, 1mM GSSG and 4°C treatments. Nuclear localization of GFP-ANAC089 protein after the corresponding treatments is indicated by arrows. Scale bars 50 µm.

Table S3 (related to STAR Methods). Primers used in this study.

Markers for fine mapping of *ANACO089*

Markers	Forward primer	Reverse primer	Enzyme
MWD9	CTTGTACAGTAGCTGCATTG	GTAAAACCGTGGGAGAAAC	*Eco*R V
MWD9-25.3	ACACGGGTTTAGGTCACA	ACCTCTCTAGACAAAAGCCA	*Acc* I
At5g22250	TATGATTCCATGACTAGA	TATAACTAAACCTTGCAG	*Hinf* I

Primers for production of *pANAC089:ANAC089Δ*C-1 and *ANAC089* cDNA

Name	Forward primer	Reverse primer
Compl	CACCATCTCTTGAAAAAATCTCC	TTCTAGATAAAACAACATTGC
pANAC089	CACCATCTCTTGAAAAAATCTCC	GAAGGCTCGCGCGTATACAAC
cDNA	ATGGACACGAAGGCGGTTGG	AAGAGCTCGAGCATACACTG

Primers for Q RT-PCR

Gene	Forward primer	Reverse primer
Q2-ANAC089	CACTGAGAAGAAAAATCCTTCAGGTT	AAAAAGAGCGCGTAAGAAATCG
NIT4	AGTACCATGCTTCTGCCATTG	CCATTAACGCTAATCGTTCCA
18S rRNA	CAGATACCGTCCTAGTCTCAACCA	CAGCGGAGTCCTATAAGCAACAT