RopGAP4-Dependent Rop GTPase Rheostat Control of *Arabidopsis* Oxygen Deprivation Tolerance

Airica Baxter-Burrell, Zhenbiao Yang, Patricia S. Springer, Julia Bailey-Serres*

Transient soil flooding limits cellular oxygen to roots and reduces crop yield. Plant response to oxygen deprivation involves increased expression of the alcohol dehydrogenase gene (*ADH*) and ethanolic fermentation. Disruption of the *Arabidopsis* gene that encodes Rop (RHO-like small G protein of plants) guanosine triphosphatase (GTPase) activating protein 4 (*ROPGAP4*), a Rop deactivator, elevates *ADH* expression in response to oxygen deprivation but decreases tolerance to stress. Rop-dependent production of hydrogen peroxide via a diphenylene iodonium chloride–sensitive calcium-dependent reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is necessary for induction of both *ADH* and *RopGAP4* expression. Tolerance to oxygen deprivation requires Rop activation and *RopGAP4*-dependent negative feedback regulation. This Rop signal transduction rheostat balances the ability to increase ethanolic fermentation with survival.

Plant endurance of transient flooding requires increased production of adenosine triphosphate through glycolysis and regeneration of nicotinamide adenine dinucleotide through ethanolic fermentation (*1*, *2*). Signal transduction processes that control changes in gene expression in O_2-deprived cells involve oscillations in cytosolic free Ca^{2+} (*3–6*). To identify the genes involved in regulating the expression of the sole alcohol dehydrogenase gene (*ADH*) of *Arabidopsis thaliana*, we screened lines carrying a gene-trap transposon (*DsG*) (*7*) for increased β-glucoronidase (GUS) histochemical staining and altered induction of ADH specific activity in response to O_2 deprivation under low light (*8*) (see supplementary methods). We identified a line that displayed elevated GUS staining throughout the seedling vasculature in response to low O_2 (Fig. 1A) but with no apparent abnormalities under control conditions. This line contained a single *DsG* transposon inserted into the first exon of *RopGAP4* (GTPase activating protein; 49 kD) (Fig. 1B) [GenBank accession number AC008153; MIPS At3g11490 (Munich Information Center for Protein Sequences identifier for *Arabidopsis ROPGAP4* on chromosome 3); BAC F24K9.16 (Bacterial Artificial Chromosome number F29 and gene identifier #16), position 61811], resulting in a translational fusion within the CRIB (Cdc42/Rac-interactive binding) motif at the amino terminus of RopGAP4 (Fig. 1B). We designated this mutant allele *ropgap4-1*.

RopGAPs were identified in a yeast two-hybrid system based on interaction with the RHO-like small G-protein of plants, Rop (*9*). RopGAPs possess a conserved GAP-like domain and a CRIB motif that enhances Rop interaction, allowing for efficient GTP hydrolysis (*9*). Rop signaling controls intracellular Ca^{2+} gradients and actin cytoskeletal dynamics required for tip growth of pollen (*10–16*) and polar growth of root hairs (*17*). Activation of Rop signaling is implicated in defense responses and developmental processes involving hydrogen peroxide (H_2O_2) (*18–20*), whereas inactivation of Rop signaling is necessary for abscisic acid–induced closure of leaf stomata (*21*).

RopGAP4 mRNA accumulation increased dramatically in response to O_2 deprivation in wild-type (WT) seedlings, as detected by reverse transcriptase–polymerase chain reaction (RT-PCR) (Fig. 1C). *RopGAP4* mRNA was not detectable in *ropgap4-1* seedlings, which indicates that the *DsG* insertion resulted in a loss-of-function mutation.

ropgap4-1 allowed us to consider whether Rop signaling is involved in regulating *ADH* expression in response to O_2 deprivation. *ropgap4-1* seedlings showed a more rapid and dramatic increase in *ADH* mRNA accumulation and ADH specific activity threefold higher than WT after 12 hours of O_2 deprivation; paradoxically, they were more sensitive to the stress (Figs. 1C and 2A; Table 1). After 24 hours of O_2 deprivation, *ADH* mRNA and specific activity levels dropped dramatically and *ropgap4-1* seedlings were unable to recover upon reoxygenation. Seedlings of a line expressing a dominant negative form of Rop2 [*35S::DN-rop2* (T20N)] (*22*) showed no detectable induction of *ADH* mRNA or specific activity after O_2 deprivation and increased stress sensitivity. This confirms that signaling through the Rop GTPase is mandatory for activation of *ADH* expression, a prerequisite for low O_2 tolerance (*8*, *23*). In a line expressing a constitutive active form of Rop2 [*35S::CA-rop2* (G15V)] (*22*), ADH specific activity was higher under control conditions and inducible by O_2 deprivation. The limited induction of ADH in *CA-rop2* versus the excessive induction in *ropgap4-1* can be explained by negative feedback regulation of Rop signaling by ROPGAP4 (see below).

We confirmed transient activation of Rop signaling by O_2 deprivation with an assay that detects Rop-GTP by interaction with Rop-interacting CRIB motif-containing pro-

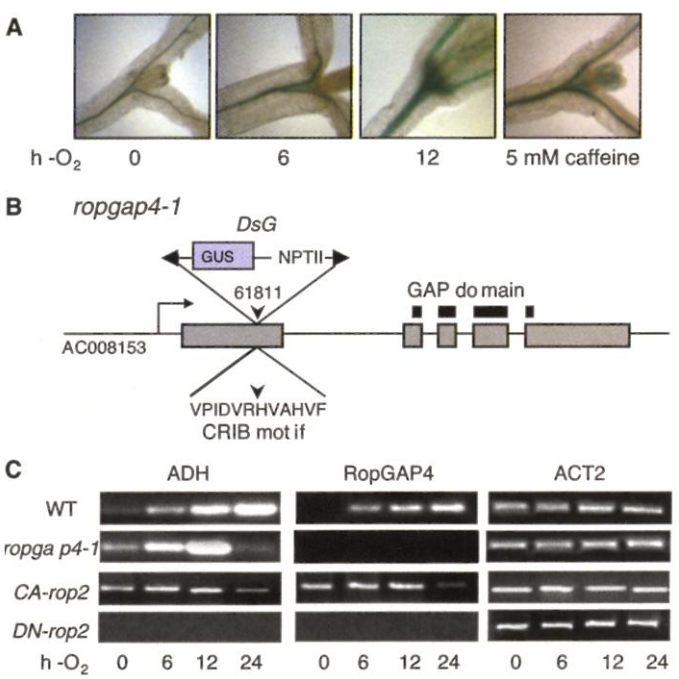

Fig. 1. Characterization of *ropgap4-1* and mRNA levels after O_2 deprivation. **(A)** Histochemical staining of 7-day-old *ropgap4-1* seedlings with 5- bromo-4-chloro-3-indolyl-beta-D-glucuronic acid after O_2 deprivation or 24-hour exposure to 5 mM caffeine. **(B)** Site and orientation of *DsG* insertion within the CRIB motif of the first exon of *RopGAP4* in *ropgap4-1*. A, Ala; D, Asp; F, Phe; H, His; I, Ile; P, Pro; R, Arg; V, Val. **(C)** RT-PCR detection of *ADH*, *RopGAP4*, and actin (*ACT2*) mRNA in WT, *ropgap4-1*, *CA-rop2*, and *DN-rop2* seedlings after O_2 deprivation.

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tein (RIC1) (*24*). Figure 2B compares the level of Rop in total cell extracts (Rop-GTP and Rop-GDP) with RIC1-interacting Rop (Rop-GTP) over 36 hours of O_2 deprivation. Rop-GTP levels increased in WT seedlings after 1.5 hours, increased through 12 hours, and then decreased. Rop-GTP levels were constitutively high in *ropgap4-1* seedlings and increased in response to low O_2 but showed no decrease even after 36 hours. O_2 deprivation promotes activation of Rop, and RopGAP4 appears to negatively regulate this activation in WT seedlings.

Cotyledons of *ropgap4-1* seedlings turned brown upon reoxygenation, whereas those of *CA-rop2*, *DN-rop2*, and WT remained green, which led us to suspect that *ropgap4-1* seedlings succumb to O_2 deprivation and reoxygenation as a result of oxidative stress. *ropgap4-1* seedlings may fail to control the production of reactive oxygen species, because overexpression of a constitutive active form of Rop results in increased production of H_2O_2 via a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in several plant species (*18–20*), and a GAP negatively regulates Rac GTPase activation of NADPH oxidase in mammals (*25*). We tested whether the response to O_2 deprivation was affected by treatment of seedlings with diphenylene iodonium chloride (DPI), which inhibits production of superoxide by flavin-containing NADPH oxidases and the resultant accumulation of H_2O_2. In all four genotypes, DPI reduced ADH activity under control and low O_2 conditions, which indicates that ADH induction requires a DPI-sensitive NADPH oxidase (Fig. 2A). DPI also reduced the duration of stress that WT seedlings survived from >24 to <12 hours (Table 1). DPI treatment reduced ADH induction in *ropgap4-1* seedlings and increased their survival after O_2 deprivation, which reveals that the inability to down-regulate DPI-sensitive NADPH oxidase reduces stress tolerance. Consistently, survival after O_2 deprivation was improved in *CA-rop2* and impaired in *DN-rop2* seedlings in the presence of DPI.

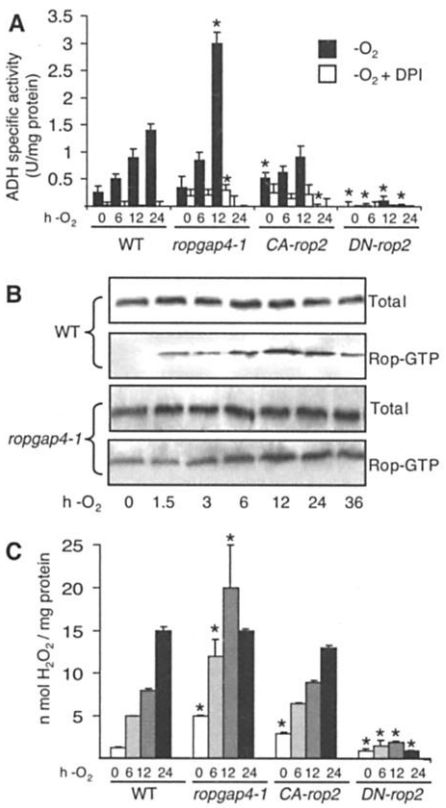

Fig. 2. Rop signaling and H_2O_2 production regulate ADH expression. (**A**) ADH specific activity in seedlings after O_2 deprivation in the absence or presence of 30 μM DPI. (**B**) Rop-RIC1 interaction assay on extracts from WT and *ropgap4-1* seedlings after O_2 deprivation. Immunoblot shows detection of levels of total Rop (Rop-GTP and Rop-GDP) in crude extracts or Rop-GTP obtained by pull-down through interaction with RIC1-maltose binding protein. Data are representative of three independent experiments. (**C**) H_2O_2 levels after O_2 deprivation. In (A) and (C), values are mean ± SE of three independent experiments. Asterisk indicates a significant difference from WT at the same time point ($P < 0.01$; Student's t test).

H_2O_2 levels increased in response to O_2 deprivation in WT, *ropgap4-1*, and *CA-rop2* seedlings but did not change significantly in *DN-rop2* seedlings (Fig. 2C), which supports a role of Rop signaling in H_2O_2 production. In WT seedlings, H_2O_2 level and ADH specific activity increased coordinately over 24 hours of stress. H_2O_2 levels in *ropgap4-1* seedlings under control conditions and after 6 and 12 hours of O_2 deprivation were significantly higher than in WT seedlings, consistent with ADH specific activity data. High H_2O_2 in the mutant may contribute to reduced stress tolerance. In *CA-rop2* seedlings, H_2O_2 levels correlated with constitutively high ADH specific activity under control conditions but were not clearly responsible for intolerance of low O_2.

ropgap4-1 seedlings have constitutively high levels of Rop-GTP but near normal levels of ADH specific activity until they are deprived of O_2, which indicates that accumulation of Rop-GTP is insufficient for induction of ADH. An increase in cytosolic free Ca^{2+} due to organellar efflux or apoplastic influx is necessary for activation of *ADH* expression in *Arabidopsis* (*3*). Treating maize cells with low levels of caffeine stimulates *ADH1* expression and promotes an increase in cytosolic free Ca^{2+}, similar to that observed in response to anoxia (*4*, *5*). Caffeine treatment under nonstress conditions induced ADH specific activity to significantly higher levels than the maximal level observed in response to low O_2 in all four genotypes (Fig. 3A). DPI effectively blocked the caffeine-stimulated increase in ADH specific activity and the concomitant increase in H_2O_2 (Fig. 3, A and B). The caffeine-promoted increase in ADH specific activity, consistent with O_2 deprivation, was dramatic in *ropgap4-1* and limited in *CA-rop2* seedlings. In *DN-rop2* seedlings, the caffeine-stimulated induction may result from a Rop-independent mechanism or interaction between a Ca^{2+} signal and the residual activity of endogenous Rops. Topical application of a H_2O_2 regenerating system, glucose and glucose oxidase, resulted in a rapid and efficient increase in ADH specific activity in WT seedlings (Fig. 4A), which confirms that H_2O_2 is a second messenger in *ADH* regulation.

These results reveal that O_2 deprivation stimulates a Rop signal transduction pathway, activating a DPI-sensitive NADPH oxidase that results in increased H_2O_2 production, which acts as a second messenger in the induction of *ADH* expression (fig. S1). An increase in cytosolic free Ca^{2+} appears to be necessary to complete this Rop-mediated signal. This could be due to the binding of Ca^{2+} by the plasma membrane DPI-sensitive NADPH oxidase gp91*phox* subunit (*26*) or to a Ca^{2+}-dependent DPI-sensitive NAD(P)H dehydrogenase/oxidase of the inner mitochondrial membrane (*27*).

The attenuation of Rop signal transduction is also necessary for tolerance of O_2 deprivation. Several lines of evidence indicate that Rop signaling drives this attenuation by activating *RopGAP4* expression. (i) Low O_2 promoted *RopGAP4* mRNA accumulation in WT but not *DN-rop2* seedlings (Fig. 1C). (ii) GUS activity increased in *ropgap4-1* seedlings in response to low O_2 and caffeine, but it was blocked by DPI

Table 1. Effect of O_2 deprivation and DPI treatment on seedling survival. +, Addition of 30 μM DPI in 3% dimethyl sulfoxide solvent; –, addition of solvent. Data are mean ± SE from three independent experiments.

Oxygen deprivation (hours)	Viable seedlings 48 hours after treatment (%)							
	WT		*ropgap4-1*		*CA-rop2*		*DN-rop2*	
DPI	–	+	–	+	–	+	–	+
0	100	100	100	100	100	100	100	90 ± 3
6	100	80 ± 5	90 ± 2	100	100	100	100	70 ± 4
12	100	0	0	50 ± 5	69 ± 5	100	100	0
24	80 ± 6	0	0	0	0	0	0	0

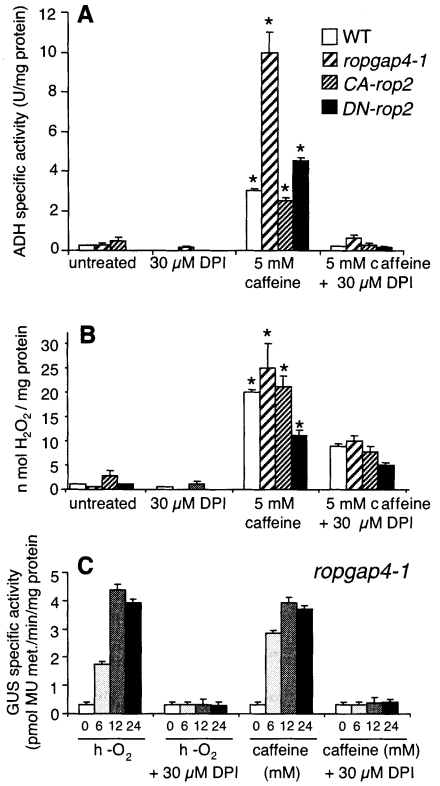

Fig. 3. ADH activity is stimulated by caffeine treatment via Rop-induced and DPI-sensitive H_2O_2 production. (**A**) ADH specific activity in seedlings treated with caffeine and/or DPI for 24 hours. (**B**) H_2O_2 levels in seedlings analyzed in (A). Values are mean ± SE of three independent experiments. Asterisk indicates significant difference from the maximal level detected after O_2 deprivation ($P < 0.01$; Student's t test). (**C**) GUS specific activity in *ropgap4-1* seedlings after O_2 deprivation, caffeine treatment, and DPI treatment. Values are mean ± SE of three independent experiments.

(Figs. 1A and 3C). (iii) Application of a H_2O_2 regenerating system elevated GUS activity in *ropgap4-1* seedlings (Fig. 4B). (iv) *RopGAP4* mRNA levels were constitutively elevated in *CA-rop2* seedlings (Fig. 1C).

Thus, a Rop rheostat regulates the production of H_2O_2 that is required to trigger the expression of beneficial genes (for example, *ADH*) and the avoidance of H_2O_2-induced cell death. Rop signaling is controlled by negative feedback regulation through the stimulation of *RopGAP4* transcription by H_2O_2. The termination of Rop signaling by RopGAP4 would alleviate oxidative stress and limit consumption of carbohydrate reserves via glycolysis and ethanolic fermentation. The reduced O_2 deprivation tolerance of the *DN-rop2*, *CA-rop2*, and *ropgap4-1* seedlings underscores the requirement for a fully functional Rop rheostat. We propose that a Rop rheostat is critical to developmental processes and environmental stress responses that use H_2O_2 as a second messenger or enhance H_2O_2 accumulation, including the response to abscisic acid, auxin, pathogen infection, and numerous abiotic stresses. Manipulation of the Rop signal transduction rheostat may enhance the productivity of crops that undergo transient submergence or soil waterlogging.

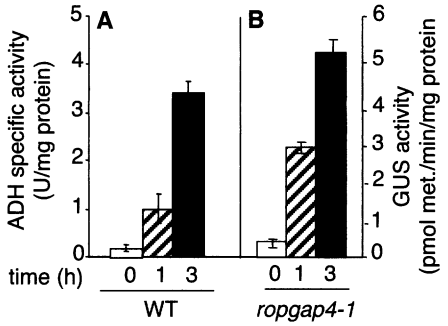

Fig. 4. ADH activity and *ROPGAP4* expression is induced by a H_2O_2-regenerating system. ADH specific activity in WT (**A**) and GUS specific activity in *ropgap4-1* seedlings (**B**) treated with glucose and glucose oxidase for up to 3 hours.

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Supporting Online Material

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Materials and Methods
Fig. S1
References and Notes

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Comparative Genome Sequencing for Discovery of Novel Polymorphisms in *Bacillus anthracis*

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Comparison of the whole-genome sequence of *Bacillus anthracis* isolated from a victim of a recent bioterrorist anthrax attack with a reference reveals 60 new markers that include single nucleotide polymorphisms (SNPs), inserted or deleted sequences, and tandem repeats. Genome comparison detected four high-quality SNPs between the two sequenced *B. anthracis* chromosomes and seven differences among different preparations of the reference genome. These markers have been tested on a collection of anthrax isolates and were found to divide these samples into distinct families. These results demonstrate that genome-based analysis of microbial pathogens will provide a powerful new tool for investigation of infectious disease outbreaks.

On 4 October 2001, the Centers for Disease Control reported a highly unusual case of inhalational anthrax in a photo editor at a West Palm Beach, Florida, media organization (*1*). This turned out to be the first in a series of letter-based attacks over several weeks. The attacks resulted in five fatalities (including the first-diagnosed victim) and