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

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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 O2-deprived cells involve oscillations in cytosolic free Ca2+ (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 of Arabidopsis. We characterized lines carrying an ADH-specific transposon (DsG) (7) for increased β-glucoronidase (GUS) histochemical staining and altered induction of ADH specific activity in response to O2 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 O2 (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 translation-termination (T20N) (9), resulting in a loss-of-function mutation.

RopGAP4 mRNA accumulation increased dramatically in response to O2 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 allowed us to consider whether Rop signaling is involved in regulating ADH expression in response to O2 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 O2 deprivation; paradoxically, they were more sensitive to the stress (Figs. 1C and 2A; Table 1). After 24 hours of O2 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 O2 deprivation and increased stress sensitivity. This confirms that signaling through the Rop GTPase is mandatory for activation of ADH expression, a prerequisite for low O2 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 O2 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 O2 deprivation with an assay that detects Rop-GTP by interaction with Rop-interacting CRIB motif-containing proteins. 2026
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 O2 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 O2, but showed no decrease even after 36 hours. O2 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 O2 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 H2O2 via a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in several plant species (16–20), and a GAP negatively regulates Rac GTPase activation of NADPH oxidase in mammal (25). We tested whether the response to O2 deprivation was affected by treatment of seedlings with dihydrogen ion ions chloride (DPI), which inhibits production of superoxide by flavin-containing NADPH oxidases and the resultant accumulation of H2O2. In all four genotypes, DPI reduced ADH activity under control and low O2 conditions, which indicates that ADH induction requires a GDP-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 O2 deprivation, which reveals that the inability to down-regulate GDP-sensitive NADPH oxidase reduces stress tolerance. Consistently, survival after O2 deprivation was improved in CA-rop2 and impaired in DN-rop2 seedlings in the presence of DPI.

H2O2 levels increased in response to O2 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 H2O2 production. In WT seedlings, H2O2 level and ADH specific activity increased coordinately over 24 hours of stress. H2O2 levels in ropgap4-1 seedlings under control conditions and after 6 and 12 hours of O2 deprivation were significantly higher than in WT seedlings, consistent with ADH specific activity data. High H2O2 in the mutant may contribute to reduced stress tolerance. In CA-rop2 seedlings, H2O2 levels correlated with constitutively high ADH specific activity under control conditions but were not clearly responsible for intolerance of low O2.

ropgap4-1 seedlings have constitutively high levels of Rop-GTP but near normal levels of ADH specific activity even after deprivation of O2, which indicates that accumulation of Rop-GTP is insufficient for induction of ADH. An increase in cytosolic free Ca2+ due to or-ganellar 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 Ca2+, 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 O2 in all four genotypes (Fig. 3A). DPI effectively blocked the caffeine-stimulated increase in ADH specific activity and the concomitant increase in H2O2 (Fig. 3, A and B). The caffeine-promoted increase in ADH specific activity, consistent with O2 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 Ca2+ signal and the residual activity of endogenous Rops. Topical application of a H2O2 regenerant system, glucose and glucose oxidase, resulted in a rapid and efficient increase in ADH specific activity in WT seedlings (Fig. 4A), which confirms that H2O2 is a second messenger in ADH regulation.

These results reveal that O2 deprivation stimulates a Rop signal transduction pathway, activating a GDP-sensitive NADPH oxidase that results in increased H2O2 production, which acts as a second messenger in the induction of ADH expression (fig. S1). An increase in cytosolic free Ca2+ appears to be necessary to complete this Rop-mediated signal. This could be due to the binding of Ca2+ by the plasma membrane DPI-sensitive NADPH oxidase gp91phox subunit (26) or to a Ca2+-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 O2 deprivation. Several lines of evidence indicate that Rop signaling drives this attenuation by activating RopGAP4 expression. (i) Low O2 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 O2 and caffeine, but it was blocked by DPI.

Table 1. Effect of O2 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.

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Fig. 2. Rop signaling and H2O2 production regulate ADH expression. (A) ADH specific activity in seedlings after O2 deprivation in the absence or presence of 30 μM DPI. (B) Rop-RIC1 interaction assay on extracts from WT and ropgap4-1 seedlings after O2 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) H2O2 levels after O2 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).
H2O2. The termination of Rop signaling by Rop rheostat is critical to developmental functional Rop rheostat. We propose that a seedlings after O2 deprivation, caffeine treatment of H2O2 that is required to trigger the mRNA levels were constitutively elevated in ropgap4 mutants, and the avoidance of H2O2-induced expression of beneficial genes (for example, transcription by DN

Thus, a Rop rheostat regulates the production of H2O2 which is required to trigger the expression of beneficial genes (for example, ADH) and the avoidance of H2O2-induced cell death. Rop signaling is controlled by negative feedback regulation through the stimulation of RopGAP4 transcription by H2O2. The termination of Rop signaling by RopGAP4 would alleviate oxidative stress and limit consumption of carbohydrate reserves via glycolysis and ethanolic fermentation. The reduced O2 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 H2O2 as a second messenger or enhance H2O2 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.

### References and Notes


### 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. 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