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# An ABA inducible *WRKY* gene integrates responses of creosote bush (*Larrea tridentata*) to elevated $CO_2$ and abiotic stresses

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**Abstract**

The physiological and molecular responses of *Larrea tridentata*, an evergreen desert shrub, to elevated $CO_2$, and abiotic stresses were examined to enhance our understanding of the crosstalk of hormones, stresses, and elevated $CO_2$ in signaling. Under nonlimiting conditions of water and nutrients, elevated $CO_2$ increased both ABA and starch concentrations in leaves by two-fold. Combinations of elevated $CO_2$ and water deficit treatments further increased the concentrations of ABA, but not starch. A transcription factor, LtWRKY21, was cloned from *Larrea tridentata* to address questions regarding crosstalk at the molecular level. The expression of *LtWRKY21* was enhanced by elevated $CO_2$, water deficit, high salinity, and wounding. In addition, ABA, jasmonic acid (JA), and glucose induced the expression of *LtWRKY21*. However, cold and heat treatments decreased the wounding-induced *LtWRKY21* mRNA level. Transient expression of *LtWRKY21* suggests that this transcription factor acts as an activator of ABA signaling and as a repressor of gibberellin (GA) signaling. These results suggest that LtWRKY21 might function as a key regulator of signaling networks in *Larrea tridentata*.


*Keywords:* WRKY; Abiotic stress; $CO_2$; Global warming; *Larrea tridentata*

## 1. Introduction

The global concentration of atmospheric $CO_2$ is predicted to double by the end of this century, which will result in a 1.5–4.5 °C increase in global air temperature compared to pre-industrial times [1]. The combination of global warming and environmental stress such as drought is expected to influence the establishment, survival, and reproduction of plants, resulting in enhanced recruitment, extinction, or geographic migration of certain species [2,3]. Therefore, it is increasingly important to study how elevated $CO_2$ influences plant response to environmental stresses.

The crosstalk between elevated atmospheric $CO_2$ and abiotic stresses on regulating photosynthesis has been documented. While the rate of $CO_2$ assimilation under elevated $CO_2$ increases in the short term, for many C3 species, long-term growth under elevated $CO_2$ results in a substantial decrease in photosynthetic capacity [4]. Large increases in leaf carbohydrate concentrations occur in plants exposed to elevated $CO_2$ [4–8], and carbohydrate accumulation has long been associated with inhibition of photosynthesis [9]. Water deficit has been found to reduce down-regulation, and even promote up-regulation of photosynthesis in some species [10,11]. On the other hand, elevated $CO_2$ appears to reduce the impact of water deficit, high and freezing temperature on plants such as *Larrea tridentata, Cucumis sativus*, and three *Yucca* species [12–14].

Hormones and other small molecules could mediate the crosstalk of elevated $CO_2$ and abiotic stresses. Water deficit induces the expression of many genes in ABA dependent and independent pathways [15]. ABA is a plant hormone that modulates plant development, seed dormancy, germination, cell division and cellular responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV radiation [16–20]. This hormone induces rapid closure of stomatal pores through ion efflux from guard cells, thereby limiting water loss through transpiration [21,22]. ABA also triggers slower changes in gene expression, hence reprogramming the cell to withstand dehydration [20,23]. Sugars also have important

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hormone-like functions as primary messengers in signal transduction in addition to regulatory effects on photosynthetic activity and plant metabolism [24,25]. Genetic and phenotypic analyses of *Arabidopsis* sugar-signaling mutants suggest integration between sugar and hormonal signaling pathways. The details of these complicated interactions remain to be studied.

WRKY family members are transcription factors that have been shown to be involved in biotic and abiotic stress responses, anthocyanin biosynthesis, senescence, trichome development, starch biosynthesis, and hormone responses [26–37]. A WRKY protein consists of one or two WRKY domains; each domain is composed of a 60 amino acid region that includes the core sequence, WRKYGQK and a zinc-finger motif. Some WRKY proteins interact with the DNA sequence (T)(T)TGAC(C/T), which is known as the W-box. In spite of the strong conservation of the DNA-binding domain, the overall structures of WRKY proteins are highly divergent and can be categorized into distinct groups, which might reflect their different functions [28,33,37–40].

*Larrea tridentata* has recently received considerable attention as one of the most drought-tolerant evergreen C3 shrubs dominating the North American warm deserts [41,42]. Here, we have used *Larrea tridentata* as a desert adopted model plant to study the integration among elevated $CO_2$, stresses, and hormones. In a previous study, we found that *LtWRKY21*, which encodes a WRKY transcription factor, is expressed in seeds and interacts synergistically with ABA and transcriptional activators VP1 and ABI5 to control the expression of the ABA-inducible *HVA22* promoter [38]. Herein, we show that the expression of *LtWRKY21* in vegetative tissues was regulated by elevated $CO_2$, sugars, hormones, and environmental stresses. In addition, LtWRKY21 not only activates ABA-inducible promoters, but also represses a GA-inducible promoter.

## 2. Materials and methods

### 2.1. Chemicals and enzymes

T4 DNA ligase and Taq DNA polymerase were obtained from Promega (Madison, WI). Restriction enzymes were acquired from Promega and New England Biolab (Boston, MA). Shrimp alkaline phosphatase was purchased from Roche Diagnostics Co. (Mannheim, Germany). (±)-*cis-trans*-[G-$^3$H]ABA, amyloglucosidase, Glucose Kit 510-A, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Plant materials and stress treatments

*Larrea tridentata* seeds were purchased from Plants of Southwest (Santa Fe, NM). The hairy seed coat was removed before planting in water-saturated Metro-Mix350 soil from Hummert International Co. (Earth City, MO). Plants were grown under 80% humidity, 135 μmol $m^{-2}$ $s^{-1}$ light intensity, 25 °C at day and 22 °C at night, under a 16 h light/8 h dark cycle. Plants were watered twice daily to prevent drying. Under these conditions, *Larrea* shoots emerged from the soil in 4–5 days. Forty-five day old plants were transferred to the green house chambers under 24–30% humidity, 28–29 °C, 140–170 μmol $m^{-2}$ $s^{-1}$ light intensity, 12 h day time on average. Plants were exposed to ambient (380 μmol $mol^{-1}$) or elevated (700 μmol $mol^{-1}$) atmospheric $CO_2$ for 1 year prior to experimentation. Plants were watered every other day, and were exposed for various periods to the following treatments: water deficit (withholding water for 3–9 days), chilling (4 °C), and heat (45 °C). To determine the impact of salinity on gene expression, leaf sections were incubated in a 1/2 strength MS (Murashige and Skoog basal salt mixture) solution containing 250 mM NaCl. For the hormone and sugar treatments, leaf sections were incubated in the 1/2 strength MS solution with addition of 100 μM ABA, 100 μM JA, and 6% sugar solutions (mannitol, glucose, and sucrose), respectively.

### 2.3. ABA and carbohydrate assays

For ABA, leaf tissue was weighed, lyophilized, ground, and extracted with 5 mL of cold 80% methanol/BHT (butylated hydroxytoluene), shaken for 2 h. ABA concentrations were assayed by radioimmunoassay as described previously [43].

Starch was assayed after the method of Kerr et al. [44]. Briefly, leaf tissue (25 mg) was extracted in 80% ethanol. Samples were shaken for 30 min, centrifuged, and the supernatant removed and saved for quantification of glucose and sucrose. The pellet was washed in 80% ethanol at 85 °C until the supernatant was clear. Samples were dried, resuspended, and boiled in 1 mL of 0.2 M KOH. Polysaccharides were broken down with amyloglucosidase at 30 °C overnight. Glucose was detected enzymatically and spectrophotometrically quantified at 340 nm using Glucose Kit 510-A. The supernatant from the above leaf extraction procedure was assayed for hexoses, using the anthrone reaction [45], and for glucose (Glucose Kit, 510 A).

### 2.4. RNA gel-blot analysis

Total RNA was isolated from *Larrea tridentata* leaves as described by Wang et al. [46]. Briefly, leaves (1 g) were frozen in liquid nitrogen and ground into a powder. The frozen tissue was suspended in five volumes of homogenization buffer. The homogenate was transferred to a plastic tube, allowed to freeze slowly at −80 °C for at least 2 h and then placed in a 37 °C water bath until just thawed. The homogenate was centrifuged at 5000 × *g* for 20 min at 4 °C. The supernatant was mixed with a 1/30 volume of 3.3 M sodium acetate (pH 5.2), and ethanol was added to a final concentration of 10% (v/v). The mixture was incubated on ice for 10 min, followed by centrifugation at 5000 × *g* for 20 min. The supernatant was mixed with a 1/9 volume of 3.3 M sodium acetate, and isopropanol was added to the supernatant to a final concentration of 33% (v/v). The mixture was placed at −20 °C for 2 h, and centrifuged at 5000 × *g* for 30 min. The pellet was resuspended in 3 ml TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 7.0) and incubated on ice for 30 min followed by centrifugation. The supernatant was