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Molecular mechanisms controlling hormonal regulation of plant responses to elevated carbon dioxide and abiotic stresses

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MOLECULAR MECHANISMS CONTROLLING HORMONAL REGULATION OF PLANT RESPONSES TO ELEVATED [CO_2] AND ABIOTIC STRESSES

by

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ABSTRACT

Molecular Mechanisms Controlling Hormonal Regulation of Plant Responses to Elevated CO_2 and Abiotic Stresses

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Global climate change due to elevated atmospheric [CO_2] and abiotic stresses have a strong impact on the establishment, survival and reproduction of plants. The goal of my research is to understand the molecular mechanism by which hormones regulate plant responses to elevated CO_2 and abiotic stresses. Abscisic acid (ABA) is well known as a stress hormone, which also promotes seed formation and dormancy, and inhibits seed germination. In contrast, another hormone, gibberellin (GA) breaks seed dormancy and promotes seed germination and post-germination growth including flowering. The focus of my research is to study the functions of a group of transcription factors (called WRKY) from creosote bush (*Larrea tridentata*) and barley (*Hordeum vulgare*) in ABA- and GA-regulated developmental and physiological processes.

This study sheds light on the mechanism controlling seed dormancy and germination, as well as plant responses to elevated [CO_2] and environmental stresses.

This information will eventually help us develop a strategy to improve crop yields and to combat against global warming resulted from elevated atmospheric [CO_2] by engineering plants that can consume [CO_2] at a massively enhanced rate, thereby reducing the elevated atmospheric concentration of CO_2.

In Chapter 2, I show for the first time that like abiotic stresses, elevated [CO_2] treatments increased ABA concentrations in the leaves of *Larrea tridentata* which is an extremely drought-tolerant evergreen C3 shrub dominating the North American warm desert. I then show that a regulatory gene from *Larrea tridentata*, *LtWRKY21*, is responsive to elevated [CO_2], water deficit, high salinity, and wounding. However, cold and heat treatments decreased the wounding-induced *LtWRKY21* mRNA level. In addition, ABA, jasmonic acid (JA, another hormone), and glucose induced the expression of *LtWRKY21*. Transient expression of *LtWRKY21* suggests that this transcription factor acts as an activator of ABA signaling and as a repressor of GA signaling. These results suggest that LtWRKY21 might function as a key regulator of signaling networks in *Larrea tridentata.*

The function of LtWRKY21 in ABA signaling is further studied in Chapter 3. Our data demonstrate that LtWRKY21 interacts synergistically with ABA and transcriptional activators, VP1 and ABI5, to control the expression of the ABA-inducible *HVA22* promoter. The transactivating activity of LtWRKY21 relies on the C-terminal sequence containing the WRKY domain and a N-terminal motif that is essential for the repression activity of some regulators in ethylene signaling. In contrast, the LtWRKY21-mediated transactivation is inhibited by 1-butanol, an inhibitor of phospholipase D, and abi1-1, a dominant-negative mutant protein phosphatase. However, abi1-1 does not block the

synergistic effect of *LtWRKY21*, *VP1*, and *ABI5* co-expression. Taken together, these data support a novel model: ABA signaling is negatively regulated by a protein complex including protein phosphatases (ABI1 or ABI2) and positively regulated by transcription factors such as VP1/ABI3, ABI4, and ABI5. LtWRKY21, VP1, ABI4, and ABI5 may form a complex that functions downstream of ABI1 to control ABA-regulated gene expression.

Chapter 4 presents the role of *HvWRKY38*, an *LtWRKY21* homologous gene, in seeds that are tolerant to dessication in the ABA-promoted dormant form, but sensitive to stresses in the GA-promoted germination form. HvWRKY38 physically and functionally interacts with other transcription factors BPBF, DOF, and GAMYB to repress transcription of the GA-inducible *Amy32b* promoter. Our data suggest that the expression of *Amy32b* is modulated by protein complexes containing activators and repressors, respectively. The ratio of activators and repressors and their affinities to individual *cis*-acting elements determine the expression level of *Amy32b* and hence dormancy or germination of seeds.

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ABBREVIATIONS

2-D	two dimension
4-MU	4-methylumbelliferone
AAO3	Arabidopsis aldehyde oxidase 3
AAPK	ABA-activated protein kinase
ABA	abscisic acid
aba	aba-deficient
aba1	aba-deficient 1
aba2	aba-deficient 2
aba3	aba-deficient 3
ABAP1	ABA-binding protein1
ABAR	ABA receptor
ABF	ABRE binding factor
ABH1	ABA hypersensitive 1
ABI	ABA insensitive
abi1-1	aba insensitive1-1
abi2	aba insensitive 2
abi3	aba insensitive 3
abi4	aba insensitive 4
abi5	aba insentitive 5
ABRC	ABA response complex
ABRE	ABA response element
AFP	ABI5 binding protein
AIP2	ABI3 interacting protein 2
AKIP1	AAPK-interacting protein
Amy	amylase box
Amy32b	α-amylase 32b
ANP1	Arabidopsis NPK1-like protein kinase 1
AO	Aldehyde oxidase
AP2	apetala 2
At	*Arabidopsis thaliana*
AtCPK	*Arabidopsis thaliana* calcium-dependent protein kinase
ATHB6	*Arabidopsis thaliana* homeobox 6
AtMPK	*Arabidopsis thaliana* MAP kinase
BSA	bovine serum albumin
bHLH	basic Helix-Loop-Helix

BiFC	bimolecular fluorescence complementation
BLAST	basic local alignment search tool
bp	base pair
BPBF	barley prolamine box binding factor
bZIP	basic leucine zipper
Ca^{2+}	calcium ion
cADPR	cyclic adenosine diphosphate ribose
CAM	crassulacean acid metabolism
CaM	calmodulin
CaMBD	calmodulin-binding domain
CaMV	cauliflower mosaic virus
CBF	C-repeat binding factor
CBL	calcineurin B-like protein
CCaMKs	calmodulin-dependent protein kinases
CDPK	calcium-dependent protein kinase
CE	coupling element
cGMP	cyclic guanosine monophosphate
CHD3	chromodomain helicase-DNA-binding protein 3
ChIP	chromatin immuno-precipitation
CHLH	Mg-chelatase H subunit
CIPK	CBL-interacting protein kinase
Clp	caseinolytic protease
COL1A1	collagen, type I, alpha 1
COR	cold-regulated protein
CO_2	carbon dioxide
CRT	C-repeat
CTAB	cetyltrimetylammonium bromide
CTR1	constitutive triple response 1
cyt	cytoplasm
DAE	downstream amylase element
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DOF	DNA-binding with one finger
DRE	dehydration-responsive element
DREB	DRE binding protein
DTT	dithiothreitol
EAR	ERF-associated amphiphilic repression
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-tetra-acetic acid
EIN2	ethylene insensitive 2
EmBP	Em binding protein

EMSA	electrophoretic mobility-shift assay
ER	endoplasmic reticulum
ERD	early responsive to dehydration
ERF	ethylene-responsive element binding factor
EST	expressed sequence tag
FACE	free-air CO_2 enrichment
FCA	flowering locus CA
FLC	flowering locus C
FL-cDNA	full-length cDNA
FUS3	fusion 3
FY	flowering locus Y
GA	gibberellin
Ga	*Gossypium arboreum*
GAI	gibberellin insensitive
GAMYB	GA-responsive Myb protein
GARC	GA responsive complex
GARE	GA response element
gca2	growth control by aba2
GCR2	G protein-coupled receptor 2
GFP	green fluorescence protein
GGDP	geranylgeranyl diphosphate
GID1	gibberelin insensitive dwarf 1
GID2	gibberelin insensitive dwarf 2
gin6	glucose insensitive 6
GPA1	G protein α subunit
GRAS	GAI, RGA, and Scarecrow
g_s	stomatal conductance
GSA	beta-O-D-glucosylsalicylic acid
GSE1	GA sensitivity
GST	glutathione S-transferase
GUS	β-glucuronidase
HD	homeodomain protein
HIF-1α	hypoxia-inducible factor 1α
HIS	histidine
HMM	hidden Markov model
HRT	*Hordeum* repressor of transcription
HSI	HvSPY-interacting
Hv	*Hordeum vulgare*
HVA1	*Hordeum vulgare* ABA responsive gene 1
HVA22	*Hordeum vulgare* ABA responsive gene 22
HvMCB1	*Hordeum vulgare???*

HvMYBS3	*Hordeum vulgare???*
HXK	hexokinase
HYL1	hyponastic leaves 1
InsP3	1,4,5 triphosphate
InsP6	myo-inositol hexakisphosphate
IPTG	isopropylthio-β-galactoside
isi3	impaired sugar induction 3
JA	jasmonic acid
K^+	potassium ion
KEG	keep on going
KGM	kinase associated with GAMYB
kin1	cold inducible 1
LEA	late embryogenesis abundant
LEC1	leafy cotyledon 1
Lt	*Larrea tridentata*
lti	low-temperture-induced
LUC	luciferase
LZ	leucine-zipper motif
MALDA-TOF	matrix-associated laser desorption/ionization time-of-flight
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MCSU	molybdenum co-factor sulfurase
MIF1	MINI ZINC FINGER 1
MINI	miniseed
miR159	microRNA159
MS	Murashige and Skoog basal medium
MUG	4-methylumbelliferyl-β-D-glucuronide trihydrate
MYB	myeloblastosis
MYBRS	MYB recognition sequence
MYC	myelocytomatosis
MYCRS	MYC recognition sequence
NAC	NAM, ATAF1, 2, and CUC2
NAM	no apical meristem
NCED	9-*cis*-epoxycartenoid dioxygenase
NLS	nuclear localization signal
NO	nitric oxide
NOS	nopaline synthase
NPK	Nicotiana protein kinase
Npr/Nim1	nonexpressor of PR genes/ noninducible immunity 1
Nt	*Nicotiana tabacum*
NtAQP1	*Nicotiana tabacum* aquaporin 1

O2S	opaque-2 binding sequence
OCT	Ser/Thr O-linked N-acetylglucosamine transferase
O-GLCNAc	O-linked N-acetylglucosamine
ORF	open reading frame
Os	*Oryza sativa*
ost1	open stomata 1
PA	phosphatidic acid
PBF	prolamine-box binding factors
Pc	*Petroselinum crispum*
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
pI	isoeletric point
PI3P	inositol 1,3,4,5 tetrakisphosphate
PKABA1	ABA induced protein kinase 1
PKL	pickle
PKS3	SOS2-like protein kinase 3
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethyl sulfonyl fluoride
Poly(dIdC)	poly(deoxyinosinic-deoxycytidylic) acid
PPA	phosphatidic acid
PR	pathogensis related
PSIG	pounds per square inch gauge
Pyr	pyrimidine box
RAB	response to ABA
RAB21	response to ABA 21
RBCS	Rubisco small subunit
RD29A	responsive to dehydration 29A
RGA	repressor of ga1-3
RNAi	RNA interference
ROS	reactive oxygen species
RPB5	RNA polymerase II subunit 5
RY/Sph	purine pyrimidine/sphingomyelin
RT-PCR	reverse transcription PCR
Rubisco	ribulose 1:5 bisphosphate carboxylase/oxygenase
S1P	sphingosine-1-phosphate
SAD	scutellum and aleurone-expressed DOF
SCF	skip-cullin-F-box complex
SCaBP5	SOS3-like Ca^{2+} binding protein
SDR1	short-chain dehydrogenase/reductase 1
SDS	sodium dodecyl sulfate

SE	standard error
SIRK	senescence-induced receptor kinase
sis5	sugar insensitive 5
SLN1	slender barley 1
SLR1	slender rice 1
sly1	sleepy 1
SnRK	sucrose nonfermenting1-related protein kinase
SOS	salt overly sensitive
SPF1	sweet potato factor 1
SPH	sphingosine
SPHK	sphingosine kinase
Sp1	specificity protein 1
SPY	spindly
SSC	standard saline citrate
S-type	slow-type
sun6	sucrose uncoupled 6
SUSIBA	sugar signaling in barley
TAP-MS	tandem affinity purification-mass spectrometry
T-DNA	transferred DNA
TEV	tobacco etch virus
TRAMP	tomato-riping-associated membrane protein
Tris	trishydroxymethylaminomethane
UBI	ubiquitin
Va	*Vitis aestivalis*
VP1	viviprarous 1
WIZZ	wound-induced leucine zipper zinc finger
WUE	Water use efficiency
YFP	yellow fluorescence protein
ZEP	zeaxanthin epoxidase
Zn-F	zinc finger motif

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CHAPTER 1

GENERAL INTRODUCTION

Water shortage and global warming because of elevated atmospheric CO_2 concentration have drawn much attention to the scientific community. The global concentration of CO_2 is predicted to reach 700 ppm by the end of this century, which will cause a 1.5 to 4.5 ^{o}C increase in global air temperature compared to pre-industrial times (Crowley, 2000). The combination of global warming and drought stress are expected to influence the plant growth, development, and reproduction, as well as the global ecosystem via changes and shift in species composition (Clark et al., 1998; Smith et al., 2000). Therefore, it is increasingly important to study how elevated [CO_2] influences plant responses to environmental stresses.

Plants, as sessile organisms, have evolved diverse mechanisms to circumvent unfavourable growth conditions, among them the formation of the seed is one of the most successful strategies. A seed represents an important developmental structure linking parental and progeny generations. In most plants, a seed consists of three distinct parts: embryo, endosperm, and seed coat. Seed formation is an intricate process that can be roughly divided into proper embryogenesis, maturation, and dormancy. While the growing seed is accumulating its major storage reserves, changes are also occurring in its content of plant hormones, such as auxin, cytokinins, GAs, and ABA. Genetic and physiological studies have shown the important roles of ABA and GAs in regulating seed

dormancy and germination. Mounting evidence suggest that dormancy maintenance depends on high ABA:GA ratios, and dormancy release involves a net shift to increased GA biosynthesis and ABA degradation, resulting in low ABA:GA ratios. This is followed by GA promotion of seed germination (Ali-Rachedi et al., 2004; Cadman et al., 2006).

The general focus of my research is to study the crosstalk of signaling pathways mediating plant responses to elevated [CO_2], stresses, and hormones, mainly ABA and GA.

Plant responses to elevated [CO_2]

Atmospheric CO_2 concentration, currently 372 μmol mol^{-1}, is approximately 38% higher than the pre-industrial level (about 270 μmol mol^{-1}), and by the middle of this century it is predicted to reach 550 μmol mol^{-1} and to surpass 700 μmol mol^{-1} by the end of the century (Long et al., 2004). Plants sense and respond to elevated [CO_2] through increased photosynthesis and reduced stomatal conductance. All other effects on plants and ecosystems are derived from these two fundamental responses (Long et al., 2004; Ainsworth and Rogers, 2007).

The direct increase in photosynthesis due to elevated [CO_2] results from two properties of Rubisco (ribulose 1:5 bisphosphate carboxylase/oxygenase): 1) The enzyme is not saturated by present atmospheric [CO_2], so that elevated [CO_2] can increase the velocity of carboxylation and net photosynthesis. 2) [CO_2] competitively inhibits the oxygenation reaction that leads to photorespiration (Long et al., 2004; Ainsworth and Rogers, 2007). However, for many C_3 species, the rate of [CO_2] assimilation by photosynthesis at elevated [CO_2] only increases in the short term. Long-term growth at

elevated [CO_2] results in a substantial decrease in photosynthetic capacity because carbohydrate accumulation inhibits photosynthesis (Long and Drake, 1992; Jang et al., 1997; Moore et al., 1999 Obrist et al., 2001; Rogers et al., 2004).

The control of Rubisco content involves a number of mechanisms that act on transcriptional, post-transcriptional, translational, and post-translational events (Berry et al., 1986; Shirley and Meagher, 1990; Moore et al., 1999; Paul and Foyer, 2001; Rolland et al., 2002; Gupta et al., 2005; Taylor et al., 2005; Wostrikoff and Stern, 2007). It has been demonstrated that hexokinase (HXK), as a glucose sensor, modulates gene expression and multiple plant hormone-signaling pathways (Jang et al., 1997; Leon and Sheen, 2003; Rolland et al., 2006). Carbohydrate-dependent regulation of photosynthetic gene expression is thought to be mediated by HXK that functions as a sugar sensor at ambient and elevated [CO_2]. It is suggested that HXK may associate with kinase/phosphatase to initiate a signaling response and results in the repression of promoter activities of *RBCS* (*Rubisco small subunit*) and a number of photosynthetic genes (Jang and Sheen, 1994; Jang et al., 1997; Moore et al., 1999).

Stomatal conductance (g_s) and possibly stomatal density have been showed to decrease under long term exposure to elevated [CO_2] (Woodward and Kelly, 1995; Teng et al., 2006; Ainsworth and Rogers, 2007). However, in FACE (free-air [CO_2] enrichment) experiments, the decrease in g_s at elevated [CO_2] does not appear to be caused by a significant change in stomatal density (Ainsworth and Rogers, 2007). It is likely that the changes in stomatal aperture rather than density determine the response of g_s to elevated [CO_2].

Guard cells regulate stomatal apertures, thereby controlling the rate of [CO_2] uptake and transpirational water loss and hence influencing not only photosynthesis but also the water status of plants (Fan et al., 2004; Israelsson et al., 2006). Stomatal movements are determined by turgor pressure changes in guard cells. ABA triggers rises in guard cell Ca^{2+} concentration (McAinsh et al., 1990) and activate outward rectifying K^+ channels and S-type anion channels activities (Schroeder, 1988; Linder and Raschke, 1992; Lemtiri-Chlieh and MacRobbie, 1994; Romano et al., 1998). These changes collectively depolarize the membrane potential of guard cells and cause stomatal closure (Schroeder et al., 2001; Ainsworth and Rogers, 2007).

Abscisic acid

ABA, a 15-carbon sesquiterpenoid carboxylic acid, is a small, lipophilic plant hormone that modulates plant development, seed dormancy, germination, cell division, photosynthesis, and cellular responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV radiation (Addicott and Carns, 1983; Zeevaart and Creelmann, 1988; Mccarty, 1995; Rock, 2000; Finkelstein et al., 2002; Xiong and Zhu, 2003; Xie, 2005; Marion-Poll A, 2006). It is ubiquitous in lower and higher plants (Zeevaart, 1999; Marion-Poll A, 2006). ABA regulates multiple physiological processes, such as inducing the rapid closure of stomatal pores by ion efflux from guard cells, thereby limiting water loss through transpiration (McAinsh et al., 1990; MacRobbie, 1998) and by triggering slower changes in gene expression, which is thought to reprogram the cell to withstand dehydration stresses (Leung and Giraudat, 1998). In developing seeds, ABA levels peak during late embryogenesis when storage proteins and

nutritive reserves accumulate, and thereafter decline during desiccation (Finkelstein et al., 2002; Nambara and Marion-Poll, 2003). In stressed vegetative tissues, ABA levels rise several-fold to 40-fold within hours and decrease after rehydration (Zeevaart, 1999). This modulation is due at least in part to the biosynthesis and catabolism of ABA (Qin and Zeevaart, 1999).

The general framework of the ABA biosynthetic pathway in plants has been elucidated by physiological, biochemical, and molecular studies (Finkelstein et al., 2002; Seo and Koshiba, 2002; Xiong and Zhu, 2003; Marion-Poll A, 2006). Synthesized from C_{40} caroteniods in plastid, the first step of the ABA-specific synthetic pathway is the epoxidation of zeaxanthin to all-*trans*-violaxanthin by Zeaxanthin epoxidase (ZEP), which is known as *ABA1* in Arabidopsis and *ABA2* in tobacco (Rock and Zeevaart, 1991; Marin et al., 1996). The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin (Schwartz et al., 1997). The xanthoxin is then transported to the cytosol, where it is converted to ABA by a two-step reaction via ABA-aldehyde (Seo and Koshiba, 2002). Arabidopsis *aba3* mutant lacks the activity of aldehyde oxidase (AO), which impairs the later steps of ABA biosynthesis (Bittner et al., 2001).

The research on the molecular mechanism of ABA response has been unfolded dramatically in the past years, revealing a complex network (Figure 1-1). Both soluble and membrane bound receptors for ABA have been recently found (Razem et al., 2006; Shen et al., 2006; Liu et al., 2007). Two soluble receptors have been identified: 1) the Arabidopsis RNA-binding protein FCA (Flowering locus A) is a homologue of the putative ABA-binding protein1 (ABAP1) purified from barley aleurone membranes

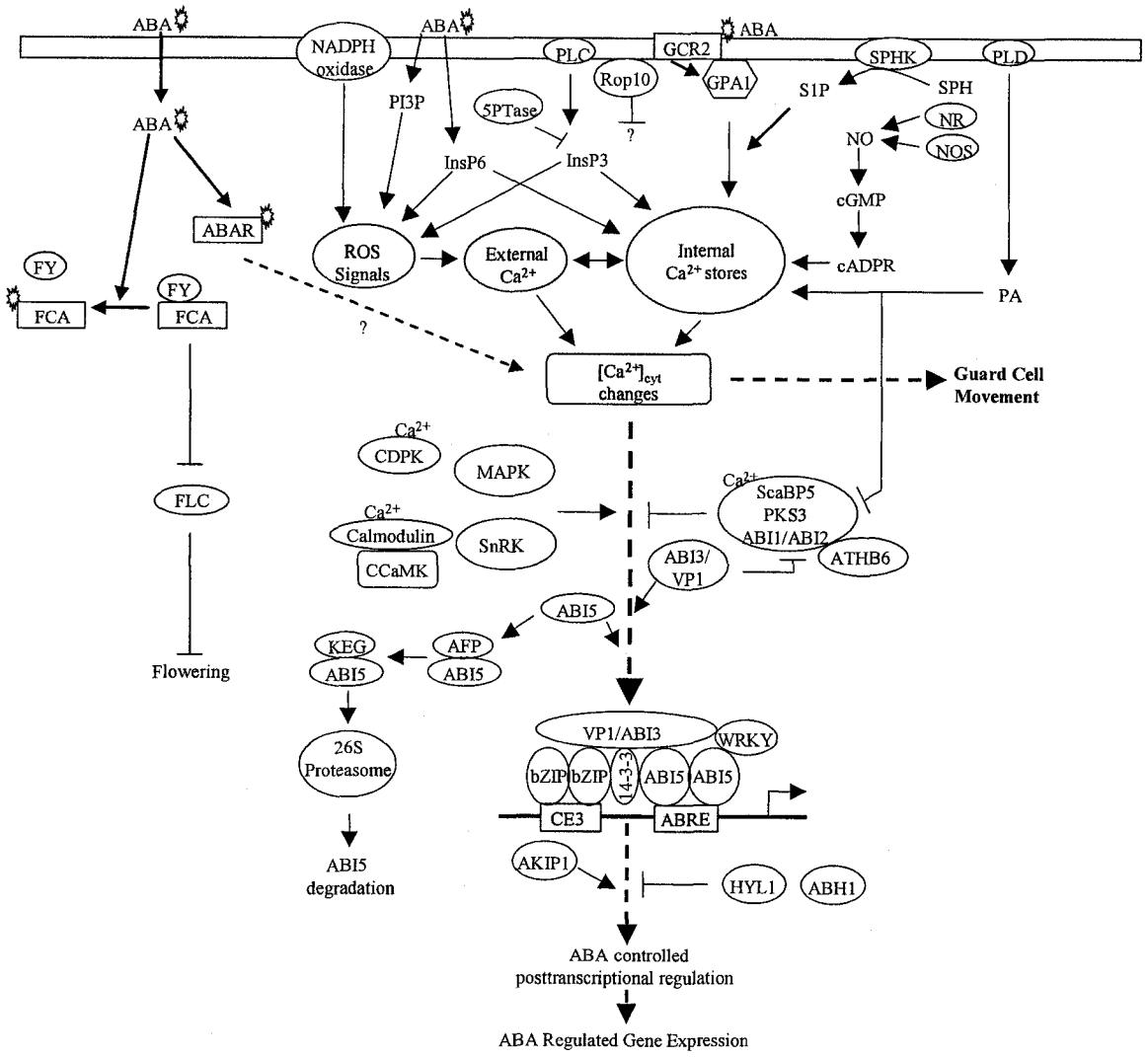

Figure 1-1. An integrated schematic model of ABA signaling networks in plants.

Figure 1-1. (continued)
ABA signaling is perceived by GCR2 (G protein-coupled receptor 2), a plasma membrane ABA receptor. The binding of ABA to the GCR2 leads to the dissociation of the GCR2-GPA1 complex. Two soluble receptors, FCA and ABAR, are also involved in ABA perception. Binding of ABA to FCA disassociates the FCA-FY complex, hence delaying the flowering. The components directly interacting with ABAR are still unknown, it probably functions by modulating $[Ca^{2+}]_{cyt}$. Secondary messengers including sphingosine-1-phosphate (S1P), cyclic ADP ribose (cADPR), inositol 1,4,5 triphosphate (InsP3), myo-inositol hexakisphosphate (InsP6), phosphatidic acid (PA) and NO (nitric oxide), promote ABA response by releasing Ca^{2+} from internal storages. Reactive oxygen species (ROS), which is produced by NADPH-oxidase or enhanced by secondary messengers (PI3P, inositol 1,3,4,5 tetrakisphosphate, InsP6 and InsP3), stimulates Ca^{2+} influx by activating Ca^{2+} channels on the plasma membrane. $[Ca^{2+}]_{cyt}$ changes eventually regulate guard cell movement and gene expression. Ca^{2+} signaling is negatively regulated by protein phosphatases (ABI1 or ABI2), a protein kinase (CIPK15/PKS3), a Ca^{2+}-binding protein (CBL/ScaBP5) and a homeodomain leucine zipper protein (ATHB6). In turn, ABI1/ABI2 is repressed by PA, ROS, and ABI3/VP1. This pathway is positively regulated by a MAP kinase cascade, sucrose nonfermenting1-related protein kinases (SnRKs), calcium-dependent protein kinases (CDPKs) and probably Ca^{2+}-calmodulin-dependent protein kinases II (CCaMKs). ABRC consists of an ACGT-box (G-box) and a coupling element (only ABRC3 is shown here). ABI3/VP1 and 14-3-3 enhance the binding affinity of ABI5 to ABRE and CE elements, forming a transcriptional complex that likely include WRKY proteins. ABI5 binding protein (AFP) and a novel RING E3 ligase, KEEP ON GOING (KEG), promote the degradation of ABI5 by 26S proteasomes. Three RNA binding proteins, one functioning as a positive regulator (AKIP1) and two as negative regulators (HYL1 and ABH1), mediate ABA response at the posttranscriptional level. This figure is updated and modified from Xie et al (2005) and Li et al (2006).

(Razem et al., 2004; Razem et al., 2006). FCA interacts with an RNA 3'-end processing factor FY (Flowering locus Y), promoting flowering by down-regulating the mRNA levels of the flowering repressor Flowering Locus C (FLC). ABA abolishes interaction of FCA with FY by binding to FCA, causing a delay in flowering (Razem et al., 2006). 2) ABAR/CHLH, encodes a Mg-chelatase H subunit (Shen et al., 2006), which positively mediates ABA signaling in seed germination, post-germination growth, and stomatal movement. Down-regulation of ABAR expression by RNAi decreased the levels of positive regulators of ABA signaling such as ABI4 and ABI5, and enhanced the levels of negative regulators such as ABI1 and CIPK15. However, components directly interacting with ABAR are still unclear (Shen et al., 2006). Recently, a G protein-coupled receptor 2 (GCR2) is shown to function as a plasma membrane ABA receptor (Liu et al., 2007). GCR2 genetically and physically interacts with the G protein α subunit GPA1 to mediate ABA responses in Arabidopsis. The binding of ABA to the GCR2 leads to the release of the G protein and the dissociation of the heterotrimeric complex into Gα and a Gβγ dimer, which in turn activate downstream ABA effectors and trigger the ABA responses. The plasma membrane-localized receptor may be the major player for perceiving extracellular ABA and mediating the classic ABA signaling (Liu et al., 2007). Secondary messengers involved in ABA responses include Ca^{2+}, pH, cyclic GMP (cGMP), cyclic ADP-ribose (cADPR), 1,4,5-triphosphate (InsP3), phosphatidic acid (PA), and sphingosine-1-phosphate (S1P) (Rock, 2000; Himmelbach et al., 2003; Xie et al., 2005; Li et al., 2006). ABA-induced mobilization of $[Ca^{2+}]_{cyt}$ from internal stores is controlled by many other intercellular messengers such as S1P, cADPR, InsP3, InsP6, PA, and NO. Reactive oxygen species (ROS), which is produced by NADPH-oxidase or enhanced by secondary

messengers (PI3P, InsP6 and InsP3), stimulates Ca^{2+} influx by activating Ca^{2+} channels on the plasma membrane. $[Ca^{2+}]_{cyt}$ changes eventually regulate guard cell movement and downstream gene expression (Xie et al., 2005; Li et al., 2006).

Phospholipases, protein kinases, and protein phosphatases are involved in the early events of ABA signaling (Merlot et al., 2001; Hallouin et al., 2002; Lu et al., 2002). ABA-induced sphingosine kinase (SPHK) converts sphingosine (SPH) into sphingosine-1-phosphate (S1P) which acts on a G protein (Coursol et al., 2003) and its receptor GCR1 (Pandey et al., 2004) to mobilize calcium (Ng et al., 2001). ABA enhances the activities of phospholipase C (PLC), phospholipase D (PLD), and ADPR cyclase to produce inositol 1,4,5-trisphosphate (InsP3) (Sanchez and Chua, 2001), PA (Ritchie and Gilroy, 2000), and cADPR (Sanchez et al., 2004), respectively. Arabidopsis protein phosphatases 2C, such as ABI1 and ABI2, function as negative regulators of ABA signaling (Merlot et al., 2001; Himmelbach et al., 2003; Leonhardt et al., 2004). The activities of protein phosphatases are modulated by secondary messengers (PA and Ca^{2+}) and protein kinases. PA binds to and inhibits ABI1 activity in addition to regulating $[Ca^{2+}]_{cyt}$ (Zhang et al., 2004a). ABI1 and ABI2 physically interact with PKS3 (or its homologue CIPK3), a Ser/Thr protein kinase. This kinase is also associated with the calcineurin B-like Ca^{2+} binding protein, SCaBP5 (or its homologue CBL), forming a complex that negatively controls ABA sensitivity (Guo et al., 2002; Kim et al., 2003). Another calcium sensor (CBL9) functions as a negative regulator of ABA signaling and biosynthesis (Pandey et al., 2004). In contrast, the protein phosphatase 2A functions as a positive regulator of ABA signaling (Kwak et al., 2002). Protein kinases also can function as positive regulators of ABA signaling. Two Arabidopsis calcium-dependent protein kinases

(CDPKs), AtCPK10 and AtCPK30, activate an ABA-inducible barley promoter in the absence of the hormone (Cheng et al., 2002). ABA and H_2O_2 activate the Arabidopsis mitogen-activated protein kinase kinase kinase, ANP1 (Arabidopsis NPK1-like protein kinase), which initiates a phosphorylation cascade involving two mitogen-activated protein kinases (MAPK), AtMPK3 and AtMPK6 (Kovtun et al., 2000). Sucrose nonfermenting1-related protein kinases (SnRKs) function as activators of ABA signaling in rice (Kaneko et al., 2004), wheat (Johnson et al., 2002b), and Arabidopsis (Fujii et al., 2007). It is likely that SnRKs activate ABRE-driven gene expression through the phosphorylation of ABFs (ABA response element binding factors) (Fujii et al., 2007).

Several types of *cis*-acting elements are involved in ABA responses, such as an ACGT- core (ACGTGGC, ACGT-box, also named G-Box or ABRE), CEs (CE1, CCACC, CE3, GCGTGTC), RY/Sph (CATGCA(TG)), AT-rich elements (CAATTATTA), Myb and Myc binding sites (YAAC(G/T)G and ACACGCATGTG, respectively) (Shen et al., 1993). The combination of the ACGT-box and the *CE* forms an ABA response complex (ABRC), which has been shown to be the smallest ABA-responsive promoter unit (Shen et al., 1996). ABREs and CE3 are bound by bZIP proteins (Guiltinan et al., 1990; Kim et al., 1997; Choi et al., 2000; Finkelstein and Lynch, 2000; Kang et al., 2002; Casaretto and Ho, 2003); CE1 by ABI4 (Niu et al., 2002); RY/Sph elements by those containing B3 domains (McCarty et al., 1991; Giraudat et al., 1992; Bobb et al., 1997; Suzuki et al., 1997; Rohde et al., 1998; Bailey et al., 1999; Ezcurra et al., 2000); AT-rich elements by homeodomain leucine zipper proteins (Himmelbach et al., 2002; Johannesson et al., 2003); MYC sites by AtMYC (Abe et al., 1997) and MYB sites by AtMYB (Abe et al., 1997; Abe et al., 2003).

Physiological, genetic, and transgenic analyses of *abi3*, *abi4*, and *abi5* mutants show cross-regulation of expression, suggesting that these genes function in a combinatorial network rather than a regulatory hierarchy controlling seed development and ABA responses (Soderman et al., 2000). VP1/ABI3 has been shown to potentiate ABA-inducible gene expression by forming DNA-binding complex with bZIP, 14-3-3, zinc finger proteins, and the RNA polymerase II subunit RPB5 (Schultz et al., 1998; Hobo et al., 1999b; Jones et al., 2000; Kurup et al., 2000). Although VP1/ABI3 binds to the Sph/RY element (Suzuki et al., 1997) to activate the *C1* promoter in the absence of ABA (Kao et al., 1996), VP1/ABI3 also can enhance the transcription of the ABRC-containing promoters that lacks an Sph/RY element (Shen et al., 1996; Hobo et al., 1999a; Gampala et al., 2002; Casaretto and Ho, 2003). In addition to interacting with VP1, 14-3-3 also interacts with HvABI5 in mediating ABA signaling (Schoonheim et al., 2007).

ABA signaling is also regulated at the posttranscriptional level. AtABH1 and AtCBP20 form a dimeric Arabidopsis mRNA cap-binding complex, negatively regulating ABA response (Hugouvieux et al., 2002). Mutations in the dsRNA binding protein HYL1, lead to enhanced levels of ABI5 and MAPK (Lu and Fedoroff, 2000). Another RNA binding protein, AKIP1, is a substrate of the protein kinase AAPK (Li et al., 2002). It functions as a positive regulator of ABA signaling. It is still unknown how these RNA binding proteins regulate ABA responses. Furthermore, protein degradation is also part of ABA signaling (Hare et al., 2003; Zhang et al., 2005). ABI5 binding protein (AFP) promotes the degradation of ABI5 by 26S proteasomes (Lopez-Molina et al., 2003). A novel RING E3 ligase, KEEP ON GOING (KEG), is reported to be involved in the ABI5 ubiquitylation (Stone et al., 2006). It will be interesting to determine whether the

ubiquitin/26S proteolytic pathway is also involved in the degradation of the repressors of ABA signaling.

Plant responses to drought

Plants frequently encounter environmental conditions that adversely affect growth, development, and productivity (Bray et al., 2000). Approximately 60% of the earth's land area belongs to arid or semi-arid zone, and the percentage is expected to increase as a result of global warming due to elevated [CO_2] and human activity (Shao et al., 2006). Most plants encounter at least transient decreases in relative water content at some stage of their life, and many also produce highly desiccation-tolerant structures such as seeds, spores, or pollen. Besides, physiological drought also occurs during cold and salt stresses that cause the water deficit in the living cells. Plants respond to the drought stress by displaying complex, quantitative traits that involve the functions of many genes. They encode different kind of proteins: water channel proteins involved in altering cellular water potential; the enzymes required for the biosynthesis of various osmoprotectants such as mannitol, trehalose, fructans, proline, glycine betaine, and polyamines; protective proteins such as late embryogenesis abundant (LEA) proteins, osmotin, chaperones, and mRNA-binding proteins; proteases and ubiquitin for protein turnover; the detoxification proteins such as glutathione reductase, ascorbate peroxidase, and superoxide dismutase; and protein kinases, phospholipases, and transcription factors, which are involved in further regulation of signal transduction and gene expression (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Ramanjulu and Bartels, 2002; Valliyodan and Nguyen, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007; Sreenivasulu et al., 2007).

Water deficit causes a decrease in turgor pressure at the cellular level. A change in the osmotic potential across the plasma membrane may be a major trigger of water-stress responses at the molecular level. The perception of cellular water deficit needs to be translated into biochemical, metabolic, and physiological stress adaptations (Ingram and Bartels, 1996; Bajaj et al., 1999; Zhu, 2001; Chinnusamy et al., 2004). In fact, not all of these responses are mediated by ABA. Several genes are induced by drought, salt, and cold in *aba* (ABA-deficit) or *abi* (ABA-insensitive) *Arabidopsis* mutants (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). These genes include *rd29A*, *kin1*, *cor6.6* (*kin2*) and *cor47* (*rd17*). The expression of a drought-inducible gene for *rd29A/lti78/cor78* was extensively analyzed (Yamaguchi-Shinozaki and Shinozaki, 1994; Narusaka et al., 2003). A 9 bp conserved sequence, TACCGACAT, termed the dehydration-responsive element, is essential for the regulation of the induction of rd29A under drought, low-temperature, and high-salt stress conditions, but does not function as an ABRE. DRE-related motifs have been reported in the promoter region of many drought- and cold-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 2000). There are several drought-inducible genes that do not respond to either cold or ABA treatment, which suggests that there is another pathway in the dehydration-stress response. These genes include *rd19* and *rd21*, which encode different thiol proteaseas and *erd1* which encodes a Clp protease regulatory subunit (Nakashima et al., 1997).

As shown in Figure 1-2, at least four signal-transduction pathways have been reported to be involved in plant responses to drought stress: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). These pathways lead to activation or synthesis of transcription factors such as MYB/MYC and bZIP

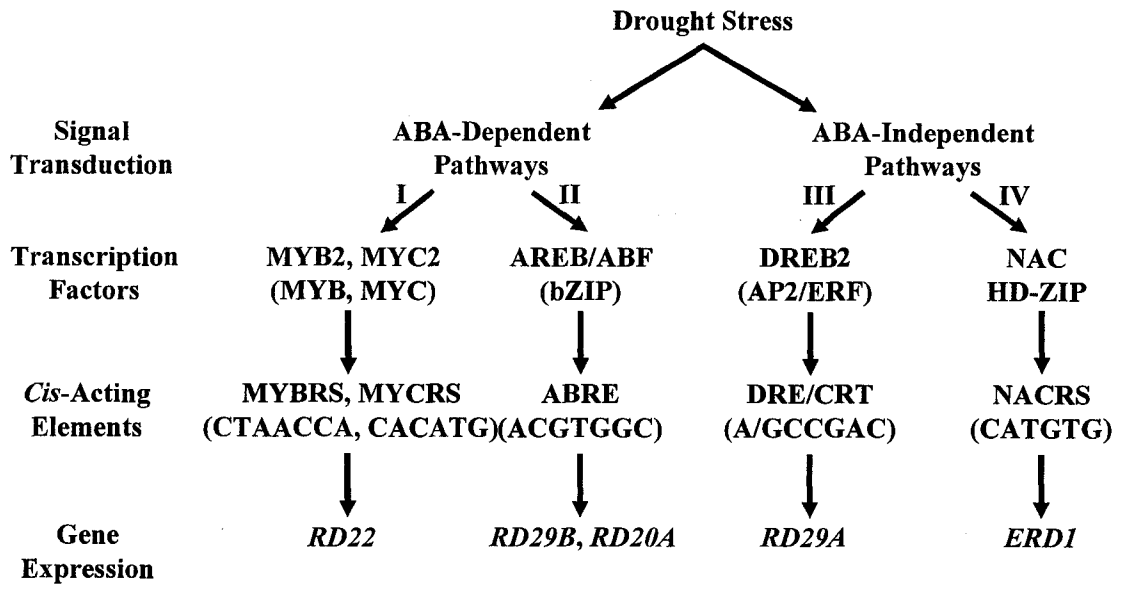

Figure 1-2. Signal transduction pathways involved in Arabidopsis responses to drought. At least four signal-transduction pathways exist in plant responses to drought: two are ABA-dependent (I and II) and two are ABA-independent (III and IV). In ABA-dependent pathways, ABRE functions as a major ABA-responsive element. AREB/ABF are bZIP transcription factors involved in this process. MYB2 and MYC2 activate the expression of *RD22* gene via MYBRS and MYCRS. In one of the ABA-independent pathways, drought response relies on a *cis*-acting element, DRE that is bounded by DREB2, an AP2 transcription factor. Another ABA-independent pathway is mediated by NAC and HD-ZIP transcription factors. The figure is modified from Shinozaki and Yamaguchi-Shinozaki (2007).

(Bajaj et al., 1999; Shinozaki and Yamaguchi-Shinozaki, 2007). ABA-dependent pathway I requires protein synthesis to activate transcription factors MYC/MYB and/or bZIP, which bind to DNA regions other than ABRE (ABA-responsive element) such as MYBRS (MYB recognition sequence) or MYCRS (MYC recognition sequence) in the promoter region of *RD22* and enhances the expression of the *RD22* gene (Abe et al., 1997). ABA-dependent pathway II activates bZIP, a transcription factor that turns on the gene expression of *RD20A* and *RD29B* through binding to ABRE (Fujita et al., 2005). ABA-independent pathway III induces gene expression through activation of DREB2 (drought-response-element-binding protein), which binds to the DRE (drought response element) motif and leads to induction of drought-induced genes (Sakuma et al., 2006). In ABA-independent pathway IV, drought induces the expression of NAC, which cooperates with a zinc-finger homeodomain protein in regulating the expression of *ERD1* (*EARLY RESPONSE TO DEHYDRATION STRESS 1*) (Tran et al., 2004). Instead of acting in a parallel manner, these four signaling pathways cross-talk and converge to activate stress-response gene expression.

Crosstalk of signaling pathways mediating responses to elevated [CO_2], drought, and ABA

The crosstalk between elevated atmospheric [CO_2] and drought stress on regulating photosynthesis has been documented. Elevated [CO_2] increases photosynthesis and reduces stomatal conductance. Reduced stomatal conductance decreases water loss through transcription, thereby increasing water-use efficiency in plants (Wullschleger et al., 2002; Fan et al., 2004; Ainsworth and Rogers, 2007). Elevated [CO_2] appears to

reduce the impact of water deficit and increase drought tolerance in plants such as *Eucalyptus*, *Fagus sylvatica*, and *Larrea tridentata*, (Roden and Ball, 1996; Heath, 1998; Hamerlynck et al., 2000). On the other hand, drought has been found to reduce down-regulation and even promote up-regulation of photosynthesis in some species (Oechel et al., 1995; Huxman et al., 1998).

The mechanisms underlying plant responses to the combination of elevated [CO_2] and drought are not fully understood. Genetic and phenotypic analyses of Arabidopsis sugar-signaling mutants have demonstrated complicated interactions between sugar and ABA signaling pathways (Rolland et al., 2002; Rolland et al., 2006). The HXK-mediated glucose signaling pathway is connected to the ABA pathway. Arabidopsis mutants *gin6* (*glucose insensitive 6*), *isi3* (*impaired sugar induction 3*), *sis5* (*sugar insensitive 5*), and *sun6* (*sucrose uncoupled 6*) are allelic to *abi4* (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). Besides, over-expression of Arabidopsis ABF3 and ABF4 shows both ABA and glucose oversensitive phenotypes (Kang et al., 2002). A putative ABI4-binding site, named the S-box (CACCTCCA), was found in RBCS promoter (Acevedo-Hernandez et al., 2005). The level of GUS was not significantly decreased by ABA treatment when introducing *RBCS-GUS* construct into *abi4* mutant background, suggesting ABI4 is essential for the down-regulation of RBCS expression by ABA (Acevedo-Hernandez et al., 2005). It remains unclear whether other ABA signal molecules are involved in mediating the expression of Rubisco.

In higher plants, water loss and [CO_2] uptake are tightly regulated by stomata on the leaf epidermis. Aquaporins are a complex family of channel proteins that facilitate the transport of water along transmembrane water potential gradients. Aquaporins can

regulate the hydraulic conductivity of membranes and potentiate a 10-20 fold increase in water permeability (Maurel and Chrispeels, 2001). Several aquaporin genes are up-regulated by dehydration, such as *rd28* from Arabidopsis (Taji et al., 1999) and the gene encoding tomato-riping-associated membrane protein (TRAMP) (Fray et al., 1994). A tobacco aquaporin NtAQP1 has been found to act as a [CO_2] transporter (Uehlein et al., 2003) and the expression of soybean aquaporin was up-regulated by elevated [CO_2] (Ainsworth et al., 2006). These data suggest that aquaporin might facilitate both [CO_2] and water transport across the plasma membrane.

Circumstantial evidence suggest the crosstalk of drought, ABA, and [CO_2] signaling. Drought triggers the production of ABA, which in turn causes stomatal closure and induces expression of stress-related genes (Shinozaki and Yamaguchi-Shinozaki, 2007). Both ABA and elevated [CO_2] induce the increase of cytosolic free calcium in guard cells (Webb et al., 1996), suggesting ABA is involved in plant responses to elevated [CO_2] and drought. Studies of ABA-insensitive mutants from Arabidopsis reveal the interaction between ABA and [CO_2] signal transduction pathways. Webb & Hetherington (1997) observed that *abi1-1* and *abi2-1* mutants fail to respond to [CO_2] and extracellular calcium. Also, an ABA-insensitive mutant *growth control by aba2* (*gca2*) are strongly insensitive to high [CO_2] (Young et al., 2006). However, ABA-insensitive mutant *ost1* (*open stomata 1*) disrupted ABA induction of stomatal closure but did not affect stomatal regulation by [CO_2] (Mustilli et al., 2002), suggesting ABA and [CO_2] signaling are not totally convergent in controlling stomatal closure.

Several transcription factors from soybean including putative MYB, AP2, and HD-ZIP are up-regulated under elevated [CO_2] conditions (Ainsworth et al., 2006).

Members of these transcription factor families also mediate plant responses to drought and ABA (Shinozaki and Yamaguchi-Shinozaki., 2007). Therefore, it is likely these elevated [CO_2] inducible genes are involved in the cross-talk among elevated [CO_2], drought, and ABA.

Gibberellins

Gibberellins are a large family of tetracyclic diterpene plant hormones. GA controls many aspects of plant growth and development, including stem elongation, transition to flowering, pollen tube elongation, seed development, and seed germination (Olszewski et al., 2002; Sun and Gubler, 2004). During seed germination and seedling growth, GA stimulates the production of hydrolytic enzymes such as endo-β-mannase and α-amylases to weaken the seed coat or the growth-constraining endosperm layer surrounding the embryo, mobilizes seed nutrient storage reserves in the endosperm to provide energy for germination, stimulates embryo expansion and hypocotyl elongation, and activates the vegetative growth of the embryo to produce new shoots and roots (Bewley and Black, 1994; Still and Bradford, 1997).

Over 136 GAs have been found in plants and fungi, but in most plant species, only GA_1, GA_3, and GA_4 are bioactive (Olszewski et al., 2002). The synthesis of bioactive GAs essentially consists a three-step process: 1) the production of *ent*-kaurene in the proplastid, 2) the synthesis of $GA_{12/53}$ in the ER, and 3) the formation of active GAs in the cytoplasm by successive oxidation steps from $GA_{12/53}$ (Olszewski et al., 2002).

Significant progress has been made in understanding the signal transduction mediating GA responses. A soluble GA receptor (Gibberelin Insensitive Dwarf1, GID1)

has been identified in rice (Ueguchi-Tanaka et al., 2005). In Arabidopsis, three soluble GA receptors, AtGID1a, AtGID1b, and AtGID1c, have been identified (Nakajima et al., 2006). A schematic model is shown in Figure 1-3: GA is perceived by GID1. The activated GID1 binds to the negative regulator, such as a DELLA protein (GAI, RGA, SLR1, and SLN1), triggering the ubiquitylation of the DELLA protein by an E3 ubiquitin ligase SCF complex and degradation of DELLA protein by the ubiquitin-26S proteasome pathway (Itoh et al., 2003; Sun and Gubler, 2004; Griffiths et al., 2006; Nakajima et al., 2006; Willige et al., 2007). Studies of constitutive GA-responsive mutants reveal that Arabidopsis *SPY* (SPINDLY) and its barley ortholog *HvSPY* encode a Ser/Thr O-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OCT), which is a repressor of GA signaling (Jacobsen et al., 1996; Robertson et al., 1998). SPY increases the activity of DELLA proteins such as Arabidopsis RGA and rice SLR1 by GlcNAc modification (Shimada et al., 2006; Silverstone et al., 2007). HvSPY interacts with two barley transcription factors, HSImyb and HSINAC, which act downstream of HvSPY as negative regulators for GA response (Robertson, 2004). Studies of GA-unresponsive dwarf mutants have identified positive regulators in GA signaling, such as barley *GSE1* (Chandler and Robertson, 1999) and Arabidopsis *SLY1* and *PICKLE* (Ogas et al., 1997; Steber et al., 1998). GSE1 appears to modulate the GA response by promoting the degradation of SLN1. However, the protein that *GSE1* encodes is still unknown (Gubler et al., 2002). The *SLY1* encodes an F-box protein, a component of the SCF(SLY1) E3 ubiquitin ligase that targets the DELLA protein for degradation (McGinnis et al., 2003; Griffiths et al., 2006). The *PKL* gene encodes a CHD3 chromatin-remodeling factor, which negatively regulates embryo-specific gene transcription (Henderson et al., 2004).

GA might also be recognized by an unknown receptor on the plasma membrane, leading to the activation of G-proteins (Hooley, 1998) and enhancement of the concentrations of the cytoplasmic cGMP (Penson et al., 1996) and Ca^{2+} (Gilroy and Jones, 1992), which modify downstream intermediates. During seed germination, these early GA responses via unknown mechanisms change the ratio of positive and negative regulators of transcription, resulting in the induction of GA-stimulated genes (Rubio-Somoza et al., 2006a; Xie et al., 2006).

Several *cis*-acting elements for the GA response of high-pI and low-pI α-amylase genes have been defined (Skriver et al., 1991; Gubler and Jacobsen, 1992; Lanahan et al., 1992; Rogers and Rogers, 1992; Rogers et al., 1994; Tanida et al., 1994). These motifs interact with various transcription factors controlling seed germination. In the low-pI α-amylase promoter, *Amy32b*, five elements, namely O2S/W-box, pyrimidine (Pyr) box, GA response element (GARE), amylase box (Amy), and the down-stream amylase element (DAE), are essential for a high level of GA-induced expression (Lanahan et al., 1992; Rogers et al., 1994; Rogers and Rogers, 1992; Gómez-Cadenas et al., 2001a). Each of these elements may be bound by one or more transcription factor(s). For example, both a transcription activator (GAMYB) and a transcription repressor (HRT) can directly interact with GARE (Gubler et al., 1995; Raventós et al., 1998). Several DOF proteins, OsDof3, SAD, and BPBF, bind to the Pyr box with SAD and OsDof3 functioning as activators and BPBF as a repressor (Diaz et al., 2002; Isabel-LaMoneda et al., 2003; Washio, 2003). R1MYB transcription factors, such as HvMYBS3 and HvMCB1, bind to the Amy box with HvMYBS3 functioning as an activator and HvMCB1 as a repressor (Rubio-Somoza et al., 2006a; Rubio-Somoza et al., 2006b). WRKY proteins, such as

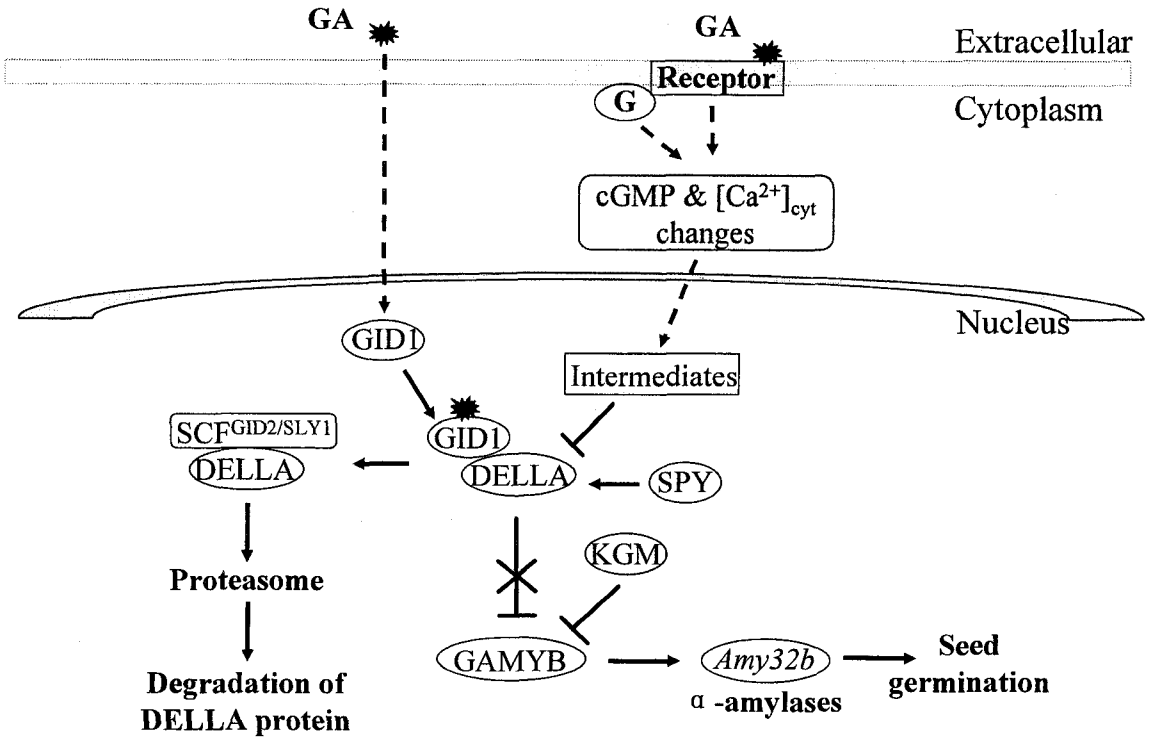

Figure 1-3. A schematic model of GA signaling during seed germination.
GA is perceived by a receptor on plasma membrane. The activated G protein enhances the concentration of cGMP and Ca^{2+} in the cytoplasm, and modifies downstream intermediates. GA also passes through membranes and is perceived by a nucleic localized receptor, GID1. The activated GID1 binds to the negative regulator DELLA proteins in a GA-dependent manner, triggering the degradation of DELLA proteins by proteasomes through an F-box protein (GID2 or SLY1) mediated pathway. Induced GAMYB promotes the expression of hydrolases, such as amylases, which leads to seed germination. SPY and KGM function as negative regulator on GA pathway. The figure is updated and modified from Xie (2006).

OsWRKY71 binds to the W-box (Zhang et al., 2004b; Xie et al., 2006). It is still not reported how these repressors and activators interact with each other in regulating gene expression.

Crosstalk of GA and ABA signaling

Although the antagonism of GA and ABA has long been studied as an important factor controlling seed maturation, dormancy, and germination (Shen et al., 1996; Bethke et al., 1997; Nambara and Marion-Poll, 2003; Sun and Gubler, 2004), our knowledge is still limited on the molecular interactions between GA and ABA response pathways. It has been demonstrated that ABA acts downstream of SLN1 in aleurone cells. This conclusion is based on two observations: 1) the expression of α-amylase gene in the *SLN1* knock-out mutant is still responsive to ABA; and 2) GA-induced SLN1 degradation is not inhibited by ABA (Fu et al., 2002). The crosstalk of GA and ABA signaling is mediated by secondary messengers. For instance, application of phosphatidic acid (PA) to barley aleurone cells inhibits GA-induced α-amylase production and promotes the expression of ABA-inducible amylase inhibitors and RAB (response to ABA) proteins (Ritchie and Gilroy, 1998). The discovery of an ABA inducible protein kinase (PKABA1) provides a link between GA and ABA signal transduction pathways (Gómez-Cadenas et al., 1999; Gómez-Cadenas et al., 2001b). Transient over-expression of PKABA1 inhibits the GA-induced expression of α-amylase and *HvGAMYB*, but not HvGAMYB-transactivated α-amylase gene expression (Gómez-Cadenas et al., 2001b). Constitutive expression of α-amylase and *HvGAMYB* in the *sln1* mutant is also repressed by ABA and PKABA1. These data indicate that PKABA1 mediates ABA suppression of α-amylase

expression upstream from the formation of GAMYB but downstream from the site of action of the SLN1 (Gómez-Cadenas et al., 2001b). However, *PKABA1* RNA interference does not hamper the inhibitory effect of ABA on the expression of α-amylase, suggesting that a PKABA1-independent pathway may also exist (Zentella et al., 2002). Evidence is emerging that several transcription factors are involved in mediating the cross-talk of GA and ABA signaling. The maize VP1 specifically activates the expression of ABA pathways while inhibiting the expression of GA-induced genes (Hoecker et al., 1995). SPY acts as a negative regulator of GA signaling in Arabidopsis and barley (Jacobsen et al., 1996; Robertson et al., 1998). Intriguingly, overexpression of SPY in aleurone cells stimulates the expression of an ABA-regulated dehydrin promoter, suggesting that SPY might be a positive regulator of ABA signaling. MINI ZINC FINGER 1 (MIF1), a putative zinc finger protein from Arabidopsis, suppresses GA signaling while enhances ABA response (Hu and Ma, 2006).

WRKY transcription factors

The first WRKY protein (SPF1) was identified from sweet potato (Ishiguro and Nakamura, 1994). Similar proteins were subsequently found in a variety of plant species (Ulker and Somssich, 2004). The name of the WRKY family is derived from the WRKY domain, an approximately 60 amino acid region that is highly conserved among family members.

A WRKY protein consists of one or two WRKY domains; each domain is composed of a WRKY core sequence and a zinc-finger motif. The WRKY domain binds specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W-

box (Figure 1-4). 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 4 distinct groups, which might reflect their different functions (Duan et al., 2007; Ulker and Somssich, 2004; Xie et al., 2005; Xie et al., 2006; Yamasaki et al., 2005; Zhang and Wang, 2005; Zou et al., 2004). The WRKY family has 74 members in Arabidopsis and about 100 members in rice (Ulker and Somssich, 2004; Xie et al., 2005; Qu and Zhu, 2006; Ross, 2007). Fewer *WRKY* genes have been found in ferns (*Ceratopteris richardii*) and mosses (*Physcomitrella patens*), but no WRKY gene has been found in animals (Ulker and Somssich, 2004). Furthermore, only double-domain *WRKY* genes are identified in green alga (*Chlamydomonas reinhardtti*), slime mold (*Dictyostelium discoideum*), and the protist (*Giardia lamblia*), indicating that *WRKY* genes might originate from an ancestral gene containing two WRKY domains at about 1.5-2 billion years ago (Ulker and Somssich, 2004). A phylogenetic analysis suggests that numerous *WRKY* gene duplications occurred after the divergence of the monocotyledons from dicotyledons some 50 to 80 million years ago (Zhang and Wang, 2005).

WRKY family members have been shown to be involved in biotic and abiotic stress responses, anthocyanin biosynthesis, senescence, trichome development, starch biosynthesis, and hormone responses (Dellagi et al., 2000; Du and Chen, 2000; Eulgem et al., 2000; Hara et al., 2000; Huang and Duman, 2002; Sun et al., 2003; Zhang et al., 2004b; Luo et al., 2005; Park et al., 2005; Ulker et al., 2007). The important role of WRKY proteins is to mediate plant response to biotic and abiotic stresses. Expression analysis of *WRKY* genes in Arabidopsis shows that 49 out of 72 tested *WRKY* genes are differentially regulated by pathogen infection or SA treatment (Dong et al., 2003).

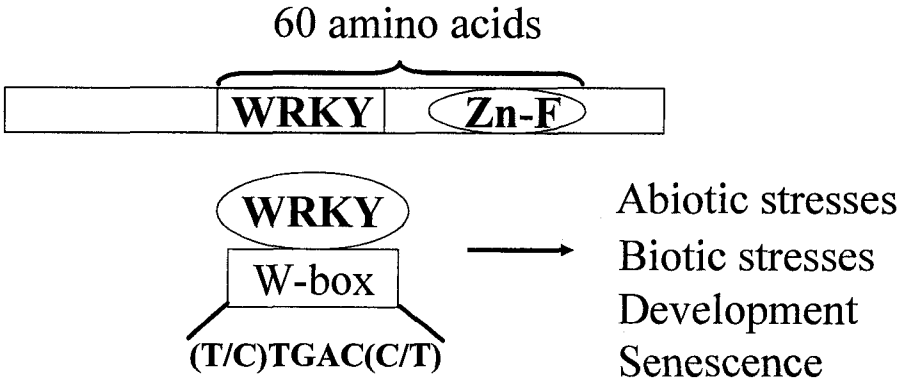

Figure 1-4. A schematic diagram of a WRKY protein and its functions.
A WRKY protein contains one or two conserved WRKY domain(s); each domain is composed of a WRKY motif and a zinc-finger motif. The WRKY domain binds to W-box. WRKY proteins have been reported to be involved in mediating various physiological processes such as abiotic/ biotic stress responses, plant development, and senescence.

WRKY proteins also mediate plant responses to environmental stresses. In the desert legume *Retama raetama*, a WRKY transcription factor was found to be associated with dormancy, extreme drought tolerance, and cold acclimation (Pnueli et al., 2002). Its homologous tobacco gene is induced under drought and heat shock (Rizhsky et al., 2002). *WIZZ* from tobacco participates in early stages of the wound response (Hara et al., 2000). *AtWRKY40* is responsive to ABA, cold, drought, high salinity, and pathogens (Seki et al., 2002; Dong et al., 2003). *HvWRKY38* is involved in cold and drought responses (Mare et al., 2004). These results suggest that stresses might play a central role in the expansion of the *WRKY* gene family in higher plants.

Functional diversity is the character of WRKY proteins. Arabidopsis WRKY75 is a modulator of phosphate acquisition and root development (Devaiah et al., 2007). MINI3/AtWRKY10 has been shown to mediate the regulation of seed size in Arabidopsis (Luo et al., 2005). TTG2/AtWRKY44 plays a key role in trichome development (Johnson et al., 2002a). A WRKY protein in cotton, GaWRKY1, is involved in regulating sesquiterpene biosynthesis (Xu et al., 2004). SUSIBA2, a WRKY protein in barley, is involved in sugar signaling by binding to the sugar-responsive elements of the *isol* promoter (Sun et al., 2003). AtWRKY70 was identified as a connection node of SA, JA, and ethylene signaling (Li et al., 2004; Ulker et al., 2007). A *WRKY* gene from creosote bush (*Larrea tridentata*), *LtWRKY21*, encodes an activator of the ABA signaling (Zou et al., 2004). Rice *WRKY51* and *WRKY71* encode transcriptional repressors of GA signaling (Zhang et al., 2004b; Xie et al., 2006).

Larrea tridentata (creosote bush)

Plants growing in North America desert can be basically categorized into C3, C4, and CAM (crassulacean acid metabolism) plants depending on their photosynthetic pathways (Smith et al., 1997). C3 plants use the C3 pathway (Calvin cycle) with CO_2 initially being incorporated into three-carbon molecules while C4 and CAM plants use both C4 and C3 pathways with CO_2 initially being incorporated into four-carbon molecules (Raven et al., 2005). In C4 plants, the two pathways are spatially separated. At first, CO_2 is fixed to phosphoenolpyruvate (PEP) by PEP carboxylase forming malate or aspartate in the cytosol of mesophyll cells via the C4 pathway. Then, malate or aspartate is transported to the bundle sheath cells and generates CO_2, which is reduced to carbohydrate via the C3 pathway. However, in CAM plants, the two pathways are temporally and spatially separated. In CAM plants, CO_2 is fixed to phosphoenolpyruvate (PEP) by PEP carboxylase forming malate in the cytosol when the stomata are open. During the day, the stored malate is transported to the chloroplast and decarboxylated, producing CO_2 and pyruvate; the CO_2 enters the Calvin cycle when the stomata are closed. Usually, C4 plants have higher water use efficiency (WUE) than C3 plants because the high activity of PEP carboxylase enables them to reduce stomatal aperture thereby conserving water for the same amount of CO_2 gained for photosynthesis. Also, CAM plants have higher WUE than C3 plants due to stomata opening at night when transpiration rates are lower. It has been reported that a CAM plant loses 50 to 100 g of water for every gram of CO_2 gained, compared with the 250 to 300 g and 400 to 500 g of water lose for a C4 and a C3 plant, respectively (Taiz and Zeiger., 1998). As a true drought-resisting C3 plant, *Larrea tridentata* dominates large areas of arid land in the

warm desert. *Larrea tridentata* remains metabolically active during all times of the year in the desert and maintains photosynthetic capacity at very low water potentials. *Larrea tridentata* has been reported to have a moderately high WUE among desert plants (Lajtha and Whitford, 1989; Smith et al., 1997). It remains unclear about the molecular mechanisms underlying its resistance to drought. One possibility is that *Larrea tridentata* has unique branches of signaling transduction pathways mediating drought tolerance. Another possibility is that *Larrea tridentata* has transcription factors that are homologous to those in other plants. However, unique domains are evolved during evolution of these transcription factors, which open bottlenecks for controlling the expression of drought resistance genes.

The scope of the dissertation

The goal of this research is to study how *WRKY* genes mediate ABA and GA signaling in regulating seed dormancy, germination, and plant responses to elevated [CO_2] and environmental stresses. My research starts from the identification of a high [CO_2] and stress inducible *WRKY* gene from *Larrea tridentata*, followed by functional analyses of *WRKY* genes in ABA and GA signaling.

In Chapter 2, I report that a *WRKY* gene from *Larrea tridentata*, *LtWRKY21*, is induced by elevated [CO_2] and abiotic stresses. LtWRKY21 acts as an activator of ABA signaling and as a repressor of GA signaling. These results suggest that LtWRKY21 might function as a connection node integrating signaling pathways in *Larrea tridentata.*

Chapter 3 focuses on the function of LtWRKY21 in the ABA signal transduction pathway. LtWRKY21 interacts synergistically with ABA and transcriptional activators

VP1 and ABI5 to control the expression of the *HVA22* promoter. This synergistic effect is not blocked by ABI1. Based on our observations, we propose a model that LtWRKY21, VP1, and ABI5 form a complex that function downstream of ABI1 to control ABA-regulated expression of genes.

Chapter 4 presents the functional analysis of *HvWRKY38*, an *LtWRKY21* homologous gene in barley, in GA signaling. I show that HvWRKY38 physically and functionally interacts with the transcriptional activator HvGAMYB. HvWRKY38 blocks GA-induced expression of *Amy32b* by interfering with the binding of HvGAMYB to the *cis*-acting elements in the *α-amylase* promoter. Furthermore, I show that the ratios of repressors (HvWRKY38 and BPBF) to activators (HvGAMYB and SAD) control the expression level of *Amy32b*. Based on the data, a hypothetical model is proposed for the control of the *Amy32b* gene expression in aleurone cells.

The contributions and significance of this dissertation study are summarized in Chapter 5. Future directions will focus on: 1) studying the gene expression pattern in *Larrea tridentata* under elevated [CO_2] and environmental stresses at a genome-wide level using the microarray technique; 2) investigating the functions of WRKY proteins in mediating plant responses to elevated [CO_2] and environmental stresses by using over-expression and knockout lines in Arabidopsis; and 3) identifying protein complexes controlling GA signaling using a method that combines tandem affinity purification with mass spectrometry.

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CHAPTER 2

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*.

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 to 4.5 °C increase in global air temperature compared to pre-industrial times (Crowley, 2000). 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 (Clark et al., 1998; Smith et al., 2000). 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 (Moore et al., 1999). Large increases in leaf carbohydrate concentrations occur in plants exposed

to elevated [CO_2] (Long and Drake, 1992; Obrist et al., 2001; Poorter et al., 1997; Moore et al., 1999; Rogers et al., 2004), and carbohydrate accumulation has long been associated with inhibition of photosynthesis (Jang et al., 1997). Water deficit has been found to reduce down-regulation, and even promote up-regulation of photosynthesis in some species (Oechel et al., 1995; Huxman et al., 1998). 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 (Hamerlynck et al., 2000; Loik et al., 2000; Taub et al., 2000).

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 (Shinozaki and Yamaguchi-Shinozaki, 2000). 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 (Addicott and Carns, 1983; Zeevaart and Creelmann, 1988; Mccarty, 1995; Rock, 2000; Finkelstein et al., 2002). This hormone induces rapid closure of stomatal pores through ion efflux from guard cells, thereby limiting water loss through transpiration (McAinsh et al., 1990; MacRobbie, 1998). ABA also triggers slower changes in gene expression, hence reprogramming the cell to withstand dehydration (Leung and Giraudat, 1998; Finkelstein et al., 2002). Sugars also have important hormone-like functions as primary messengers in signal transduction in addition to regulatory effects on photosynthetic activity and plant metabolism (Rolland et al., 2002; Rook et al., 2006). 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 (Dellagi et al., 2000; Du and Chen, 2000; Eulgem et al., 2000; Hara et al., 2000; Kim et al., 2000; Huang and Duman, 2002; Sun et al., 2003; Ulker and Somssich, 2004; Zhang et al., 2004; Luo et al., 2005; Park et al., 2005; Xie et al., 2005). 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 (Eulgem et al., 2000; Ulker and Somssich, 2004; Zou et al., 2004; Xie et al., 2005; Zhang and Wang, 2005; Xie et al., 2006).

Larrea tridentata has recently received considerable attention as one of the most drought-tolerant evergreen C3 shrubs dominating the North American warm deserts, (Smith et al., 1997; Nowak et al., 2004). 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 (Zou et al., 2004). 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.

Materials and methods

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).

Plant material 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 to 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 one 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 ½ strength MS (Murashige and Shoog basal salt mixture) solution containing 250 mM NaCl. For the hormone and sugar treatments, leaf sections were incubated in the ½ strength MS solution with addition of 100 μM ABA, 100 μM JA, and 6% sugar solutions (mannitol, glucose, and sucrose), respectively.

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 (Smit et al., 1990).

Starch was assayed after the method of Kerr et al. (1984). 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 (Hansen and Moller, 1975), and for glucose (Glucose Kit, 510A).

RNA gel-blot analysis

Total RNA was isolated from *Larrea tridentata* leaves as described by Wang et al. (2000). Briefly, leaves (1g) 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

5,000 × 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% (vol/vol). The mixture was incubated on ice for 10 min, followed by centrifugation at 5,000 × 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 5,000 × 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 mixed with a 1/4 volume of 10 M LiCl and kept in refrigerator at 4 °C overnight. The following day the sample was centrifuged at 10,000 × g for 30 min. The pellet was resuspended in 1.5 ml TE buffer, following which 1.5 volume of 5 M potassium acetate were added and the mixture was incubated for 3 h on ice and centrifuged at 10,000 × g for 30 min. The pellet was resuspended in 1 ml TE buffer, incubated on ice for 1 h, and centrifuged to remove any undissolved material. The clear supernatant was mixed with a 1/9 volume of 3.3 M sodium acetate and two volumes of ethanol, and placed at –20 °C for 2 h. The RNA pellet was then obtained by centrifugation at 10,000 × g for 30 min, and was washed with 500 ul ethanol followed by centrifugation at 10,000 × g for 10 min. The pellet was air-dried and resuspended in DEPC-treated water. Northern blot analysis was performed as previously described (Zou et al., 2004), and the integrated density of each band was quantified using ImageJ from National Institutes of Health.

Construct preparation and transient expression assays

Three types of DNA constructs were used in the transient expression experiments: reporter, effector, and internal control. Plasmid *HVA1-GUS* (Shen et al., 1993), and

Amy32b-GUS (Lanahan et al., 1992) were used as the reporter constructs. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by the constitutive maize ubiquitin promoter (Bruce et al., 1989), was used as an internal control construct to normalize GUS activities of the reporter construct. *LtWRKY21* effector construct was prepared as follows: The *LtWRKY21* effector gene was amplified by PCR using two primers: TTAGGCGCGCCATGGCATATCCTTCTTGG and TTAGGCGCGCCTCACCAATTTCCTCCAGG. The AscI site was included to facilitate cloning. The PCR product was confirmed by sequencing, and then cloned into the AscI site of the intermediate construct containing the *UBI* promoter and *NOS* terminator (Zhang et al., 2004) to generate *UBI-LtWRKY21*.

The detailed description of transient expression procedure with the barley (*Hordeum vulgare*) aleurone system and the particle bombardment technique have been published before (Shen et al., 1993). Briefly, de-embryonated half-seeds of Himalaya barley were imbibed for 2.5-3 days, and then the pericarp and testa were removed. The DNA mixture (in 1:1 molar ratio) of *HVA1-GUS* and *UBI-Luciferase* or *Amy32b-GUS* and *UBI-Luciferase*, along with or without an effector construct, was bombarded into barley embryoless half-seeds (four replicates per test construct). After incubation for 24 h with various treatments, GUS assays and luciferase assays were performed as previously described (Shen et al., 1996).

Results

Elevated [CO_2] enhanced ABA concentrations in leaves of *Larrea tridentata*

Elevated [CO_2] decreases stomatal conductance (Hamerlynck et al., 2000; Wullsch-leger et al., 2002), and ABA induces rapid closure of stomatal pores in response to water deficit stress (Leckie et al.,1998; MacRobbie, 1998). To investigate the interaction be-tween elevated [CO_2] and water deficit stress on ABA production, ABA concentrations in the leaves of *Larrea tridentata* in ambient and elevated [CO_2] under well watered and water deficit were quantified by radioimmunoassy. The ABA concentrations in the leaves of well watered *Larrea tridentata* grown in ambient [CO_2] were determined to be 2.5 pmol per mg of dry weight (Figure 2-1). Water deficit led to a 3.5-fold increase in ABA concentrations under ambient [CO_2] conditions. The ABA concentration doubled in leaves of plants grown under elevated [CO_2]. The combination of elevated [CO_2] and water deficit resulted in a 6-fold increase in ABA compared to the well-watered plants grown under ambient [CO_2].

Elevated [CO_2] enhanced starch concentrations in leaves of *Larrea tridentata*

Plants produce sugars by photosynthesis for subsequent metabolism or storage in heterotrophic tissues. Sucrose is the main transportable form of sugar while starch is the storage form of energy and carbon (Buchanan, 2002). Recent genetic and molecular analyses have revealed a connection between sugar and hormones (Leon and Sheen, 2003; Rook et al., 2006). ABA biosynthesis and signaling genes appear to be responsive to carbohydrates. It is possible that elevated [CO_2] enhances ABA levels via sugar response

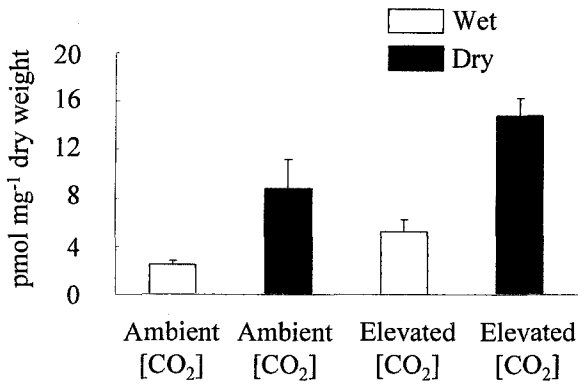

Figure 2-1. Elevated [CO_2] enhanced ABA concentrations in leaves of *Larrea tridentata*. Plants were grown under ambient (350 μmol/mol) and elevated (700 μmol/mol) [CO_2] for one year. For water deficit treatments, water was withheld 3 days. Data are means ± SE of four replicates.

pathways. To test this hypothesis, we measured starch, sucrose and glucose concentrations in leaves of *Larrea tridentata* grown under either ambient or elevated [CO_2] and under water deficit by withholding water for 3, 5, 7, to 9 days, representing mild to severe water deficit. The starch concentration in the leaves of *Larrea tridentata* grown under ambient [CO_2] and watered regularly was 90 μg per milligram of dry weight (Figure 2-2A). *Larrea tridentata* plants grown under elevated [CO_2] had higher starch concentrations. However, under water-limited conditions, starch concentrations of plants grown under either ambient or elevated [CO_2] decreased. The sucrose and glucose concentrations were 115 and 20 μg per milligram of dry weight for cultured plants (Figure 2-2B and 2C). No significant effect of elevated [CO_2] and water deficit on sucrose and glucose concentrations in leaves could be detected.

The expression of *LtWRKY21* is induced

by elevated [CO_2] and abiotic stresses

With 43 Arabidopsis stress-inducible genes (Seki et al., 2002) as queries, we searched the *Larrea tridentata* expression sequence tag database and identified a WRKY gene (*LtWRKY21*) that functions as an activator of ABA signaling pathway (Zou et al., 2004). The *LtWRKY21* cDNA is 945-bp in length, encoding a polypeptide with 314 amino acids with a WRKY domain containing a WRKYGQK motif (from amino acid residue 166 to residue 172) and a zinc-finger motif (C-X_{4-5}-C-X_{22-23}-H-X_1-H).

To study the expression pattern of the *LtWRKY21* gene, total RNA was isolated from the leaves of well-watered and water deficit *Larrea* plants grown under ambient or elevated [CO_2]. At the onset of the treatment, the steady-state mRNA level of *LtWRKY21* is barely detectable (Figure 2-3A, lane 1). However, the level of *LtWRKY21* increased by

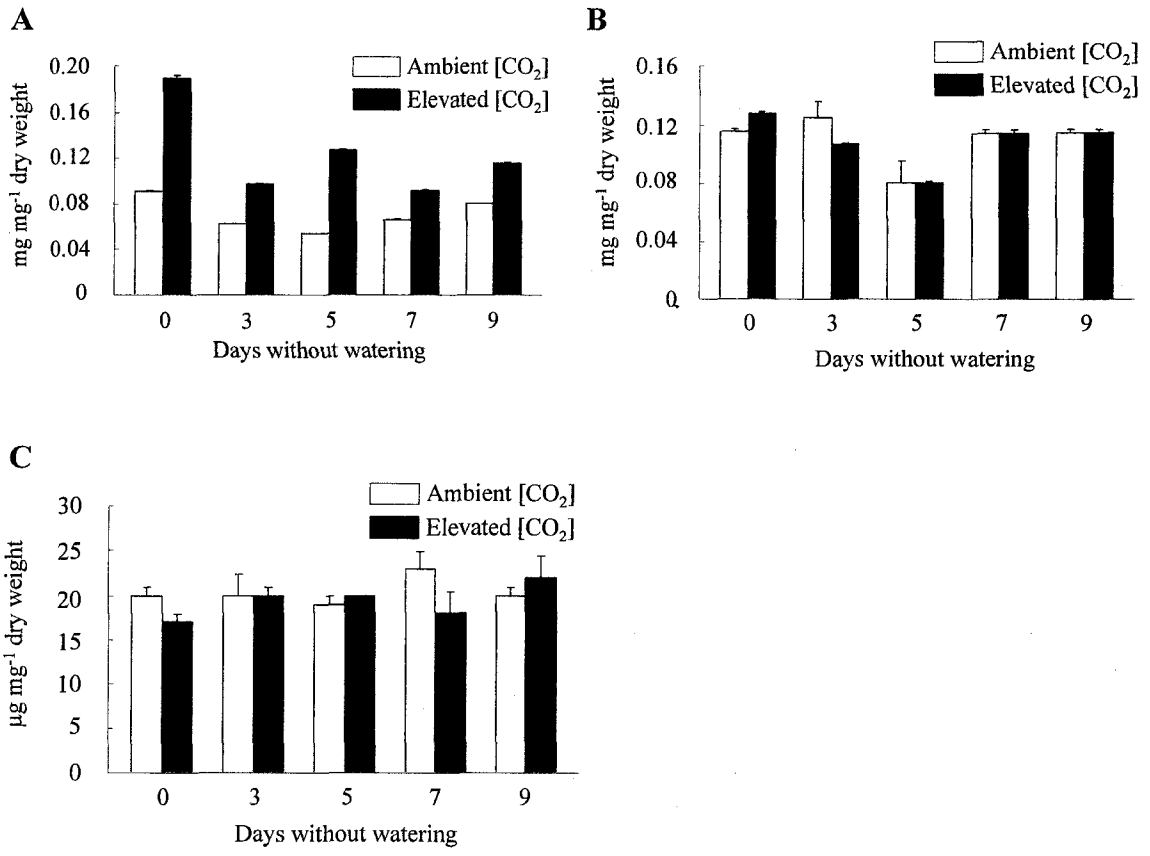

Figure 2-2. Elevated [CO_2] enhanced starch concentrations in leaves of *Larrea tridentata*. Starch concentration (A), sucrose concentration (B), and glucose concentration (C) from growing leaves of one-year-old plants. Data are means ± SE of eight replicates.

three-fold after 3 days of water deficit (compare lane 2 with lane 1), and decreased during days 5, 7 and 9 under ambient [CO_2]. In contrast, elevated [CO_2] led to a two-fold induction of *LtWRKY21* mRNA level (compare lane 7 with lane 6); the level remained high even after 5-, 7- and 9-days of water deficit.

To further investigate whether other abiotic stresses have an effect on the expression of *LtWRKY21* gene, total RNA was isolated from detached leaves incubated in a 250 mM NaCl solution, 4 °C, or 45 °C for 4 and 8 h, respectively. Stronger signals were detected in controls after incubation for 4 and 8 h (Figure 2-3B), indicating that this gene is wounding-inducible. High salinity treatment further increased the *LtWRKY21* mRNA level by 40% (compare lane 5 with lane 3). However, cold or heat treatments, for 4 and 8 h, appeared to promote the degradation of *LtWRKY21* mRNA levels induced by wounding (compare lanes 6 and 8 with lane 2, and lanes 7 and 9 with lane 3).

LtWRKY21 is ABA, JA, and glucose-inducible in vegetative tissues

ABA and JA are two plant hormones mediating plant response to stress (Kunkel and Brooks, 2002; Xiong and Zhu, 2003). The direct interactions between sugar and hormonal signaling have been demonstrated (Rolland et al., 2002; Rook et al., 2006). Since elevated [CO_2] and water deficit enhanced ABA and starch levels (Figures 2-1 and -2), as well as increased *LtWRKY21* mRNA levels in leaves of *Larrea tridentata* (Figure 2-3), it is possible that ABA, JA and sugars have effects on the expression of the *LtWRKY21* gene. To test this hypothesis, total RNA was isolated from detached leaves incubated in solutions of ABA or JA for 1, 2, and 3 h, respectively. Northern analysis revealed that the steady-state mRNA level of *LtWRKY21* was enhanced by wounding,

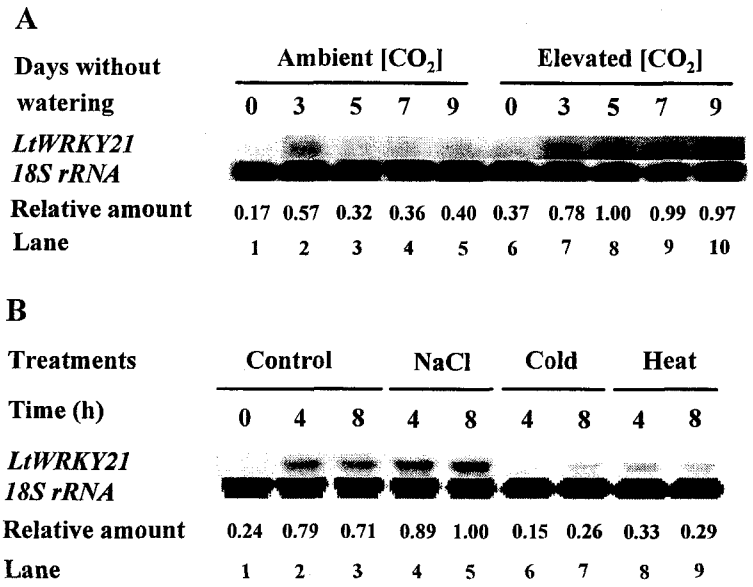

Figure 2-3. Northern blot analysis of the *LtWRKY21* gene in response to [CO_2] and abiotic stresses.
Total RNA was extracted from leaves of *Larrea tridentata* grown under ambient or elevated [CO_2] and under well watered or water deficit conditions (A), or leaves of *Larrea tridentata* treated with 250 mM NaCl, 4 °C or 45 °C (B). The RNA blots were probed with either the cDNA clone of *LtWRKY21* or *18S rRNA* as a control. Values below the blot represent the ratio of *LtWRKY21* to *18S rRNA*, with the maximum value set to 1.

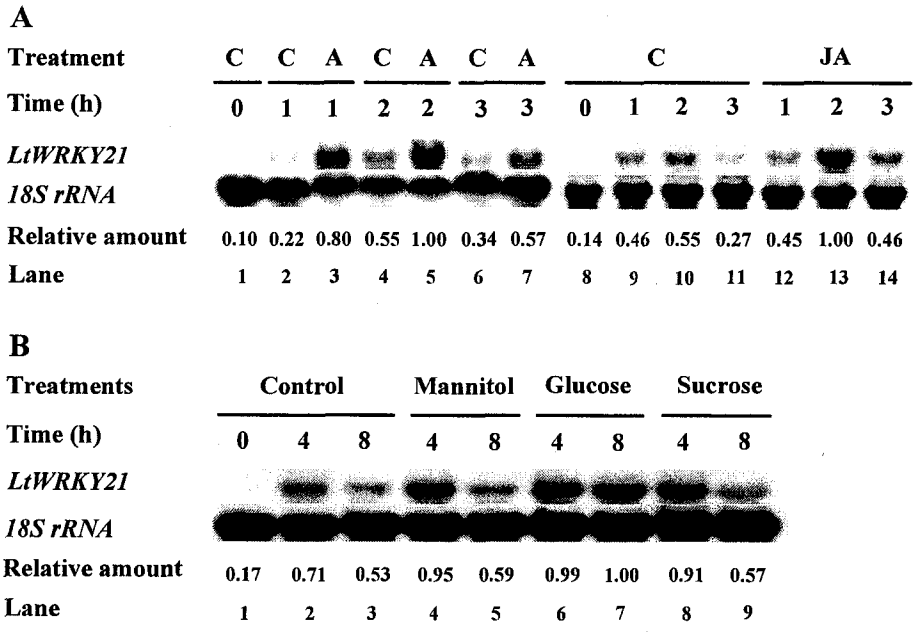

Figure 2-4. LtWRKY21 is ABA-, JA-, and glucose-inducible in vegetative tissues. Total RNA was isolated from leaves of *Larrea tridentata* treated with ABA or JA (A) for 1, 2, and 3 hr and 6% of mannitol, glucose and sucrose for 4 and 8 hr (B). The RNA blots were probed with either *LtWRKY21* or *18S rRNA* as a control. Values below the blot represent the ratio of *LtWRKY21* to *18S rRNA*, with the maximum value set to 1. C: Control; A: ABA treatment; JA: jasmonic acid treatment.

and was further increased (by 82%) in response to exogenous ABA (Figure 2-4A, compare lane 5 with lane 4) and JA treatments (Figure 2-4A, compare lane 13 with lane 10). Furthermore, after 8 h of incubation in 6% glucose, the expression of *LtWRKY21* was increased by 89% (Figure 2-4B, compare lane 7 with lane 3). However, 6% of mannitol or sucrose treatments for 8 h had little effect on the abundance of *LtWRKY21* mRNA (Figure 2-4 B, compare lanes 5 and 9 with lane 3).

LtWRKY21 activates ABA induction of the *HVA1* promoter and represses GA induction of the *Amy32b* α-amylase promoter

In our previous work, we demonstrated that LtWRKY21 activates the ABA-inducible *HVA22* promoter (Zou et al., 2004). To confirm the function of LtWRKY21 on ABA signal transduction pathway, another reporter construct was tested; *HVA1-GUS*, which contains the *GUS* reporter gene driven by the ABA responsive promoter *HVA1* (Shen et al., 1996). Although both *HVA*1 and *HVA22* are ABA inducible, they have a different promoter structure (Shen et al., 1996). The *HVA1-GUS* and the *UBI- LtWRKY21* (the *LtWRKY21* effector gene driven by the constitutive maize ubiquitin promoter) constructs were co-introduced into aleurone cells by particle bombardment. In the absence of ABA, a very low level of GUS activity was detected (Figure 2-5). The exogenous ABA (20 μM) treatment resulted in a 21-fold enhancement of GUS activity over that found in the ABA-untreated control. Interestingly, *LtWRKY21* synergistically interacted with ABA to transactivate the expression of the *HVA1* promoter, leading to a 54-fold induction. ABA and GA have been considered to play essential and often antagonistic roles in regulating seed germination, plant growth, development, and stress responses (Karssen et al., 1983; Koornneef and Karssen, 1994; Razem et al., 2006), it is

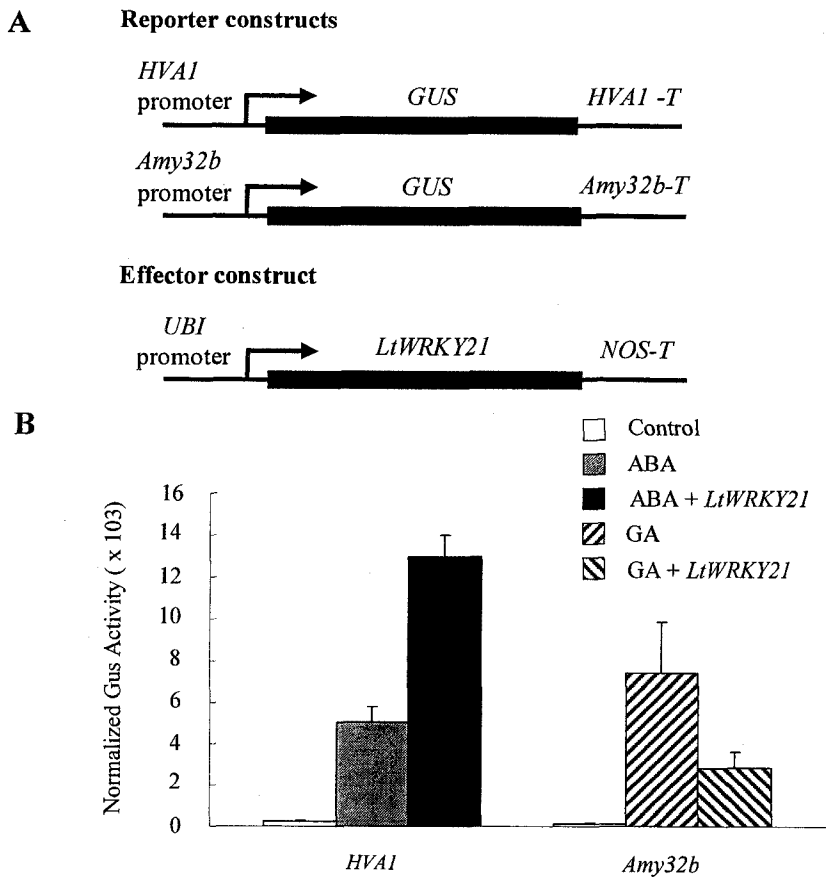

Figure 2-5. LtWRKY21 activates ABA induction of the *HVA1* promoter and represses GA induction of the *Amy32b* α-amylase promoter.
(A) Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment.
(B) The reporter construct, *HVA1-GUS*, or *Amy32b-GUS* and the internal construct, *UBI-luciferase*, were cobombarded into barley half-seeds either with (+) or without (-) the effector construct (*UBI-LtWRKY21*) by using the same amount of effector and reporter constructs (1.43 μg per shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. Bars indicate GUS activities ± SE after 24 h of incubation with (+) or without (-) 20 μM ABA or 1 μM GA. Data are means ± SE of four replicates.

therefore possible that LtWRKY21 is involved in crosstalk between ABA and GA. To test the function of LtWRKY21 on the GA signal transduction pathway, a reporter construct (*Amy32b-GUS*) containing the *GUS* reporter gene driven by the GA responsive *Amy32b* α-amylase promoter (Lanahan et al., 1992) and the effector construct (*UBI-Lt-WRKY21*) were co-introduced into aleurone cells by particle bombardment. GA (1 μM) treatment resulted in a 59-fold enhancement of GUS activity over controls (Figure 2-5). However, when both GA and *LtWRKY21* were introduced, the GA induction was attenuated to 22-fold only. Taken together, these data suggest that LtWRKY21 functions as an activator of the ABA signaling pathway and a repressor of the GA signaling pathway.

Discussion

In addition to common environmental stresses such as drought, cold and salt stress, plants are also experiencing the global warming resulted from elevated atmospheric [CO_2]. Ecological studies have clearly shown that long-term exposure to elevated [CO_2] resulted in enhanced recruitment, extinction, or geographic migration of certain species (Clark et al., 1998; Smith et al., 2000). The long term goal of our study is to understand the molecular foundation underlying these plant population changes. Alternations in concentrations of and sensitivity to hormones play a pivotal role in mediating plant responses to stresses, as have been well demonstrated for ABA (Zeevaart, 1999; Xiong and Zhu, 2003). Here, we show for the first time that like abiotic stresses, elevated [CO_2] treatments increased ABA concentrations in the leaves of *Larrea tridentata* (Figure 2-1). We then showed that a regulatory gene from *Larrea tridentata*,

LtWRKY21, is responsive to elevated [CO_2], water deficit, high salinity and wounding (Figure 2-3 and -4). Finally, we showed that LtWRKY21 enhanced the expression of an ABA-inducible gene and inhibited that of an ABA-repressible gene (Figure 2-5). Together, these data illustrate a mode of action that is involved in changes of ABA concentrations, induction of a key regulator gene and modulations (enhancement and inhibition) of the downstream genes.

High [CO_2] is shown to enhance the level of ABA (Figure 2-1). This enhancement might be due to increased biosynthesis of ABA, or decreased catabolism of ABA, or both. Expression of ABA biosynthetic genes, *ZEP*, *AAO3* and *MCSU* are up-regulated by ABA, drought and salt stresses (Audran et al., 1998; Seo et al., 2000; Iuchi et al., 2001; Xiong et al., 2001a; Xiong et al., 2001b; Xiong et al., 2002), likely through a Ca^{2+}-dependent protein phosphorylation and dephosphorylation cascade (Xiong and Zhu, 2003). Webb et al. (1996) found that elevated [CO_2] treatments increased cytosolic free calcium in guard cell. It remains to be determined whether there is a link between elevated [CO_2] induced calcium increase and activation of the ABA biosynthesis genes. Another possibility is that high [CO_2] modulates ABA levels by way of carbohydrate chemistry. Several ABA biosynthesis genes are induced by glucose (Cheng et al., 2002). The *gin1* (*glucose insensitive 1*) mutant is known to be allelic to *aba2* (Laby et al., 2000; Rook et al., 2001), an ABA-deficient Arabidopsis mutant (Schwartz et al., 1997). Increased atmospheric CO_2 concentration is known to primarily affect components of photosynthesis through plant secondary metabolism, at least in part as a response to changes in carbohydrate metabolism. Under twice-ambient [CO_2], leaf soluble carbohydrate content increased by roughly 15%, and starch content by 57% in 27 C3 species

(Poorter et al., 1997). However, our data show that there was no change in glucose or sucrose concentrations in leaves of *Larrea tridentata* (Figure 2-2B and 2C). Similar results have been found in potato leaves (Angelica et al., 2005), where soluble sugars concentration was found to be stable irrespective of the [CO_2] treatment. However, in this study, the concentration of starch approximately doubled in leaves of *Larrea* under twice ambient [CO_2] (Figure 2-2A). It will be interesting to study whether starch regulates any of ABA biosynthesis genes.

Similar responses of stomata to ABA and elevated [CO_2] have been observed. Stomatal conductance and possibly stomatal density have been showed to decrease under long term exposed to elevated [CO_2] (Woodward and Kelly, 1995; Wullschleger et al., 2002). Both ABA and elevated [CO_2] induced increases in guard cell cytosolic free calcium (Webb et al., 1996). Drought increased internal [CO_2] concentration, thereby counteracting photosynthetic down-regulation by elevated [CO_2] in *Larrea tridentata* (Huxman et al., 1998). Elevated [CO_2] appears to reduce the impact of drought, high temperature on *Larrea* by increasing the stomatal conductance and net photosynthetic rate (Hamerlynck et al., 2000). Our work suggests stomatal responses to elevated [CO_2] might be mediated by ABA.

As key switches of signaling networks, transcription factors are good targets for studying the molecular mechanism underlying the interactions of elevated [CO_2] and abiotic stress. We focused on *WRKY* genes because this gene family mediates biotic and abiotic stress responses, and hormone responses (Du and Chen, 2000; Eulgem et al., 2000; Hara et al., 2000; Kim et al., 2000; Huang and Duman, 2002; Ulker and Somssich, 2004; Zhang et al., 2004; Xie et al., 2005; Park et al., 2005). In the desert legume *Retama*

raetama, a WRKY transcription factor was found to be associated with dormancy, extreme drought tolerance, and cold acclimation (Pnueli et al., 2002). Its homologous tobacco gene is induced under drought and heat shock (Rizhsky et al., 2002). *WIZZ* from tobacco participates in early stages of the wound response (Hara et al., 2000). *LtWRKY21* reported in this study is a homologue of the Arabidopsis *AtWRKY40* (Eulgem et al., 2000), and the barley *HvWRKY38* (Mare et al., 2004). *AtWRKY40* is responsive to ABA, cold, drought, high salinity, and pathogens (Seki et al., 2002; Dong et al., 2003). *HvWRKY38* is involved in cold and drought responses (Mare et al., 2004). A soybean *WRKY* gene is expressed at a higher level under elevated [CO_2] (Ainsworth et al., 2006). In this study, we showed that that *LtWRKY21* is induced by elevated [CO_2], wounding, drought, and salt stresses (Figures 2-3 and -4). It is also induced by ABA and JA, two hormones well known to mediate plant responses to environmental stresses (Figure 2-5). However, cold and heat stresses promoted the degradation of wounding-induced LtWRKY21 mRNA or suppress the expression of *LtWRKY21* that is induced by wounding (Figure 2-4). Together these data suggest that LtWRKY21 might function as a node integrating stress signaling in *Larrea tridentata.*

Studying genes that are induced or suppressed by LtWRKY21 revealed potential signaling components downstream of this key transcription factor. Because a functional assay system is not yet available for *Larrea tridentata*, we preformed the functional study in aleurone cells of barley seeds, which are also desiccation tolerant and responsive to ABA and other hormones. In a previous study, LtWRKY21 was showed to control the expression of an ABA-inducible *HVA22* promoter (Zou et al., 2004). Here, we showed that LtWRKY21 also enhanced the expression of *HVA1*, another ABA- and stress-

inducible gene whose promoter contains an ABRE element similar to that in HVA22, but a different coupling element (Shen et al., 1996). The other role of ABA is to suppress genes that are induced by GA to prevent seeds from germinating under unfavorable environmental conditions. Data in Figure 2-5B showed that LtWRKY21 also suppressed the induction of the *Amy32b* amylase gene, which is suggested to be required for seed germination and post-germination growth (Gómez-Cadenas et al., 2001). Therefore, roles of LtWRKY21 include induction of genes for stress tolerance and suppression of genes for germination so that the seeds can remain in a state tolerant to desiccation and other adverse conditions.

In summary, mounting evidence indicates that plants have intricate and complex webs in signal transduction pathways in response to diverse environments. Some transcription factors act as integration nodes, receiving signals and generating integrated plant responses (Rolland et al., 2002). *LtWRKY21* is induced by ABA, JA, glucose, elevated [CO_2], water deficit, high salinity, and wounding. Furthermore, wounding induction of the *LtWRKY21* gene expression is suppressed by cold and heat stresses. These data suggest that LtWRKY21 might function as a node integrating stress signaling in *Larrea tridentata*.

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CHAPTER 3

A *WRKY* GENE FROM CREOSOTE BUSH ENCODES AN ACTIVATOR OF THE ABSCISIC ACID SIGNALING PATHWAY

This chapter has been published in The Journal of Biological Chemistry and is presented in the style of that journal. My work is specifically on the functional study of LtWRKY21 on ABA pathway. The complete citation is:

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Summary

The creosote bush (*Larrea tridentata*) is a xerophytic evergreen C3 shrub thriving in vast arid areas of North America. As the first step toward understanding the molecular mechanisms controlling the drought tolerance of this desert plant, we have isolated a dozen genes encoding transcription factors, including *LtWRKY21* that encodes a protein of 314 amino acid residues. Transient expression studies with the *GFP-LtWRKY21* fusion construct indicate that the LtWRKY21 protein is localized in the nucleus and is able to

activate the promoter of an abscisic acid (ABA)-inducible gene, *HVA22*, in a dosage-dependent manner. The transactivating activity of LtWRKY21 relies on the C-terminal sequence containing the WRKY domain and a N-terminal motif that is essential for the repression activity of some regulators in ethylene signaling. LtWRKY21 interacts synergistically with ABA and transcriptional activators VP1 and ABI5 to control the expression of the *HVA22* promoter. Co-expression of *VP1*, *ABI5*, and *LtWRKY21* leads to a much higher expression of the *HVA22* promoter than does the ABA treatment alone. In contrast, the LtWRKY21-mediated transactivation is inhibited by two known negative regulators of ABA signaling: 1-butanol, an inhibitor of phospholipase D, and abi1-1, a dominant negative mutant protein phosphatase. Interestingly, abi1-1 does not block the synergistic effect of *LtWRKY21*, *VP1*, and *ABI5* co-expression, indicating LtWRKY21, VP1, and ABI5 may form a complex that functions downstream of ABI1 to control ABA-regulated expression of genes.

Introduction

The phytohormone abscisic acid (ABA) modulates plant developmental processes such as seed formation, dormancy, and germination, as well as plant responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV radiation (Addicott and Carns, 1983; Zeevaart and Creelmann, 1988; Mccarty, 1995; Albinsky et al., 1999; Rock, 2000; Finkelstein et al., 2002). Plant responses to ABA are mediated at several molecular levels such as transcription, RNA processing, post-translational modification, and metabolism of the secondary messengers (Rock, 2000; Finkelstein et al., 2002; Himmelbach et al., 2003). Recent data indicate that the ABA signaling

pathways appear to be conserved among higher plant species and even bryophytes (Knight et al., 1995; Gampala et al., 2002;).

Both ABA resistant and hypersensitive mutants have been extremely valuable in helping define ABA signaling pathways. Studies of mutants in several plant species suggest that the ABA signaling is mediated by a membrane-bound metal sensor (Alonso et al., 1999; Ghassemian et al., 2000), type 2C serine/threonine protein phosphatases, PP2C (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997), a Ser/Thr protein kinase (Li and Chory, 1997; Steber and McCourt, 2001), a protein farnesyl transferase (Cutler et al., 1996), a steroid reductase (Steber and McCourt, 2001), an inositol polyphosphate 1-phosphatase (Xiong et al., 2001), and several transcription factors (Wilson et al., 1990; McCarty et al., 1991; Giraudat et al., 1992; Ishitani et al., 1998; Finkelstein et al., 1998; Németh et al., 1998; Finkelstein and Lynch, 2000; Nagpal et al., 2000). In addition, the mutant studies also suggest that RNA processing plays an important role in the regulation of ABA signaling (Kuhn and Schroeder, 2003) because several ABA response mutants are impaired in a double-stranded RNA-binding protein (Lu and Fedoroff, 2000), a mRNA CAP-binding protein (Hugouvieux et al., 2001), or a U6-related Sm-like small ribonucleoprotein (Xiong et al., 2001). In line with these reports, an ABA-induced maize glycine rich protein can bind to uridine- and guanosine-rich RNA fragments (Freire and Pages, 1995).

Several types of *cis*-acting elements are involved in ABA responses, such as the 10-bp element containing an ACGT core (ACGT-box, also named G-Box or ABRE), CE, RY/Sph, AT-rich elements, and Myb and Myc-binding sites (Finkelstein et al., 2002; Himmelbach et al., 2003). In a series of mutational analyses of two ABA responsive

barley genes, *HVA1* and *HVA22*, it was shown that in addition to the ACTG-box (*A3*, GCCACGTACA or *A2*, CCTACGTGGC), a coupling element (*CE1*, TGCCACCGG or *CE3*, ACGCGTGTCCTC) is also necessary for the ABA response (Shen and Ho, 1995; Shen et al., 1996). The combination of the ACGT-box and the *CE* forms an ABA response complex (ABRC), which has been shown to be the smallest ABA-responsive promoter unit (Shen et al., 1996). Recently, the ACGT-box is further narrowed down to be ACGTGGC, and CE1 and CE3 are narrowed down to be CCACC and GCGTGTC, respectively (Shen et al., 2004). ABREs and CE3 are bound by bZIP proteins (Guiltinan et al., 1990; Oeda et al., 1991; Chern et al., 1996; Kim et al., 1997; Kim and Thomas, 1998; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Uno et al., 2000; Kang et al., 2002; Casaretto and Ho, 2003); CE1 is bound by ABI4 (Niu et al., 2002); RY/Sph elements are bound by those containing B3 domains (McCarty et al., 1991; Giraudat et al., 1992; Hattori et al., 1994; Bobb et al., 1995; Bobb et al., 1997; Carson et al., 1997; Chandler and Bartels, 1997; Jones et al., 1997; Suzuki et al., 1997; Rohde et al., 1998; Shiota et al., 1998; Bailey et al., 1999; Ezcurra et al., 2000), AT-rich elements are bound by homeodomain leucine zipper proteins (Himmelbach et al., 2002; Johannesson et al., 2003); MYC sites are bound by AtMYC (Abe et al., 1997) and MYB sites are bound by AtMYB (Abe et al., 2003; Abe et al., 1997).

WRKY genes are known to be involved in biotic (bacterial and fungal diseases) and abiotic (heat, drought, wounding, and freezing) stress responses, anthocyanin and starch biosynthesis, senescence and trichome development, and hormone responses (Chen and Chen, 2000; Dellagi et al., 2000; Du and Chen, 2000; Eulgem et al., 2000; Hara et al., 2000; Kim et al., 2000; Hinderhofer and Zentgraf, 2001; Robatzek and

Somssich, 2001; Yu et al., 2001; Asai et al., 2002; Huang and Duman, 2002; Sun et al., 2003; Zhang et al., 2004). *WRKY* genes have either one or two WRKY domains, each containing a 60-amino acid region with a core sequence, WRKYGQK, at its N-terminal end and a novel zinc-figer-like motif. The WRKY domain binds specifically to the DNA sequence motif (T)(T)TGAC(C/T), which is known as the W-box. Despite the strong conservation of their 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 (Eulgem et al., 2000).

In this work, we identified and characterized a WRKY family transcription factor, *LtWRKY21*, from creosote bush (*Larrea tridentata*). We then co-expressed ABA-regulated reporter constructs with effector constructs encoding LtWRKY21 and other known ABA signaling regulators, such as ABI1, VP1, and ABI5, in barley aleurone layers to better define the signal transduction pathways mediating ABA signaling. Our results indicate that LtWRKY21 activates ABA-regulated transcription by interacting with VP1 and ABI5 and acting downstream of ABI1.

Experimental procedures

Construction of creosote bush cDNA libraries

Total RNA was isolated from creosote bush leaves from the Nevada Desert research center (www.unlv.edu/Climate_Change_Research/) with TRIzol® reagents (Invitrogen). The first strand cDNA was synthesized via priming of the poly(A) tail with the primer 5'-GACTAGTTCT AGATCGCGAG CGGCCGCCCT TTTTTTTTTTTTTT-3'. This primer contains four restriction sites: SpeI, XbaI, NruI, and NotI. Once the

double-stranded cDNAs were synthesized, their ends were polished with Klenow. This was followed by digestion with NotI, leaving a sticky NotI site at the 3' end and a blunt end at the 5'end. The digested fragments were then cloned into EcoRV and NotI cut pCMVSport6 vector (Invitrogen). Sequencing of the expression sequence tags was done using Applied Biosystems Prism 3730 DNA analyzer at the Nevada Genomics Center (www.ag.unr.edu/genomics/).

RNA gel blot analysis

Total RNA was isolated from creosote bush seeds with the LiCl precipitation method as described (Zhang et al., 2004). Ten μg total RNA was transferred to nylon membrane according to the method of Shen et al. (Shen et al., 1993). The gene specific fragment of *LtWRKY21* was amplified by PCR using two primers: CCTCTTAGGG CATCTAATGA AGCTTCACC and CTCTTATCGT CTTTGTCGGT TCGGACATAA TC. The gene specific fragment of 18S was amplified by PCR using GTGGTGCATG GCCGTTCTTA GTTG and ACTCGTTGGA TACATCAGTG TAGC. The membrane was probed with digoxigenin-labeled DNA by using a digoxigenie probe synthesis kit (Roche Applied Science) according to the manufacturer's instructions. After hybridization, the membrane was washed twice with 2X SSC and 0.1% SDS for 5 min at room temperature and then twice with 0.1 X SSC plus 0.1% SDS for 15 min at 50 °C. The signal was detected using the digoxigenin chemiluminescent detection kit (Roche Applied Science).

Genomic DNA isolation

Creosote bush seeds were germinated on wet Whatman paper saturated with imbibing solution (20 mM $CaCl_2$ and 20 mM sodium succinate) in dark at 26 °C.

Genomic DNA was isolated from 10-day-old seedlings. Briefly, sterile shoots were frozen in liquid nitrogen and ground into a powder. The frozen powder tissue was suspended in CTAB extraction buffer (55 mM hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA) plus 2% β-mercaptoethanol. The homogenate was incubated at 55 °C for 30-45 min, cooled to room temperature and extracted twice with an equal volume of chloroform. The DNA was precipitated with isopropanol and then redissolved in TE buffer (10 mM Tris, pH 8, 1 mM EDTA) plus RNase A (20 μg/mL). After incubation at 37 °C for 1 hr, the DNA was precipitated with ammonium acetate and ethanol, and then dissolved in TE buffer.

Effector construct preparation

Three types of DNA constructs were used in the transient expression experiments: reporter, effector, and internal control (Shen et al., 1996). Plasmid *HVA22-GUS* (Shen et al., 1993) was used as the reporter construct. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by the constitutive maize ubiquitin promoter (Bruce et al., 1989), was used as an internal control construct to normalize GUS activities of the reporter construct. *LtWRKY21* effector construct was prepared as follows: The *LtWRKY21* effector gene was amplified from a cDNA clone by PCR using two primers: TTAGGCGCGC CATGGCATAT CCTTCTTGG and TTAGGCGCGC CTCACCAATT TCCTCCAGG, which contain an AscI site to facilitate cloning. The PCR product was confirmed by sequencing, and then cloned into the AscI site of the intermediate construct containing the *UBI* promoter and *NOS* terminator (Zhang et al., 2004), generating *UBI-LtWRKY21*. The deletion and substitution mutants were prepared by oligonucleotide-directed mutagenesis with the method of Kunkel et al. (1987). Single-stranded DNA from

plasmid *UBI-LtWRKY21* was used as template. The primer CTGGCCATAT TTTCTCTAG GATATCCATCT TTAAC was used to introduce a stop codon (TAG) upstream from the WRKY domain; the primer GCCCTAAGAG GATTAAGGAC TCGAGCCAAT GACGTATCTA CCC was used to mutate the EAR motif of LtWRKY21. The *UBI-ABI5*, *35S-VP1*, and *35S-abi1-1* effector constructs have been described (McCarty et al., 1991; Armstrong et al., 1995; Casaretto and Ho, 2003).

Particle bombardment and transient expression assays

Transformation of barley (*Hordeum vulgare* L.) aleurone cells by particle bombardment was carried out as described previously (Shen et al., 1993). Briefly, de-embryonated half-seeds of Himalaya barley were imbibed for 2.5-3 days before the pericarp and testa were removed. The DNA mixture (in 1:1 molar ratio) of *HVA22-GUS* and *UBI-Luciferase*, along with or without an effector construct, was bombarded into barley embryoless half-seeds (four replicates/test construct). For each bombardment, eight prepared half seeds were arranged in a small circle (about 1.8 cm in diameter) to maximize the bombarded surface area. After bombardment treatments, GUS and luciferase assays were performed as published before (Shen et al., 1996).

Preparation of GFP fusion constructs

and confocal microscopy

The coding region of *LtWRKY21* was inserted into the AscI site of *UBI-GFP* (Zhang et al., 2004) to generate *UBI-GFP-LtWRKK21*. Barley aleurone cells were bombarded with *UBI-GFP-LtWRKY21* fusion constructs. After incubation at 24°C for 24 hr, the aleurone layers were peeled from barley half-seeds and soaked in a 5 μM SYTO17 solution (Molecular Probe, Eugene, OR). The stained samples were observed and images

of GFP fluorescence and SYTO17 staining were obtained simultaneously through a Laser Scanning Microscope (LSM 510; Carl Zeiss, Inc.) with 488-nm excitation and 505-530-nm emission wavelengths for the green fluorescence, and 633-nm excitation and 650-nm emission wavelengths for the red fluorescence in separate channels. The acquired images were processed using Paint Shop Pro 7.

Results

Northern blot analysis of the *LtWRKY21* gene expression in response to ABA treatment

In an effort to identify creosote bush stress response genes, 43 Arabidopsis stress-inducible genes (Seki et al., 2002) were collected and searched against the creosote bush expression sequence tag database. Ten creosote bush genes encoding putative stress-inducible transcription factors of different families were identified (Table 3-1). We focused on WRKY proteins because they regulate plant response to various stresses (Chen and Chen, 2000; Dellagi et al., 2000; Du and Chen, 2000; Eulgem et al., 2000; Hara et al., 2000; Kim et al., 2000; Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001; Yu et al., 2001; Asai et al., 2002; Huang and Duman, 2002), hence likely also mediating ABA responses. One of these genes, *LtWRKY21*, was studied in more detail.

To study the expression pattern of the *LtWRKY21* gene, RNA was isolated from creosote bush seeds without or with different ABA treatments for Northern analyses. The mRNA level of *LtWRKY21* was abundant in the seeds at onset of the ABA treatment. ABA treatment for 12 and 24 hr had little effect on the abundance of *LtWRKY21* mRNA

Arabidopsis	Lt Homolog	E-value
AtbHLH	LtbHLH28	E-34
AtDREB1A	LtDREB1A1	E-22
AtDREB2A	LtDREB2A2	E-19
AtERF	LtERF7	E-26
AtHomeo	LtHomeo35	E-31
AtMYB	LtMYB25	E-27
AtNAC	LtNAC30	E-90
Other	Lt42	E-50
AtWRKY	LtWRKY21	E-60
AtZINC	LtZINC20	E-107

Table 3-1. Putative stress-inducible transcription factors in *Larrea tridentata*.
Forty-three protein sequences of Arabidopsis stress-inducible genes were collected and BLAST was used to search against the *Larrea tridentata* EST database. First column represents Arabidopsis stress-inducible transcription factors. Second column represents the homologous proteins in *Larrea tridentata*. E-value stands for expectation value based on the probability that the level of alignment occurs by chance. The lower the E-value, the higher the similarity.

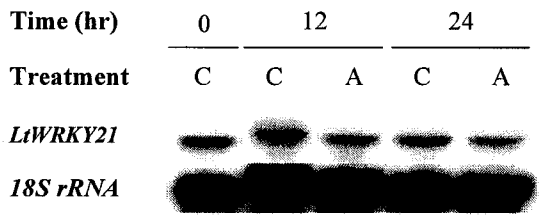

Figure 3-1. Northern analysis of the *LtWRKY21* gene expression in the seeds.
Total RNA was isolated from creosote bush seeds treated with ABA for 12 and 24 h, respectively. The RNA blot was probed with either *LtWRKY21* or *18S rRNA* as a control. C: Control; A: ABA treatment.

level (Figure 3-1), suggesting ABA has little effect on regulating *LtWRKY21* at the transcriptional and post-transcriptional levels.

Protein sequence alignment of LtWRKY21 with its homologues

To identify the open reading frame of *LtWRKY21*, both strands of the cDNA clone was sequenced. The full-length of this cDNA is 945-bp, encoding a protein of 314 amino acid residues with a hydrophilic N-terminus. The deduced amino acid sequence of LtWRKY21 and its homologues, including Arabidopsis WRKY40 (accession number At1g80840) (Eulgem et al., 2000), parsley WRKY4 (accession number AF204925.1) (Cormack et al., 2002), cotton WRKY1 (accession number AY507929.2) (Xu et al., 2004), tobacco WIZZ (accession number AB028022.1) (Hara et al., 2000), and grapevine WRKY4 (accession number AY484579.1) were aligned with the ClustalW program at the default settings. LtWRKY21 shares 44%-48% identity and 57%-62% chemical similarity at the amino acid level with these WRKY proteins (Figure 3-2). The WRKY, zinc finger motif, nucleus targeting signal sequence, and putative leucine zipper domain are highly conserved. Interestingly, among this group of homologues, only LtWRKY21 contains an EAR motif, with a consensus sequence of (L)/(F)DLN(L)/(F)XP. In LtWRKY21, this motif is LDLNLNP (Figure 3-2). EAR resides in the 59-amino acid ERF domain of ERFs (Hao et al., 1998). In Arabidospis there are five ERFs, all of which bind to the GCC box (GCCGCC). AtERF1, AtERF2, and AtERF5 function as activators of GCC box-dependent transcription in Arabidopsis leaves while AtERF3 and AtERF4 act as repressors (Fujimoto et al., 2000). Both AtERF3 and AtERF4 contain the EAR motif, which is also present in other unrelated proteins such as SUPERMAN that

A

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LtWRKY21  --MAYPSWVD TSLDLNLNPL RASNEASPMK QEMEINFMQL GIETPVKQEM QAAEIVKELN  58
AtWRKY40  MDQYSSSLVD TSLDITIG-- -------VTR MRVEEDPP-- ---------- -TSAIVEELN  38
PcWRKY4   MEYSSS-FVD TSLDINAKPL QLF--SETPI QQVQGSFIDF GMR-TSVKEE NNGAIIEELN  56
GaWRKY1   ---MEPAWVD TTLDLNINPC FRT--NKAMK REFEGDVAES -----APVKY ESGVIVEELN  50
NtWIZZ    --MEFTSLVD TSLDISFRPL PVL--DKVLK QEVQSNFTGL SRDNMLVKDE -AGDILEELN  55
VaWRKY4   MAMDSSNWMA ASLDLNANPL RLF--DDTPK KEVQDDFTGL GLKVVSLKEE ETGVIVEKLN  58

LtWRKY21  RVSAENKKIT HMLTGMCESY NTLKCQLEEY MSK------S PEKESSPSKK RKSE------  106
AtWRKY40  RVSAENKKLS EMLTLMCDNY NVLRKQLMEY VNKS---NIT ERDQISPEKK RKSP------  89
PcWRKY4   RVNTENKKLT EMLTVMCENY NTLRNNLMDY MSK----NPE PNLETTTIKK RKSVERSSTT  112
GaWRKY1   RVSAENKKIT EMLTVLCEQY YSLQHQFMEL VNKNPEIETT AAATTSSSKK RKAE------  104
NtWIZZ    RVSSENKKIT EMLTVVCENY NALRNQLMEY MNNQ---NNG VVDDSAGSRK RKAENISNPN  112
VaWRKY4   QVNAENRKLT EMLTVMCENY NALRSHVMEY ISK----NPE R--ESPSSRK RK-AESS---  108

LtWRKY21  ---SGDNSGN VIGNGGNSSS STSDEE--SC KKPKEE-AIK VKTSKIYVRT DKDDKS-ILV  159
AtWRKY40  ---AREDAFS CAVIGGVSSS SSTDQDEYLC KKQREETVVK EKVSRVYYKT EASDTT-LVV  145
PcWRKY4   SCMIKNNASS AKNND-NSSS CSTDEDHNST KKPKEE-HVK AKISRVYFRS EASDTTGLIV  170
GaWRKY1   ---WEDYGAN MIGFSGNTTT SSSDDG--SP KTPKDC--IK PKVSRVQVRT NPSDNS-LIV  156
NtWIZZ    NNNNNKNNNL DIVCGRLSSS SSSD-EESCC KKPREE-HIK TKVSVVSMRT EASDTS-LIV  169
VaWRKY4   ----NNNSNN NGVVG-NSSS SSSDED--SF KKPREE-TIK AKISRVYTRT DASDTS-LVV  159

LtWRKY21  KDGYCWRKYG QKVTRDNPCP RAYFKCSFAP SCPVKKKVQR SAEDQSLLIA TYEGEHNHPQ  219
AtWRKY40  KDGYCWRKYG QKVTRDNPSP RAYFKCACAP SCSVKKKVQR SVEDQSVLVA TYEGEHNHPM  205
PcWRKY4   KDGYCWRKYG QKVTRDNPSP RAYFKCSYAP TCPVKKKVQR SIDDQSILVA TYEGEHNHPH  230
GaWRKY1   RDGYCWRKYG QKVTRDNPCP RAYFKCSFAP SCPVKKKVQR SAEDPSILVA TYEGEHNHHE  216
NtWIZZ    KDGYCWRKYG QKVTRDNPSP RAYFRCSFAP GCPVKKKVQR SIEDQSVVVA TYEGEHNHPV  229
VaWRKY4   KDGYCWRKYG QKVTRDNPSP RAYFKCSFAP SCPVKKKVQR SVEDQSILVA TYEGEHNHPH  219

LtWRKY21  -PSQIDVTSS SS--------- RPVA---LSP LPGSACTGSS ---------- -AVTATVELT  256
AtWRKY40  -PSQIDSNNG ---------LN RHISHGGSAS TPVAANRRSS -------LTV P--VTTVDMI  247
PcWRKY4   -PAKLEPNDS SS--------N RCVT---PAS LRCSTSLNLS ---------A P--TLTLDMT  268
GaWRKY1   HHRSPPAEIS LN------SN NNTPSSNTGS GPVSSAPTKA ---------- LASTVTLELL  260
NtWIZZ    NPSKPEAAAG TATSTGSRLN VRTIGGTTAS VPCSTTLNSS GPTITLDLTE PTTVAKGDIM  289
VaWRKY4   -HGRIEP-TS GA--------N RSVN---LGS VPCASSLSSS ---------G P--AITLDLT  256

LtWRKY21  KSKSCQNTIF RP--------- KVETPDFKDY LVEQMAFSIT KDPNFTGAFA AAISGRINNQ  308
AtWRKY40  ESKKVTSPTS ---------- RIDFPQVQKL LVEQMASSLT KDPNFTAALA AAVTGKLYQQ  297
PcWRKY4   KSKKSITEDA NKKATTK--- KIDSPEFQQF LVDQMASSLT KDPSFKAALA AAISCKILQQ  325
GaWRKY1   QPAGLGGDET ER-----AAL QIDAPAIQQI LVHQMAASLS RDPNFTAALA AAISGRAV--  313
NtWIZZ    KMSSSISPTG GSSQRTTEGD HYSRPEFQQF LIEQMASSLT KDPSFKAALA AAISCKILQH  349
VaWRKY4   KPKSNSDAKA SK--------- ---SPELHHF LVEQMASSLT KDPSFKAALA AAISGRILHH  305

LtWRKY21  R-PGGNW-- 314
AtWRKY40  N-HTEK--- 302
PcWRKY4   N-QQRNGEH 333
GaWRKY1   --------- 313
NtWIZZ    NNQTSRW-- 356
VaWRKY4   N-QTEKW-- 311
```

B

Figure 3-2. Protein sequence alignment of LtWRKY21 with its homologues.

Figure 3-2. (continued)
A, The alignment of LtWRKY21 with its homologues. The deduced amino acid sequences were aligned by using ClustalW. Identical residues are shaded in black and residues chemically similar are in gray. The putative EAR motif (LDLNLNP), nuclear localization signal {K(RK)X(RK)}, and WRKY amino acid residues are labeled with rectangles, and amino acids residues potentially interacting with zinc ligands are pointed to with arrows.
B, The schematic structure of the LtWRKY21 protein showing the EAR motif, nuclear localization signals (N), WRKY motif, and zinc finger motif (Zn-F). It is not drawn to scale.

regulates flowering (Hiratsu et al., 2002). A related motif, LXLXLX, is necessary for the activity of some Aux/IAA repressors mediating auxin signaling (Tiwari et al., 2004).

The GFP:LtWRKY21 fusion protein

was localized in nuclei

To examine the subcellular localization of the LtWRKY21 protein, we used GFP as a reporter and a red fluorescent nucleic acid stain, SYTO17, for nuclear localization. *GFP* was fused in frame to the 5' end of the *LtWRKY21* coding sequence. The *UBI-GFP* or *UBI-GFP-LtWRKY21* plasmid was introduced into the barley aleurone cells by particle bombardment, and the GFP fluorescence was visualized using confocal microscopy. In control, GFP fluorescence was observed throughout the cells (Figure 3-3 A). In contrast, GFP-LtWRKY21 fusion proteins were localized exclusively in the nuclei (Figure 3-3 C), as confirmed by SYTO 17 staining (Figure 3-3 B and D).

LtWRKY21 transactivates the *HVA22* promoter

To test the function of LtWRKY21 on ABA signal transduction pathways, we used a reporter construct that contains a *GUS* reporter gene driven by the promoter of *HVA22*, an ABA-responsive gene in barley (Shen et al., 1996). The effector construct, *UBI-LtWRKY21,* was co-introduced to evaluate its effect on ABA signaling. As shown in Figure 3-4, a very low level of GUS activity was observed in the absence of ABA. The exogenous ABA (20 μM) treatment resulted in a 30-fold enhancement of GUS activity over that found with the ABA-untreated control. Expression of *UBI-LtWRKY21* resulted in a 7-fold induction in the absence of ABA. Interestingly, *LtWRKY21* synergistically interacted with ABA to transactivate the expression of the *HVA22* promoter, leading to a 47-fold induction.

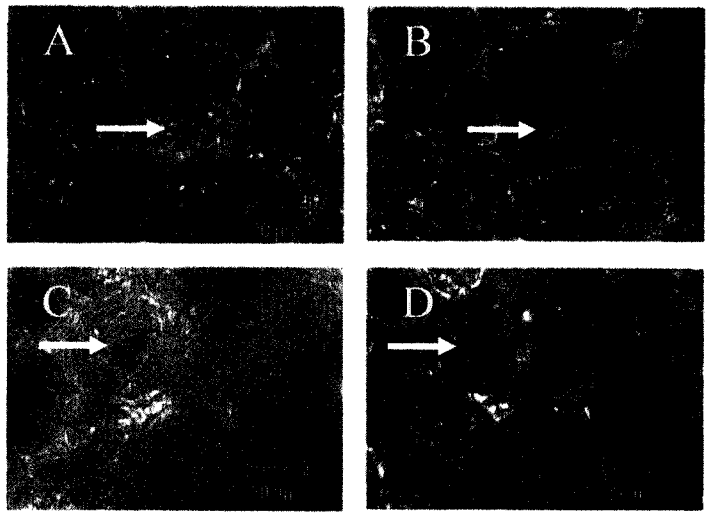

Figure 3-3. The GFP:LtWRKY21 fusion protein was localized in the nuclei.
A, The GFP fluorescence from cells bombarded with the *Ubi-GFP* construct. C, The GFP fluorescence from *Ubi-GFP-LtWRKY21*. B and D, Nuclei in the same cells as in A and C that were stained with SYTO17, respectively. The bars represent 20 μm.

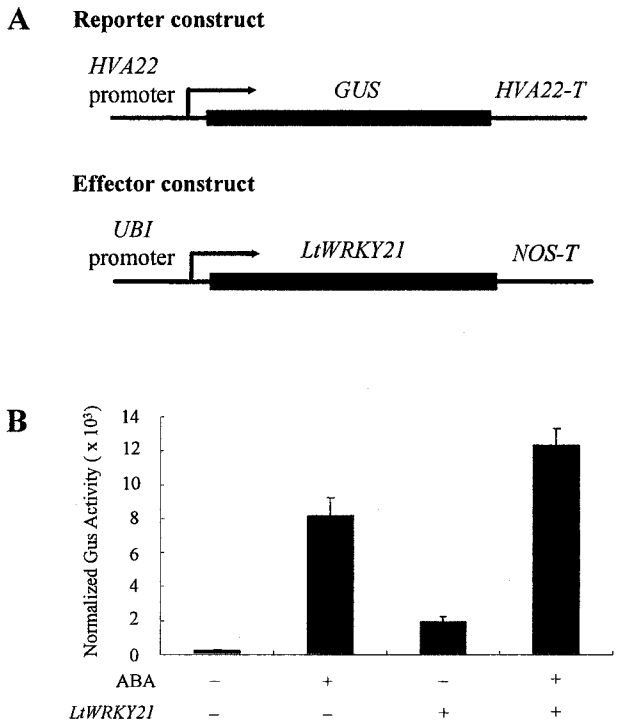

Figure 3-4. LtWRKY21 synergistically interacts with ABA to transactivate the expression of the *HVA22* promoter.

A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. *HVA22* is an ABA-responsive gene. *UBI* promoter is from the maize ubiquitin gene. *HVA22-T* represents the terminator sequence of *HVA22*.

B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (-) the effector construct (*UBI-LtWRKY21*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seed with (+) or without (-) 20 μM ABA. Data are the means ± SE of four replicates.

The activating effect of LtWRKY21 on the expression of the *HVA22* promoter is dosage-dependent

The activating effect of LtWRKY21 was further confirmed by a dosage experiment, in which the amount of reporter plasmid was always constant, whereas that of the effector varied from 0 to 100% (Figure 3-5). As expected, when the *HVA22-GUS* construct was transformed alone, the treatment with 20 μM ABA led to 31-fold induction of *HVA22-GUS*. The expression of the *HVA22-GUS* in response to ABA treatment increased gradually with the increment of the *UBI-LtWRKY21* effector construct. When the relative amount of effector to reporter was 25% and 50%, the *GUS* expression, in reference to the control (no ABA, no effector), was induced by a factor of 57 and 62, respectively. The GUS activities increased to 74-fold and reached a plateau with the higher amounts of the effector construct (75% or 100%). These data indicated that under these conditions, LtWRKY21 is a transcriptional activator of ABA signaling. To our knowledge, this is the first report of such activity by a WRKY protein.

The EAR motif at the N terminus and C-terminal region containing the WRKY domain of LtWRKY21 are essential for its transactivating activity

To further demonstrate the specificity of LtWRKY21 on activating ABA induction, mutagenesis experiments were carried out to try to change its activity. A stop codon was introduced at amino acid 165 (mutant 1), which is upstream from the WRKY domain. The purpose was to produce a truncated protein missing the WRKY domain and the rest of the C-terminal region. As shown in Figure 3-6, the expression of the *HVA22* promoter increased 35-fold after ABA treatment. The wild-type *LtWRKY21* gene alone

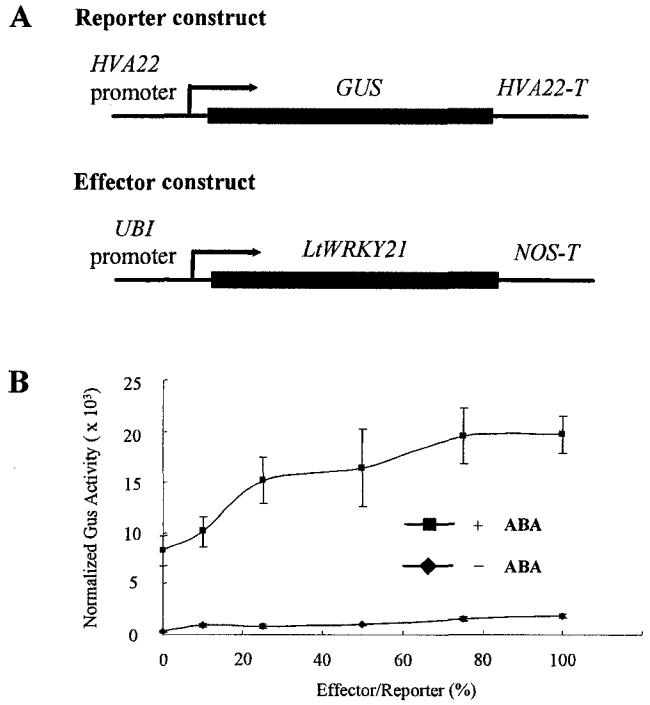

Figure 3-5. The synergistic effect of LtWRKY21 is dosage-dependent.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. The annotations are the same as in Figure 3-4.
B, The effector construct, *UBI-LtWRKY21*, was co-bombarded into barley half-seeds along with the reporter construct, *HVA22-GUS*, and the internal control construct, *UBI-luciferase*. The amount of reporter and internal control plasmid DNA was always constant (1.43 μg/shot), whereas that of the effector varied with respect to the reporter as shown in the x axis. 100% means the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seeds with (+) or without (-) 20 μM ABA. Data are the means ± SE of four replicates.

activated the expression of the *HVA22* promoter by 5-fold. ABA treatment along with *LtWRKY21* expression resulted in a 60-fold induction. However, when the LtWRKY21 mutant 1 was co-expressed, the induction of GUS was 28-fold, which is comparable with that of ABA treatment alone (35-fold, Figure 3-6).

Interestingly, the EAR motif (Figure 3-2) is reported to be present in transcriptional repressors only. The presence of such a motif in LtWRKY21 (a clear activator under the experimental conditions) is intriguing. Thus, the DLN residues at the 12^{th}-14^{th} positions were mutated to ARV (mutant 2, Figure 3-6). In the presence of ABA, wild type LtWRKY21 activated *HVA22-GUS* expression by 60-fold. However, mutation of the EAR motif decreased the induction level to 21-fold (Figure 3-6). In summary, the results presented in Figure 3-6 suggested that the EAR domain and C-terminal region of LtWRKY21 are necessary for its transactivating activity in ABA signaling.

LtWRKY21 interacts synergistically with ABA and VP1

to transactivate the expression of the *HVA22* promoter

VP1 encodes a transcription activator that up-regulates ABA responsive genes (McCarty et al., 1991). Figure 3-7 shows the results of a functional interaction of LtWRKY21 with VP1 on regulating ABA response. Expression of *VP1* promoted a small induction (2-fold) of *HVA22-GUS*, in the absence of ABA. ABA treatment along with *VP1* expression resulted in a 16-fold induction. Interestingly, expression of *LtWRKY21* also led to a 2-fold induction. ABA treatment along with *LtWRKY21* expression led to a 53-fold induction. Co-expression of *LtWRKY21* and *VP1* resulted in a 21-fold induction, which is even higher than ABA treatment along with *VP1* expression (16-fold). ABA

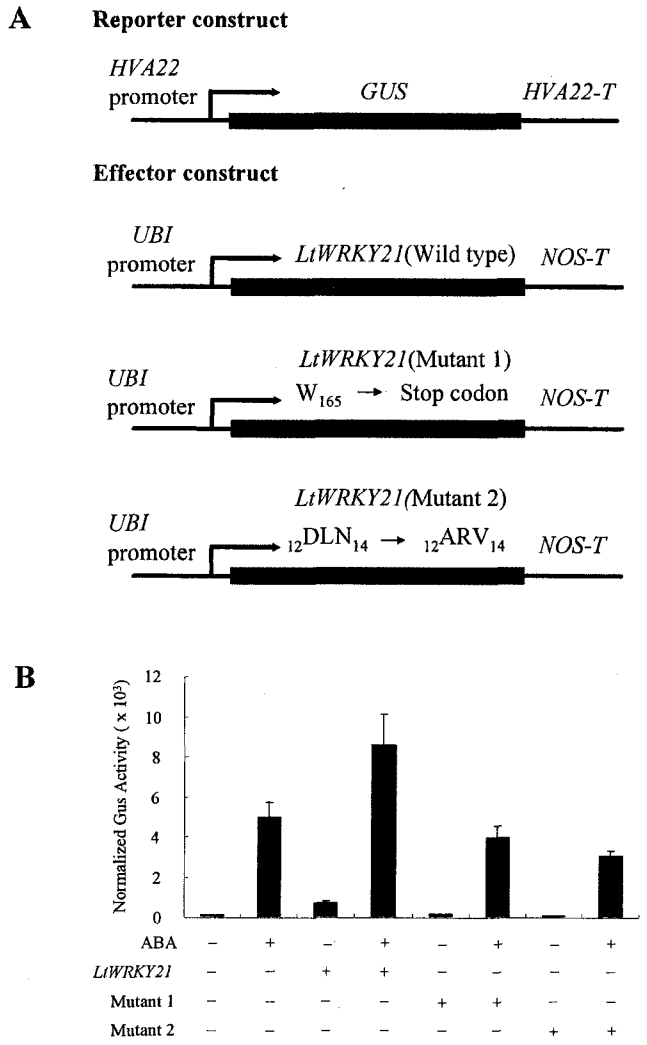

Figure 3-6. The EAR motif and C-terminal region containing the WRKY domain of LtWRKY21 are essential for its transactivating activity.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. The mutant 1 was made by introducing a stop codon at amino acid 165, which is a tryptophan in the wild type protein. The mutant 2 was made by changing residues 12-14 from aspartate-leucine-asparagine to alanine-arginine-valine.
B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (-) the effector constructs (*UBI-LtWRKY21*, *UBI-LtWRKY21* mutants) by using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seeds with (+) or without (-) 20 μM ABA. Data are the means ± SE of four replicates.

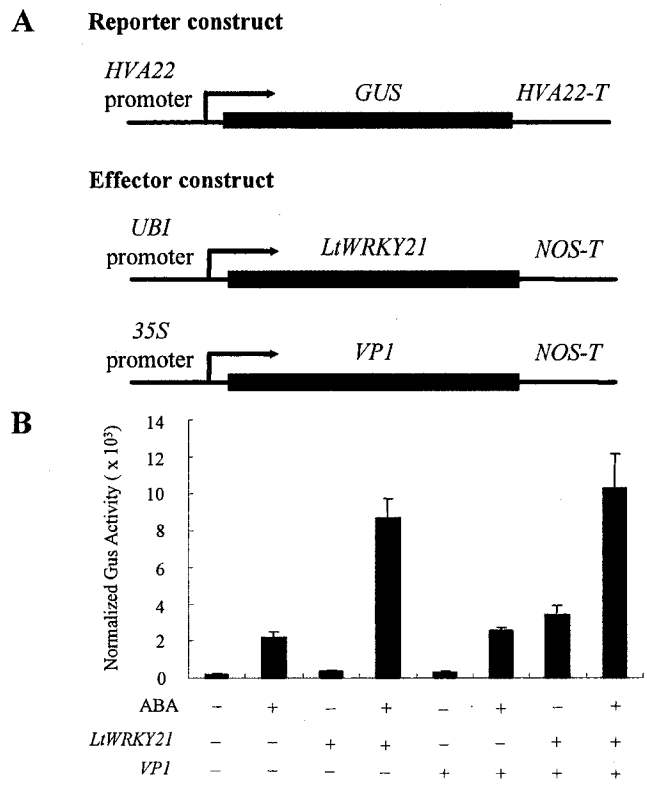

Figure 3-7. LtWRKY21 interacts synergistically with ABA and VP1 to transactivate the expression of the *HVA22* promoter.

A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. *VP1* is the *Viviparous 1* gene from maize. The *35S* promoter is from the cauliflower mosaic virus.

B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (-) the effector constructs (*UBI-LtWRKY21* or *35S-VP1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seeds with (+) or without (-) 20 μM ABA. Data are the means ± SE of four replicates.

treatment along with *VP1* and *LtWRKY21* co-expression gave a 62-fold induction (Figure 3-7).

LtWRKY21 interacts synergistically with ABA and ABI5

to transactivate the expression of the *HVA22* promoter

ABI5 encodes a transcription activator on the ABA pathway (Finkelstein and Lynch, 2000). As reported before (Casaretto and Ho, 2003), expression of *ABI5* promoted a small induction of *HVA22-GUS* in the absence of ABA (Figure 3-8). A similar level of induction (5-fold) was achieved with the expression of *LtWRKY21*. Like *ABI5*, *LtWRKY21* synergistically interacted with ABA to induce the expression of *HVA22-GUS*. Co-expression of *LtWRKY21* and *ABI5* resulted in a 21-fold induction, which is similar to ABA treatment (22-fold). The highest level of induction was achieved with the co-expression of *LtWRKY21* and *ABI5* in the presence of ABA (Figure 3-8).

1-Butanol blocks the synergistic effect

of ABA and LtWRKY21

Phospholipase D (PLD) is a phosphodiesterase that hydrolyzes phospholipids to produce phosphatidic acid. PLD has been demonstrated to be up-regulated by ABA (Ritchie and Gilroy, 1998). As reported (Gampala et al., 2001), 1-butanol, a specific inhibitor of PLD, inhibits ABA-inducible gene expression. Indeed, ABA induction of *HVA22-GUS* dropped from 32-fold to 2-fold (Figure 3-9). In this experiment, *LtWRKY21* expression led to 4-fold induction in the absence of ABA. 1-Butanol treatment decreased the induction to 1-fold. This chemical also blocks the synergistic interaction of ABA and LtWRKY21, decreasing the induction level from 61-fold to 10-fold only.

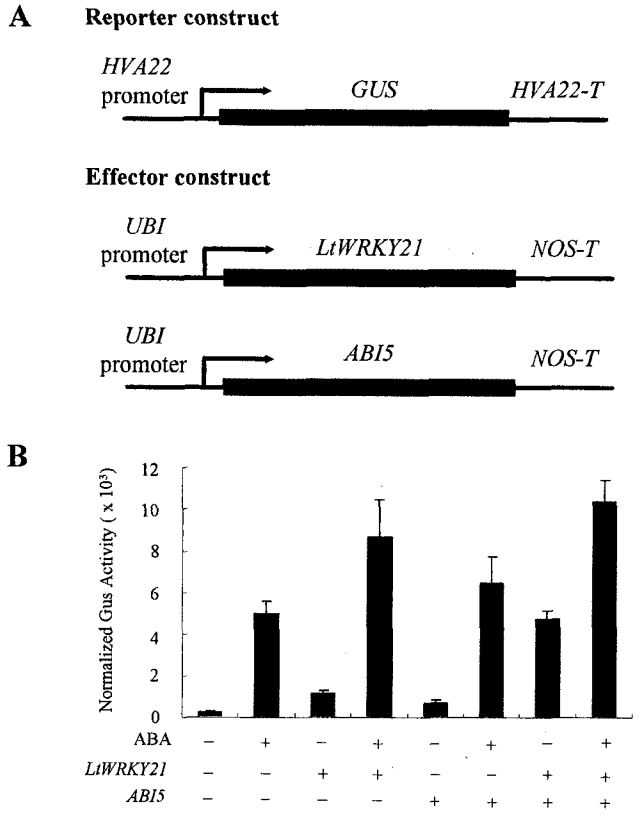

Figure 3-8. LtWRKY21 interacts synergistically with ABA and ABI5 to transactivate the expression of the *HVA22* promoter.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. *ABI5* is the *ABA Insensitive 5* gene from barley.
B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley half-seeds either with (+) or without (-) the effector constructs (*UBI-LtWRKY21* or *UBI-ABI5*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seed with (+) or without (-) 20 μM ABA. Data are means ± SE of four replicates.

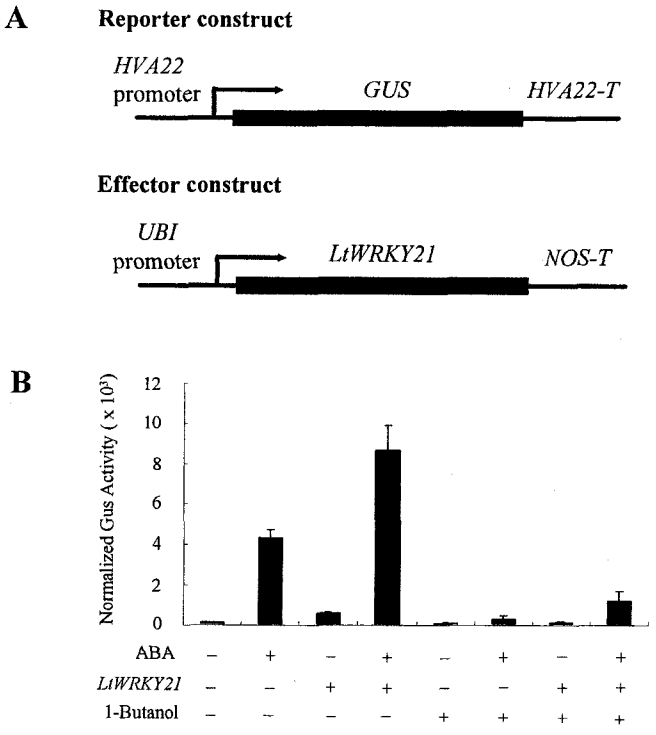

Figure 3-9. 1-Butanol blocks the synergistic effect of ABA and LtWRKY21.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. Annotations are the same as in Figure 3-4.
B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombardment into barley half-seeds either with (+) or without (-) the effector construct (*UBI-LtWRKY21*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seed with (+) or without (-) 1% 1-butanol and 20 μM ABA. Data are means ± SE of four replicates.

ABI1 blocks the synergistic effect of ABA and LtWRKY21

ABI1 encodes a protein phosphatase 2C, a negative regulator of ABA signaling (Leung et al., 1994; Meyer et al., 1994). A mutation of this gene, *abil-1*, causes a reduction of phosphatase activity (Sheen, 1998) and this mutation is dominant negative in blocking ABA responses in Arabidopsis (Meyer et al., 1994) and barley (Shen et al., 2001). Indeed, abi1-1 prevented the ABA induction of *HVA22-GUS* from 14-fold to the background level in this experiment (Figure 3-10). In the absence of ABA, abi-1 did not appear to affect the activity of LtWRKY21. However, the synergistic effect of ABA and LtWRKY21 was essentially abolished by abi1-1, with the induction level dropped from 53-fold to 3-fold.

Interaction among LtWRKY21, VP1, ABI5, and ABI1

Because abi1-1 functions upstream of ABI5 and VP1 in modulating the ABA signaling (Shen et al., 2001; Casaretto and Ho, 2003), we studied the effect of co-expressing *abi1-1*, *LtWRKY21*, *VP1*, and *ABI5* on regulating the *HVA22* promoter. As shown in Figure 3-11, co-expression of *LtWRKY21*, *VP1*, and *ABI5* led to 122-fold induction of *HVA22-GUS,* which is much higher than that of ABA treatment alone (33-fold in this experiment). ABA treatment did not further enhance the induction of the *HVA22* promoter by co-expression of *LtWRKY21*, *VP1*, and *ABI5*. Interestingly, abi-1 did not block the synergistic effect of LtWRKY21, VP1, and ABI5 on inducing the *HVA22* promoter, either in the absence or in the presence of ABA (Figure 3-11).

Discussion

The creosote bush survives exceptionally well in the arid desert where rainfall

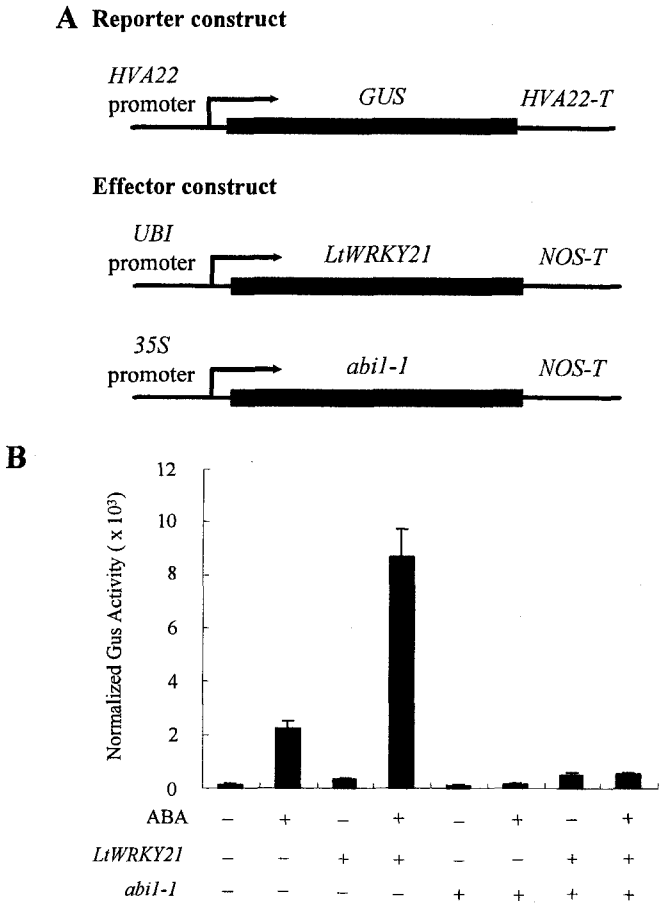

Figure 3-10. ABI1 blocks the synergistic effect of ABA and LtWRKY21.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment. The *35S* promoter is from the cauliflower mosaic virus. a*bi1-1* is the dominant mutant gene of *ABI1* from Arabidopsis.
B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombardment into barley half-seeds either with (+) or without (-) the effector constructs (*UBI-LtWRKY21* or *UBI-abi1-1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seed with (+) or without (-) 20 μM ABA. Data are means ± SE of four replicates.

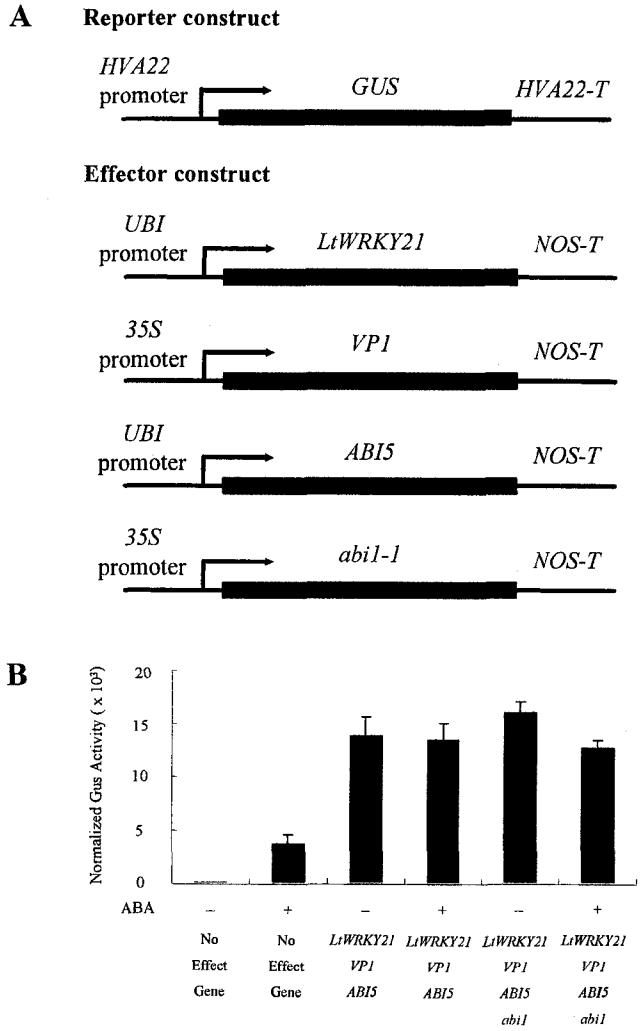

Figure 3-11. Interaction among LtWRKY21, VP1, ABI5, and ABI1.
A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment.
B, The reporter construct, *HVA22-GUS*, and the internal construct, *UBI-luciferase*, were co-bombardment into barley half-seeds either with (+) or without (-) the effector constructs (*UBI-LtWRKY21*, *UBI-ABI5*, *35S-VP1*, or *35S-abi1-1*) by using the same amount of effector and reporter constructs (1.43 μg/shot). GUS activity was normalized in every independent transformation relative to the luciferase activity. The bars indicate GUS activities ± SE after 24 h of incubation of the bombarded half-seed with (+) or without (-) 20 μM ABA. Data are means ± SE of four replicates.

events only occur a few months each year. Understanding the molecular mechanism underlying its resistance to the drought is biologically and agriculturally important. Because transcription factors are master switches of gene regulation, alternations in their expression levels, activities, and/or functions, as opposed to those of structural genes, are more likely to have broader impacts on the resistance of plants to environmental stresses and hence on the speciation of creosote bush. Therefore, we focused on the drought stress-induced transcription factors. In Arabidopsis, there are 43 stress-induced transcription factor genes that have been identified, corresponding to 11% of all stress-inducible genes (Seki et al., 2002). Among these stress-inducible proteins, there are six DREBs, two ERFs, 10 zinc fingers, four WRKYs, three MYBs, two bHLHs, four bZIPs, five NACs, and three homeodomain transcription factors (Seki et al., 2002). The protein sequences of these 43 transcription factors were collected and searched against the translated creosote bush expression sequence tag database. This effort led to the identification of ten putative stress-inducible transcription factors of different families in creosote bush. One of the ten genes is *LtWRKY21*, which is highly expressed in creosote bush seeds (Figure 3-1). LtWRKY21 contains a WRKY motif, a zinc finger motif, two nucleus targeting signal sequences, and a putative leucine zipper domain (Figure 3-2). Consistent with its role as a transcription factor, the GFP-LtWRKY21 fusion proteins were targeted to nuclei (Figure 3-3).

The homologues of LtWRKY21 are present in many other plant species such as Arabidopsis, parsley, cotton, tobacco, and grapevine, and plays a variety of roles. *AtWRKY40* is a drought and ABA response gene; *GaWRKY1*, *PsWRKY4*, and *VaWRKY4* are involved in pathogen defense; and *NtWIZZ* is a wounding-inducible gene. The goal of

this study is to address the function of *LtWRKY21* in ABA responses. It has been demonstrated that the ABA signaling machinery is conserved among higher plant species and even bryophytes (Knight et al., 1995; Shen et al., 2001; Gampala et al., 2002). For example, the promoter of *EM*, a wheat ABA-responsive gene, responds to osmotic stress and ABA in moss, and the moss transcription factors can bind to the *Em* promoter (Knight et al., 1995). Transcription factors from maize and Arabidopsis function well in barley aleurone layer (Shen et al., 2001) or rice protoplasts (Gampala et al., 2002) to regulate the expression of the wheat *Em*, Arabidopsis *AtEM6*, bean *β-Phaseolin*, and barley *HVA1* and *HVA22* promoters. Therefore, we studied the function of LtWRKY21 in barley aleurone cells and demonstrated that it acted as an activator of ABA signaling (Figures 3-4 and 5). Unlike *AtWRKY40*, *LtWRKY21* expression did not appear to be affected by external ABA applications in the seeds (Figure 3-1). Similar results were observed for other genes involved in ABA signaling such as *VP1/ABI3* and *ABI4* genes (Parcy et al., 1994; Hollung et al., 1997; Finkelstein et al., 2002). It is speculated that LtWRKY21 activity may be regulated by post-translational modifications and/or interaction with other regulators in response to ABA.

Six classes of transcription factors have been demonstrated by genetic analyses to be essential for ABA responses: VP1/ABI3, ABI4, ABI5, LEC1, LEC2, and FUS3 (Finkelstein et al., 2002). We studied the interactions of LtWRKY21 with VP1 and ABI5. Like VP1 and its Arabidopsis orthologue ABI3, ABI5 and other bZIP transcription factors function as an activator of ABA signaling (McCarty et al., 1991; Giraudat et al., 1992; Hobo et al., 1999; Choi et al., 2000; Finkelstein and Lynch, 2000; Shen et al., 2001; Casaretto and Ho, 2003; Suzuki et al., 2003). Excitingly, LtWRKY21 synergistically

interacted with VP1 (Figure 3-7) and ABI5 (Figure 3-8) in regulating ABA responses. VP1 has been shown to potentiate ABA-inducible gene expression by forming a DNA-binding complex with bZIP, 14-3-3, ring (C3HC3-type) zinc finger proteins, and RNA polymerase II subunit RPB5 (Schultz et al., 1998; Hobo et al., 1999; Jones et al., 2000; Kurup et al., 2000). Our data suggest the WRKY protein may be a new component of this complex.

WRKY proteins can bind specifically to the W box that contains a TGAC core (Ishiguro and Nakamura, 1994; Rushton et al., 1995; Zhang et al., 2004). The putative W box has been found in the promoter regions of *HVA22* (Shen et al., 1993) and *ABF* (Choi et al., 2000). However, the 49-bp promoter fragment in the reporter construct of this study does not include this W box. Similarly, this promoter does not contain the Sph I element that is bound by the C-terminal B3 domain of VP1 (Suzuki et al., 1997). Instead, only two elements are present in this promoter fragment: the ABRE that is bound by ABI5 or its related bZIP proteins (Kim and Thomas, 1998; Hobo et al., 1999; Finkelstein and Lynch, 2000; Casaretto and Ho, 2003) and CE1 that is bound by a APETALA2 domain-containing transcription factor ABI4 (Finkelstein et al., 1998; Niu et al., 2002). It should be noted that the full-length VP1 does not bind DNA specifically *in vitro*, suggesting that it interacts with other proteins that mediate DNA binding (Suzuki et al., 1997). Our preliminary data suggest that LtWRKY21 did not bind to the promoter sequence of the *HVA22-GUS* reporter construct used in this study (X. Zou and J.Q. Shen, unpublished result). Therefore, we suggest that LtWRKY21 regulates the *HVA22* promoter as a non-DNA-binding component of the transcription complex mentioned above.

Deletion and substitution studies of LtWRKY21 should lead to the identification of domains and residues that are essential for the interaction of LtWRKY21 with the remaining components of the transcription complex. Figure 3-6 showed that the C-terminal region, which contains the WRKY domain and zinc-finger motif, was required for LtWRKY21 transactivating the expression of the *HVA22* promoter. Interestingly, LtWRKY21 also contains the EAR motif, which is necessary for the repression function of AtERF3, AtERF4, and their orthologues in ethylene signaling of Arabidopsis (Fujimoto et al., 2000), wheat, and petunia plants (Ohta et al., 2001). Yet the EAR motif was essential for the transactivation activity of LtWRKY21. These data suggest that the same motif might play opposite roles in different hormonal signaling pathways and it is possible that LtWRKY21 is also involved in ethylene signaling. Transcription factors with dual activities have been found in plants. For instance, maize *VP1* promotes the ABA induction pathway yet inhibits the GA induction pathway (Hoecker et al., 1995; Shen et al., 2001). Arabidopsis *WRKY6* acts as a negative regulator of its own and *WRKY42* expression; on the other hand, it positively influences the senescence- and pathogen-defense-associated *PR1* promoter activity (Robatzek and Somssich, 2002).

Several groups of proteins such as G proteins, phospholipases, protein kinases, and protein phosphatases are involved in the early events of ABA signaling (Merlot et al., 2001; Wang et al., 2001; Hallouin et al., 2002; Lu et al., 2002). Phospholipase C and D produce inositol 1,4,5-trisphosphate and diacylglycerol or phosphatidic acid and the head group, respectively. These products of phospholipases act as secondary messengers in ABA signaling (Sanchez and Chua, 2001; Hallouin et al., 2002). The application of phosphatidic acid to barley aleurone inhibits α-amylase production and induces an ABA-

inducible amylase inhibitor and RAB (response to ABA) protein expression (Ritchie and Gilroy, 1998). On the other hand, 1-butanol, a specific inhibitor of PLD (Munnik et al., 1995), inhibits the accumulation of the RAB protein (Ritchie and Gilroy, 1998). 1-Butanol inhibits the transactivation of VP1 or ABI5 on ABA response promoters (Gampala et al., 2001). Together, these data suggest that PLD is involved in ABA signaling. Figure 3-9 shows that the synergistic effect of LtWRKY21 and ABA was also inhibited by 1-butanol. ABI1 and its dominant negative mutant abi1-1 act as a negative regulator of ABA signaling in Arabidopsis (Gosti et al., 1999), barley (Shen et al., 2001) and rice (Gampala et al., 2001). The ABI1-1 inhibitory effect is able to overcome the transactivation effect of VP1 or ABI5 in ABA signaling (Gampala et al., 2001; Shen et al., 2001), but it does not decrease the synergistic effect of VP1 and ABI5 on ABA induction, indicating that abi1-1 acts upstream of ABI5 in the ABA up-regulatory pathway (Casaretto and Ho, 2003). Here, we showed that abi1-1 inhibited the synergistic effect of ABA and LtWRKY21 (Figure 3-10), but it had little effect when *VP1*, *ABI5*, and *LtWRKY21* were co-expressed (Figure 3-11). Therefore, we suggest that LtWRKY21 may form a complex with VP1, ABI4, and ABI5 to control ABA response, and this complex functions downstream of ABI1 in ABA signaling. Experiments are ongoing to further address this question.

Acknowledgments

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CHAPTER 4

INTERACTIONS OF TWO TRANSCRIPTIONAL REPRESSORS AND TWO TRANSCRIPTIONAL ACTIVATORS IN MODULATING GIBBERELLIN SIGNALING IN ALEURONE CELLS

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Summary

Gibberellins (GAs) regulate many aspects of plant development such as germination, growth, and flowering. HvWRKY38, a WRKY transcription factor from barley, was investigated in this work to address its functions in GA signaling. Transient expression of *HvWRKY38* by particle bombardment specifically represses the GA-inducible *Amy32b* α-amylase promoter but not the ABA-inducible *HVA22* and *HVA1* promoters in barley aleurone cells. The C-terminal region containing the WRKY domain

of HvWRKY38 is essential for its repressing activity. Consistent with its role as a transcriptional repressor, double-stranded RNA interference (RNAi) of *HvWRKY38* results in de-repression of *Amy32b* expression in the absence of GA. To address how HvWRKY38 suppresses GA induction, we studied the interaction of HvWRKY38 with other transcription factors involved in GA signaling, namely, BPBF (a DOF protein), another transcriptional repressor, and two transcriptional activators HvGAMYB (a MYB protein) and SAD (a DOF protein). Data indicate that HvWRKY38 blocks inductive activities of SAD and HvGAMYB when either of these proteins is present individually. However, SAD and HvGAMYB together overcome the inhibitory effect of HvWRKY38. Interestingly, co-expression of HvWRKY38 and BPBF dramatically reduces the synergistic effect of SAD and HvGAMYB on inducing the *Amy32b* promoter in the absence of GA. The physical interaction of HvWRKY38 and HvGAMYB was revealed in the nuclei of barley aleurone cells using bimolecular fluorescence complementation (BiFC) assays. Moreover, HvWRKY38 blocks GA-induced expression of *Amy32b* by interfering with the binding of HvGAMYB to the *cis*-acting elements in the *α-amylase* promoter. These data suggest that the expression of *Amy32b* is modulated by protein complexes containing activators and repressors, respectively. The ratio of repressors (HvWRKY38 and BPBF) to activators (HvGAMYB and SAD) controls the expression level of *Amy32b*.

Introduction

Gibberellins (GAs) control many plant developmental processes, such as germination, growth, and flowering (Olszewski et al., 2002; Sun and Gubler, 2004). During

germination of cereal grains, GA is secreted from embryos into aleurone cells to promote the expression of hydrolytic enzymes, such as α-amylases, which degrade stored starches within the endosperm for seed germination and post-germination growth (Lovegrove and Hooley, 2000; Ritchie and Gilroy, 1998).

The GA signal is perceived by receptors, such as GID1 (gibberellin insensitive dwarf 1) in rice and AtGID1a, AtGID1b, and AtGID1c in Arabidopsis (Nakajima et al., 2006; Ueguchi-Tanaka et al., 2005). The activated GID1 binds to the negative regulator, such as the DELLA protein RGA, triggering the degradation of this DELLA protein by the 26S proteasomes (Itoh et al., 2003; Sun and Gubler, 2004; Griffiths et al., 2006; Nakajima et al., 2006). Studies of constitutive activated GA signaling mutants reveal that Arabidopsis *SPY* and its barley ortholog *HvSPY* encode a Ser/Thr O-linked N-acetylglucosamine (*O*-GlcNAc) transferase (OCT), which is a repressor of GA signaling (Jacobsen et al., 1996; Robertson et al., 1998). SPY increases the activity of DELLA proteins such as Arabidopsis RGA and rice SLR1, probably by attaching a GlcNAc moiety to the Ser/Thr residues of a targeted protein (Shimada et al., 2006; Silverstone et al., 2007). SPY also physically interacts with two transcriptional repressors of α-amylase expression in aleurone cells (Robertson, 2004). Studies of unresponsive GA signaling mutants have identified positive regulators in GA signaling such as Arabidopsis *SLY1* and *PICKLE* (Ogas et al., 1997; Steber et al., 1998). The *SLY1* encodes an F-box protein, a component of the SCF(SLY1) E3 ubiquitin ligase that targets the DELLA protein for degradation (McGinnis et al., 2003; Griffiths et al., 2006). The *PKL* gene encodes a CHD3 chromatin-remodeling factor, which negatively regulates embryo-specific gene transcription (Henderson et al., 2004). It is also known that the activation of G-proteins

(Hooley, 1998) and enhancement of cytoplasmic cGMP (Penson et al., 1996) and Ca^{2+} concentrations (Gilroy and Jones, 1992) follow GA treatment, although it is not entirely clear how they are linked to upstream and downstream events.

Several types of *cis*-acting elements for the GA response of high-pI and low-pI α-amylase genes have been defined (Skriver et al., 1991; Gubler and Jacobsen, 1992; Lanahan et al., 1992; Rogers and Rogers, 1992; Rogers et al., 1994; Tanida et al., 1994). These motifs interact with various transcription factors controlling seed germination. In the low-pI α-amylase promoter, *Amy32b*, five elements, namely O2S/W-box, pyrimidine (Pyr) box, GA response element (GARE), amylase box (Amy), and down-stream amylase element (DAE), are essential for a high level of GA-induced expression (Lanahan et al., 1992; Rogers and Rogers, 1992; Rogers et al., 1994; Gómez-Cadenas et al., 2001). Each of these elements may be bound by one or more transcription factor(s) of R2R3MYB, R1MYB, DOF, and zinc-finger protein families (Gubler et al., 1995; Raventós et al., 1998; Diaz et al., 2002; Isabel-LaMoneda et al., 2003; Washio, 2003; Mare et al., 2004; Peng et al., 2004; Zhang et al., 2004; Rubio-Somoza et al., 2006a; Rubio-Somoza et al., 2006b; Xie et al., 2006; Moreno-Risueno et al., 2007). However, it still remains unclear how these repressors and activators interact with each other in regulating gene expression. WRKY proteins have been shown to be involved in plant responses to biotic and abiotic stresses, as well as in anthocyanin biosynthesis, senescence, and trichome development (Eulgem et al., 2000; Ulker and Somssich, 2004). Herein, we report that the *HvWRKY38* gene encodes a negative transcription factor that is specific to the GA pathway. HvWRKY38 physically and functionally interacts with another transcriptional repressor,

BPBF, and two transcriptional activators, DOF and GAMYB, to regulate transcription of the *Amy32b* α-amylase promoter in barley aleurone cells.

Experimental procedures

RNA isolation and RT-PCR

Total RNA was isolated from barley aleurone cells with the LiCl precipitation method as described (Zhang et al., 2004). The first-strand cDNAs were synthesized using ImProm-II reverse transcriptase in a 50-μl reaction containing 2.5 μM oligo(dT) primers, 2.5 μM random heximer, and 2.5 μg of total RNA, according to the manufacturer's instructions. Five microliters of each reaction mixture was used as a template for PCR amplification in a 25-μl mixture containing 1.5 μM $MgCl_2$, 200 μM dNTPs, 5% dimethyl sulfoxide, 2.5 units of Taq DNA polymerase, and 0.4 μM primers.

Effector construct preparations

Three types of DNA constructs were used in the transient expression experiments: reporter, effector and internal control. Plasmid *Amy32b-GUS* (Lanahan et al., 1992), *HVA1-GUS*, and *HVA22-GUS* (Shen et al., 1993) were used as the reporter constructs. Plasmid pAHC18 (*UBI-Luciferase*), which contains the luciferase reporter gene driven by the constitutive maize ubiquitin promoter (Bruce et al., 1989), was used as an internal control construct to normalize GUS activities of the reporter construct. The full-length cDNA of *HvWRKY38* and *HvGAMYB* was amplified from total RNA of barley aleurone cells by RT-PCR, and cloned into the AscI site of the intermediate construct containing the *UBI* promoter and *NOS* terminator (Zhang et al., 2004) using primers P1 and P2 for preparation of *UBI-HvWRKY38*, and P3 and P4 for preparation of *UBI-HvGAMYB* (Table

4-1). The full-length cDNA of *BPBF* and *SAD* were amplified and cloned into the AscI site of the expression vector using the following primers: P5 and P6 for preparation of *UBI-BPBF*, and P7 and P8 for preparation of *UBI-SAD*. To construct *HvWRKY38* (RNAi), a gene-specific PCR product of 330 bps fragment was obtained using primers P9 and P10 cloned into BlpI and Bsu36I sites in pDT01008007 (Zhang et al., 2004) in reverse orientation. The deletion and substitution mutants were prepared by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Single-stranded DNA from plasmid *UBI-HvWRKY38* was used as a template. Primer P11 was used to introduce a stop codon (TAG) at amino acid 200, which is upstream from the WRKY domain; primer P12 was used to mutate the WRKY motif of *HvWRKY38*; and primer P13 was used to mutate the conserved histidine residues of the zinc finger motif. In order to disrupt the presumed function of HvWRKY38 Leucine zipper motif from position aa 63 to position aa 91), a double mutant construct was created in which Leu-63 and Leu-77 were changed to arginine and histidine residues by using primers P14 and P15.

Particle bombardment and transient expression assays

The detailed description of transient expression procedure with the barley (*Hordeum vulgare*) aleurone system and the particle bombardment technique have been published before (Shen et al., 1993). Briefly, de-embryonated half-seeds of Himalaya barley were imbibed for 2.5 to 3 days, and then the pericarp and testa were removed. The DNA mixture (in a 1:1 molar ratio) of *HVA1-GUS* and *UBI-Luciferase* or *Amy32b-GUS* and *UBI-Luciferase*, along with or without an effector construct, was bombarded into barley embryoless half-seeds (four replicates per test construct). After incubation for 24 h

Primers	Primer sequences	Genes/Fragments	Restriction sites
P1	AATTGGCGCGCCATGGATCCATGGATGGGCAGCCAGCC	*HvWRKY38*	AscI
P2	TAAGGCGCGCCTTAATTGATGTCCCT	*HvWRKY38*	AscI
P3	ATGGCGCGCCATGTACCGGGTGAAGAG	*HvGAMYB*	AscI
P4	GAGGCGCGCCTCATTTGAATTCCTCCGAC	*HvGAMYB*	AscI
P5	TATGGCGCGCCATGGAGGAAGTGTTTTCG	*BPBF*	AscI
P6	TTAGGCGCGCCTTACATCAGGGAGGTGCTG	*BPBF*	AscI
P7	ATTGGCGCGCCATGACATATGGAGGAGAGAG	*SAD*	AscI
P8	ATTGGCGCGCCGGTCGACCTACCACGAGC	*SAD*	Asci
P9	TATTGCTTAGCTCTCGCCGACCAGGG	*HvWRKY38 RNAi*	BlpI
P10	TTAAGCTAAGCGACTGCATTATCCTAG	*HvWRKY38 RNAi*	Bsu36I
P11	CTTCTGCCCGTACTTGCTCTAGAGGTACCCGTCCTTCAC	*HvWRKY38 Mutant 1*	
P12	GTCACCTTCTGCCCGTCCGTGCTCGAGTGGTACCCGTCCTTCAC	*HvWRKY38 Mutant 2*	
P13	CGGCGGGGGCTGGGTGAGCTCGAGCTCGCCCTCGTACGTC	*HvWRKY38 Mutant 3*	
P14	CGCTGTAGCTCCGACTCTCGAGCCGCAACCTCAGGGTC	*HvWRKY38 LZ Mutant 1*	
P15	CCCTGAGCATCTCGCCATGGCGCCGGTTCTCCTCG	*HvWRKY38 LZ Mutant 2*	
P16	ATTGGCGCGCCATGCAATGGCGCAAGTAC	*HvWRKY38 C-terminal*	AscI
P17	TTAGGCGCGCCTTAATTGATGTCCCTGGTC	*HvWRKY38 C-terminal*	AscI

Table 4-1. Primers for the preparation of constructs.
P1 to P8 are primers for preparation of effector constructs. P9 and P10 are primers for preparation of *HvWRKY38 RNAi* construct. P11 to P15 are primers for *HvWRKY38* mutagenesis studies. P16 and P17 are primers for preparation of *HvWRKY38* C-terminal region.

with various treatments, GUS assays and luciferase assays were performed as previously described (Shen et al., 1996).

Subcellular localization and bimolecular fluorescence complementation

HvWRKY38 was inserted into the AscI site in *UBI-GFP* (Zhang et al., 2004), *UBI-YFP*, *UBI-YN*, and *UBI-YC* (Xie et al., 2006) to generate *UBI-GFP:HvWRKY38*, *UBI-YN:HvWRKY38*, and *UBI-YC:HvWRKY38*. A N-terminal region of *HvWRKY38* was produced by introducing a stop codon at amino acid 200, which is upstream from the WRKY domain. The rest of C-terminal region of *HvWRKY38* was amplified using the following primers: P16 and P17 (Table 4-1). The N-terminal and C-terminal regions of HvWRKY38 were inserted into the AscI site in *UBI-YN* to produce *UBI-YN:N-HvWRKY38* and *UBI-YN:C-HvWRKY38*. The leucine zipper double mutant of *HvWRKY38* was inserted into the AscI site in *UBI-YN* to generate *UBI-YN:LZ-HvW38*. *HvGAMYB* was inserted into the AscI site in *UBI-YN* and *UBI-YC* to generate *UBI-YN:HvGAMYB* and *UBI-YC:HvGAMYB*. After incubation at 24°C for 24 h, the aleurone layers were peeled from barley half-seeds and soaked in a 5 μM SYTO17 solution (Molecular Probe, Eugene, OR). The stained samples were observed and images of GFP fluorescence and SYTO17 staining were obtained simultaneously through Laser Scanning Microscope LSM 510 (Carl Zeiss, Inc. Germany) with 488-nm excitation and 505- to 530-nm emission wavelengths for the green fluorescence, and 633-nm excitation and 650-nm emission wavelengths for the red fluorescence in separate channels. The yellow fluorescence was observed with 514-nm excitation and 530-nm emission wavelength. At least 8000 cells were checked in each sample, and fluorescence was

observed in more than 10% of total cells in positive results. The acquired images were processed using Paint Shop Pro 7 (Jasc Software, Eden Prairie, MN, USA).

Electrophoretic mobility shift assay

The full-length cDNA of *HvWRKY38* was cloned into the *AscI* site of the modified pGEX-KG (Zhang et al., 2004) to generate *GST:HvWRKY38*. The fusion constructs were then introduced into *Escherichia coli* strain Origami B DE3 (Novagen, Madison, WI, USA). Over-expression of the fusion protein was induced by 1 mM isopropylthio-β-galactoside at 25 °C in 2XYT medium for 3 h. The cell suspension was passed three times through a SLM-Aminco French pressure cell press at 1,600 PSIG. The GST fusion proteins were purified using glutathione-agarose beads (Zhang et al., 2004). The full-length cDNA of *HvGAMYB* were cloned into the *AscI* site of the modified pGEX-KG (Zhang et al., 2004) to generate *GST:HvGAMYB*. The fusion constructs were then introduced into *E. coli* BL-21 (DE3) pLysS (Novagen, Madison, WI, USA). Over-expression of the fusion protein was induced by 1 mM isopropylthio-β-galactoside at 25 °C in 2XYT medium containing 1 M sorbitol and 2.5 mM betaine (Blackwell and Horgan, 1991) for 3 h. The cell suspension was passed three times through an SLM-Aminco French pressure cell press at 1,600 PSIG. The GST fusion proteins were purified using glutathione-agarose beads (Zhang et al., 2004). A 61-bp fragment of the *Amy32b* promoter (Lanahan et al., 1992) that spans from position -171 to -111 was used as a probe. The DNA probe was labeled with α-^{32}P-dATP by a Klenow fill-in reaction. The sequences of synthetic oligonucleotides used as competitors were described previously (Zhang et al., 2004). Unless otherwise indicated, binding reactions (20 µl) contained 1.5 ng of probe, 1 µg of poly-(dIdC), 10 mM Tris-HCl (pH7.6), 50 mM KCl, 0.5 mM EDTA,

5 μM $ZnCl_2$, 0.07% Igepal CA-630, and 10% glycerol. Equal amounts of recombinant proteins (0.5 μg for each) were added into reactions and incubated at 4 °C for 20 min with labeled DNA probes in the absence or presence of competitors. After incubation, reactions were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5XTBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) buffer for 2 h. The signals were scanned with a Typhoon 9400 phosphorimager (Amersham Biosciences, NJ, USA).

Results

HvWRKY38 specifically represses GA induction of the *Amy32b* α-amylase promoter in aleurone cells

HvWRKY38 is the member of a group II WRKY transcription factors containing one WRKY domain and a zinc-finger-like motif ($C\text{-}X_{4\text{-}5}\text{-}C\text{-}X_{22\text{-}23}\text{-}H\text{-}X_1\text{-}H$), which is involved in cold and drought stress responses (Mare et al., 2004). Homologues of HvWRKY38 have been found in wild oat, Arabidopsis, rice, and creosote bush (Rushton et al., 1995; Seki et al., 2002; Zhang et al., 2004; Zou et al., 2004). HvWRKY38 is capable of binding to W-boxes/Box2 in the GA responsive *Amy32b* promoter (Mare et al., 2004). To study the function of HvWRKY38 on the GA signal transduction pathway, a reporter construct (*Amy32b-GUS*) containing the *GUS* reporter gene driven by the GA responsive *Amy32b* α-amylase promoter and an effector construct (*UBI-HvWRKY38*) were co-introduced into barley aleurone cells by particle bombardment (Figure 4-1A). The exogenous GA treatment led to a 45-fold induction of the *Amy32b-GUS* expression over the control (Figure 4-1B). However, when both GA and HvWRKY38 were

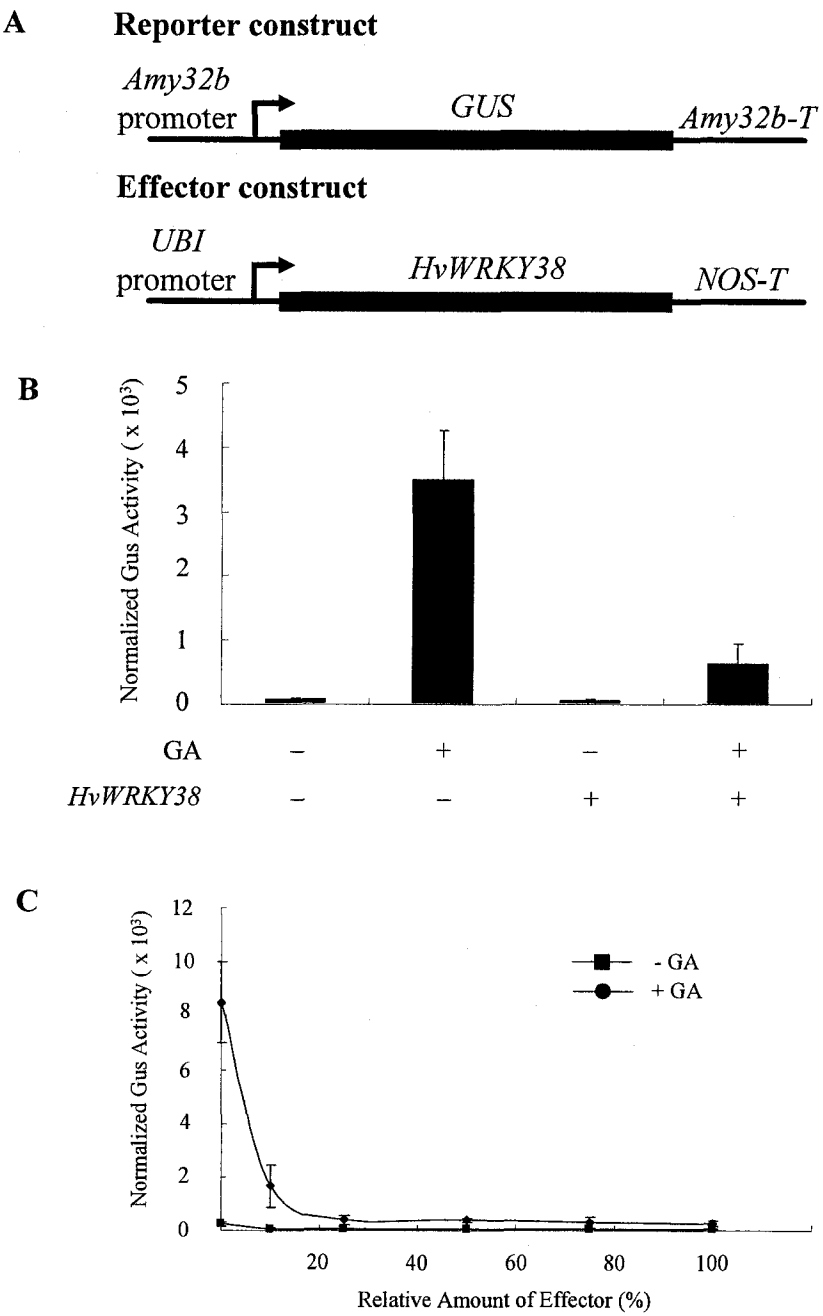

Figure 4-1. HvWRKY38 specifically represses GA induction of the *Amy32b* α-amylase promoter in aleurone cells.

A, Schematic diagram of the reporter and effector constructs used in the co-bombardment experiment.

B, The reporter construct, *Amy32b-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley aleurone cells either with (+) or without (-) the effector construct (*UBI-HvWRKY38*) by using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to

Figure 4-1. (continued)
the luciferase activity. Bars indicate GUS activities ± SE after 24 h of incubation with (+) or without (-) 1 μM GA. Data are means ± SE of four replicates.
C, The effector construct, *UBI-HvWRKY38*, was co-bombarded into barley aleurone cells along with the reporter construct, *Amy32b-GUS*, and the internal control construct, *UBI-luciferase*. The amount of reporter and internal control plasmid DNA was always constant, whereas that of effector varied with respect to the reporter as shown in the x-axis. 100% means the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the luciferase activity. The lines indicate GUS activities ± SE after 24 h of incubation of the bombarded aleurone cells with (+) or without (-) 1μM GA. Data are means ± SE of four replicates.

introduced, the GA induction of *Amy32b-GUS* was decreased to 8-fold. These data suggest that HvWRKY38 functions as a repressor of the GA signaling pathway.

To further study the effect of HvWRKY38 on the GA pathway, a dosage experiment was carried out in which the reporter plasmid concentration was kept constant, whereas the effector varied from 0 to 100% (Figure 4-1C). When the *Amy32b-GUS* construct was transformed alone, the treatment with 1 μM GA induced the expression of this gene construct by 37-fold. The expression of *Amy32b-GUS* in response to GA was reduced to 7-fold in the presence of 10% of the relative amount of effector. When the relative amounts of effector were higher than 25%, the GUS expression was reduced to 2-fold or less. These data indicate that the effect of HvWRKY38 on the repression of GA induction of the *Amy32b* α-amylase gene expression is dosage dependent. Similar dosage experiments with two ABA-inducible reporter constructs, *HVA22-GUS* and *HVA1-GUS* (Shen and Ho, 1995; Shen et al., 1996), showed that HvWRKY38 had little effect on ABA responsive promoters *HVA22* and *HVA1* (Figure 4-2 B and C). These results suggest the suppressing effect of Hv-WRKY38 is specific for the GA signal transduction pathway.

Loss of HvWRKY38 activity leads to increased expression of *Amy32b* in the absence of GA

We have demonstrated that over-expression of HvWRKY38 blocked GA induction of the *Amy32b* promoter, indicating HvWRKY38 encodes a negative regulator of the GA pathway controlling the expression of *Amy32b* in aleurone cells. It is possible that knocking down the expression of HvWRKY38 by double-stranded RNA interference (dsRNAi) could result in greater expression of the *Amy32b* promoter in the absence of

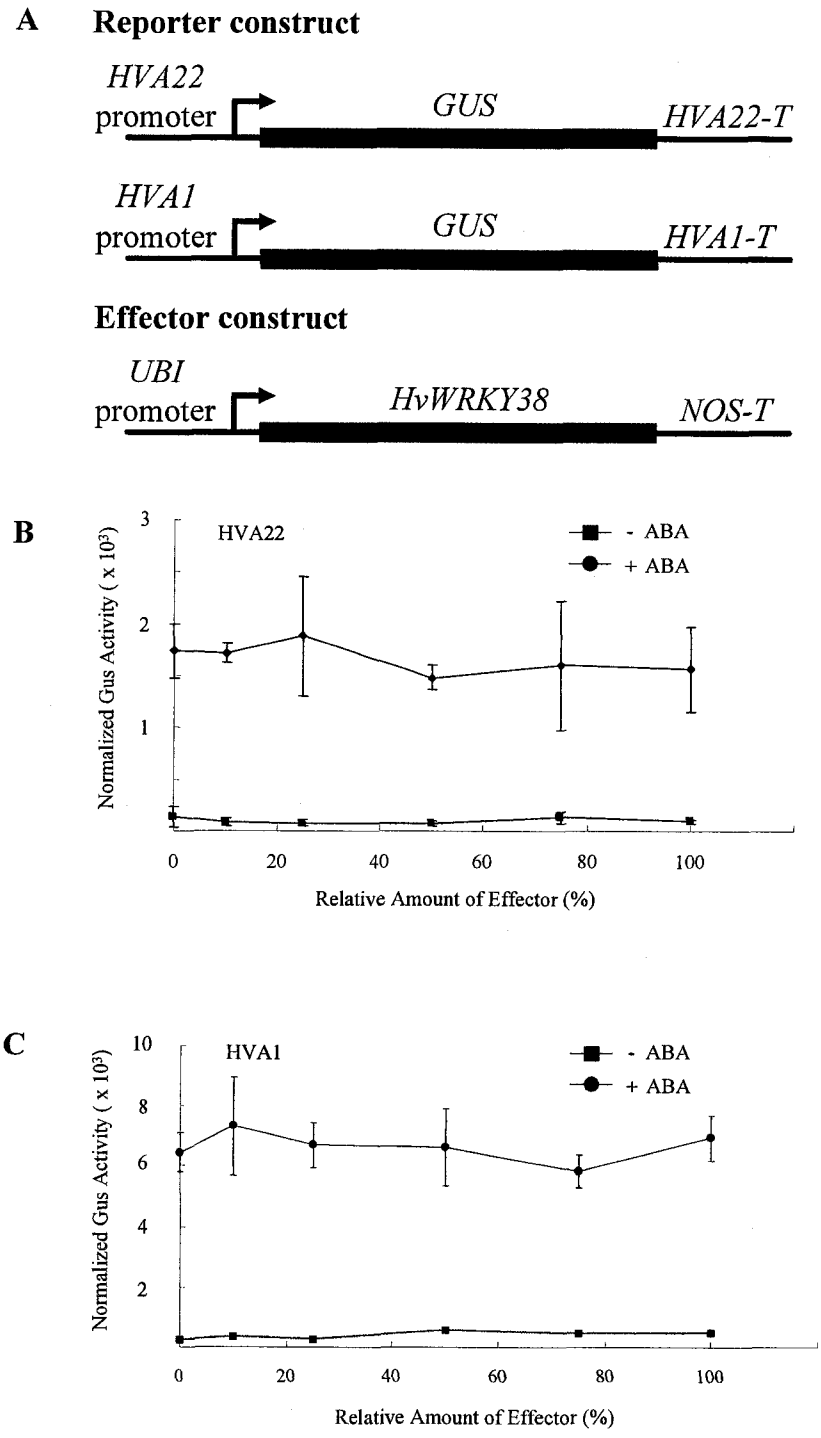

Figure 4-2. HvWRKY38 has little effect on the ABA signaling.
A. Schematic diagram of the reporter and effector constructs used in the cobombardment experiment.
B. The effector construct, *UBI-Hv-WRKY38*, was cobombardment into barley aleurone cells along with the reporter construct, *HVA22-GUS*, and the internal control construct, *UBI-luciferase*. The amount of reporter and internal control plasmid DNA was always

Figure 4-2. (continued)
constant, whereas that of effector varied with respect to the reporter as shown in the x axis. 100% means the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the luciferase activity. Lines indicate GUS activities ± SE after 24 hr of incubation of the bombarded aleurone cells with (+) or without (-) 20 μM ABA. Data are means ± SE of four replicates.
C. The effector construct, *UBI-Hv-WRKY38*, was cobombardment into barley aleurone cells along with the reporter construct, *HVA1-GUS*, and the internal control construct, *UBI-luciferase*. The amount of reporter and internal control plasmid DNA was always constant, whereas that of effector varied with respect to the reporter as shown in the x axis. 100% means the same amount of effector and reporter DNA was used. GUS activity was normalized in every independent transformation relative to the luciferase activity. Lines indicate GUS activities ± SE after 24 hr of incubation of the bombarded aleurone cells with (+) or without (-) 20 μM ABA. Data are means ± SE of four replicates.

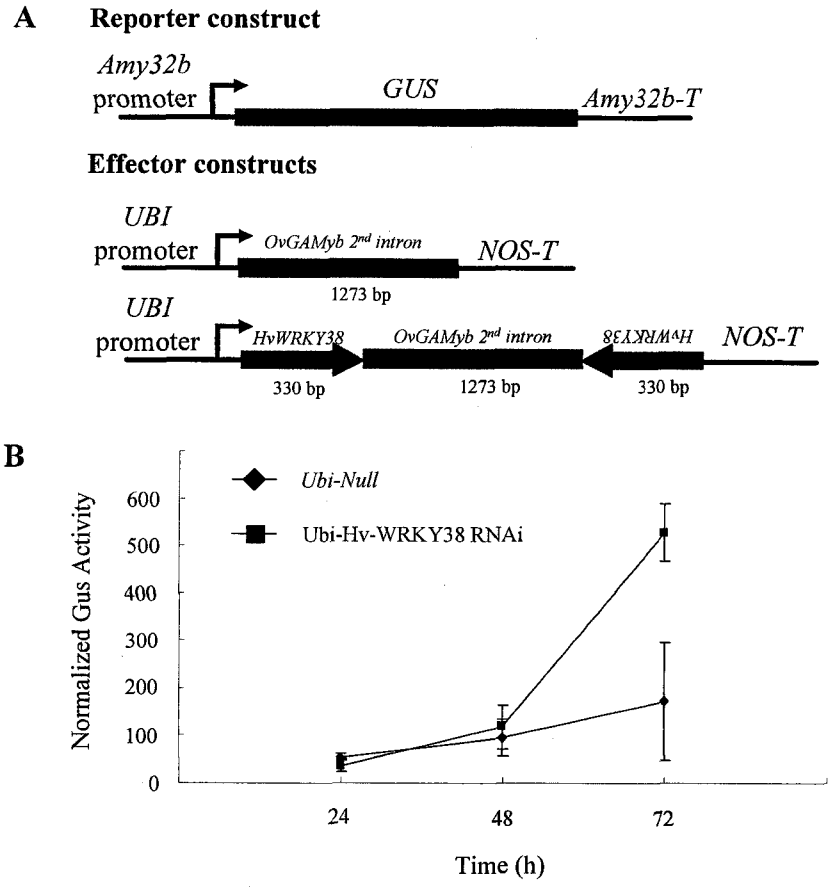

Figure 4-3. Loss of *HvWRKY38* activity leads to increased expression of *Amy32b* in the absence of GA.

A, Schemes of gene constructs. Arrowheads indicate the orientation of the gene fragments. Numbers below the effector constructs represent the size (in bp) of every segment (not drawn to scale).

B, The reporter construct, *Amy32b-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley aleurone cells with the effector constructs, *UBI-Null* or *UBI-HvWRKY38* (RNAi), by using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Lines indicate GUS activities ± SE after 24 h, 48 h, or 72 h of incubation of the bombarded aleurone cells without GA. Data are means ± SE of four replicates.

GA. To test this hypothesis, *UBI-Null* and *UBI-HvWRKY38 RNAi* constructs were prepared (Figure 4-3A). The *UBI-Null* control contains the second intron (1273-bp) of the *OsGAMyb*, which is flanked by the *UBI* promoter and *NOS* terminator (Zhang et al., 2004). The *OsGAMyb* intron functions as a loop in the RNAi construct. The *UBI-HvWRKY38 RNAi* construct contains two HvWRKY38 fragments in an opposite orientation. The *Amy32b-GUS* reporter construct was co-bombarded into aleurone cells along with *UBI-Null* or *UBI-HvWRKY38 RNAi* constructs. As shown in Figure 4-3B, co-expression of *UBI-Null* had little effect on *Amy32b-GUS* expression at 24 h, 48 h, and 72 h time points tested after bombardment. In contrast, co-expression of *UBI-HvWRKY38 RNAi* increased the level of GUS expression from *Amy32b-GUS* without GA after 72 h of incubation to three times that of the control.

The C-terminal region containing the WRKY domain of HvWRKY38 is essential for repression of the *Amy32b* promoter

To identify functional domains of HvWRKY38, mutagenesis experiments were carried out to eliminate specific domains of the polypeptide. A stop codon was introduced at amino acid 200 (mutant 1), which is upstream from the WRKY domain. The purpose was to produce a truncated protein missing the WRKY domain and the rest of the C-terminal region. The WRKY motif and zinc-finger motif were replaced in mutants 2 and 3, respectively (Figure 4-4A). The reporter construct along with one of the effector constructs encoding these mutant polypeptides were then co-bombarded into barley aleurone cells. In this experiment, the expression of the *Amy32b* promoter increased 37-fold after GA treatment (Figure 4-4B). The presence of the wild-type HvWRKY38 essentially abolished GA-induction of *Amy32b-GUS*. Although deletion of the C-

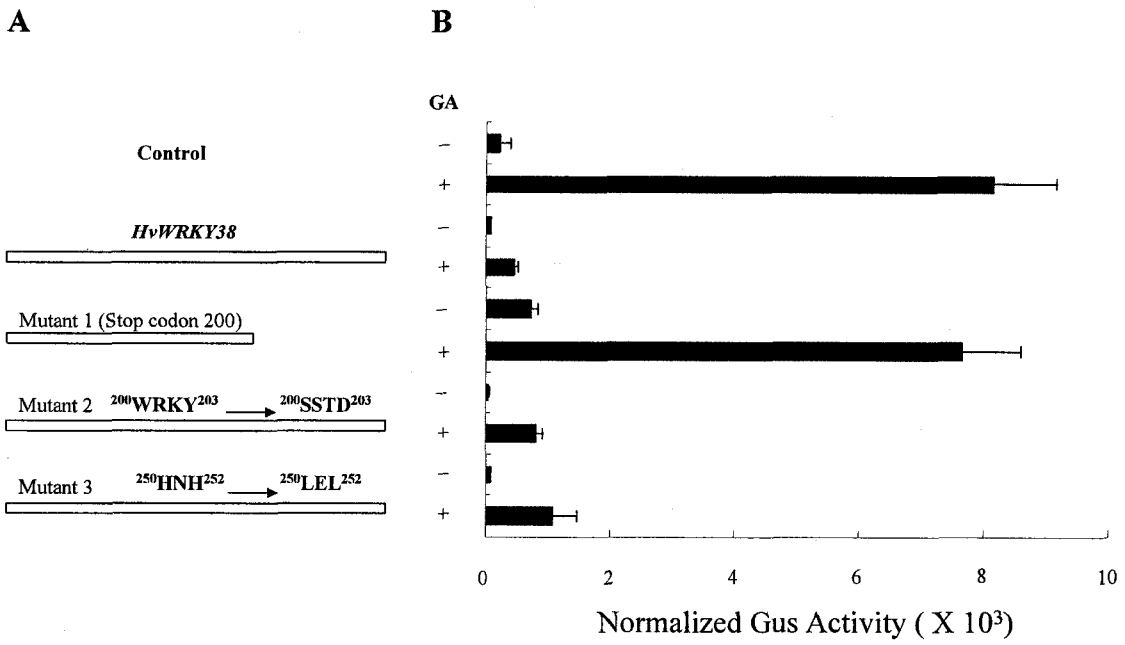

Figure 4-4. The transcriptional activity of HvWRKY38 resides in the C-terminal region containing the WRKY domain.
A, Schematic diagrams of the wild-type and mutant versions of *HvWRKY38* used in the co-bombardment experiment. Control means without effector construct.
B, The reporter construct, *Amy32b-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley aleurone cells either with (+) or without (-) the effector constructs (*UBI-HvWRKY38* or *UBI-HvWRKY38* mutants) by using the same molar ratio of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Bars indicate GUS activities ± SE after 24 h of incubation of the bombarded aleurone cells with (+) or without (-) 1 μM GA. Data are means ± SE of four replicates.

terminal region containing the WRKY domain abolished the transrepressing activity of HvWRKY38, mutations of the WRKY motif (Mutant 2) or the zinc-finger motif (Mutant 3) had little effect on its activity (Figure 4-4B). This is consistent with what reported for OsWRKY71 and OsWRKY51 (Xie et al., 2006). These data indicate that the C-terminal region containing the WRKY domain of HvWRKY38 is essential for the repression on Amy32b promoter (Xie et al., 2006).

HvWRKY38 and HvGAMYB proteins physically interact in the nuclei of aleurone cells.

To examine the subcellular localization of the HvWRKY38 protein, the *HvWRKY38* gene was fused in frame to the 3' end of a green fluorescent protein (*GFP*) gene that is driven by the constitutive maize Ubiquitin (*UBI*) promoter. The *UBI-GFP* control or *UBI-GFP:HvWRKY38* plasmid was introduced into barley aleurone cells by particle bombardment, and the GFP fluorescence was visualized with confocal microscopy. In the control, GFP fluorescence was observed throughout the cells (Figure 4-5A, panel I). In contrast, GFP:HvWRKY38 fusion proteins were localized exclusively in the nuclei (Figure 4-5A, panel III), as confirmed by staining with a red fluorescent nucleic acid stain SYTO 17 (Figure 4-5A, panels II and IV). These results suggest that HvWRKY38 is targeted to the nuclei of aleurone cells.

HvGAMYB is a positive regulator mediating the pathway between GA and α-amylase in aleurone cells (Gubler et al., 1995). To understand how these two regulators modulate the expression level of *Amy32b*, we studied whether HvWRKY38 and HvGAMYB physically interact with each other in aleurone cells. The *HvWRKY38* and *HvGAMYB* coding sequences were fused in-frame with sequences coding the N-terminal

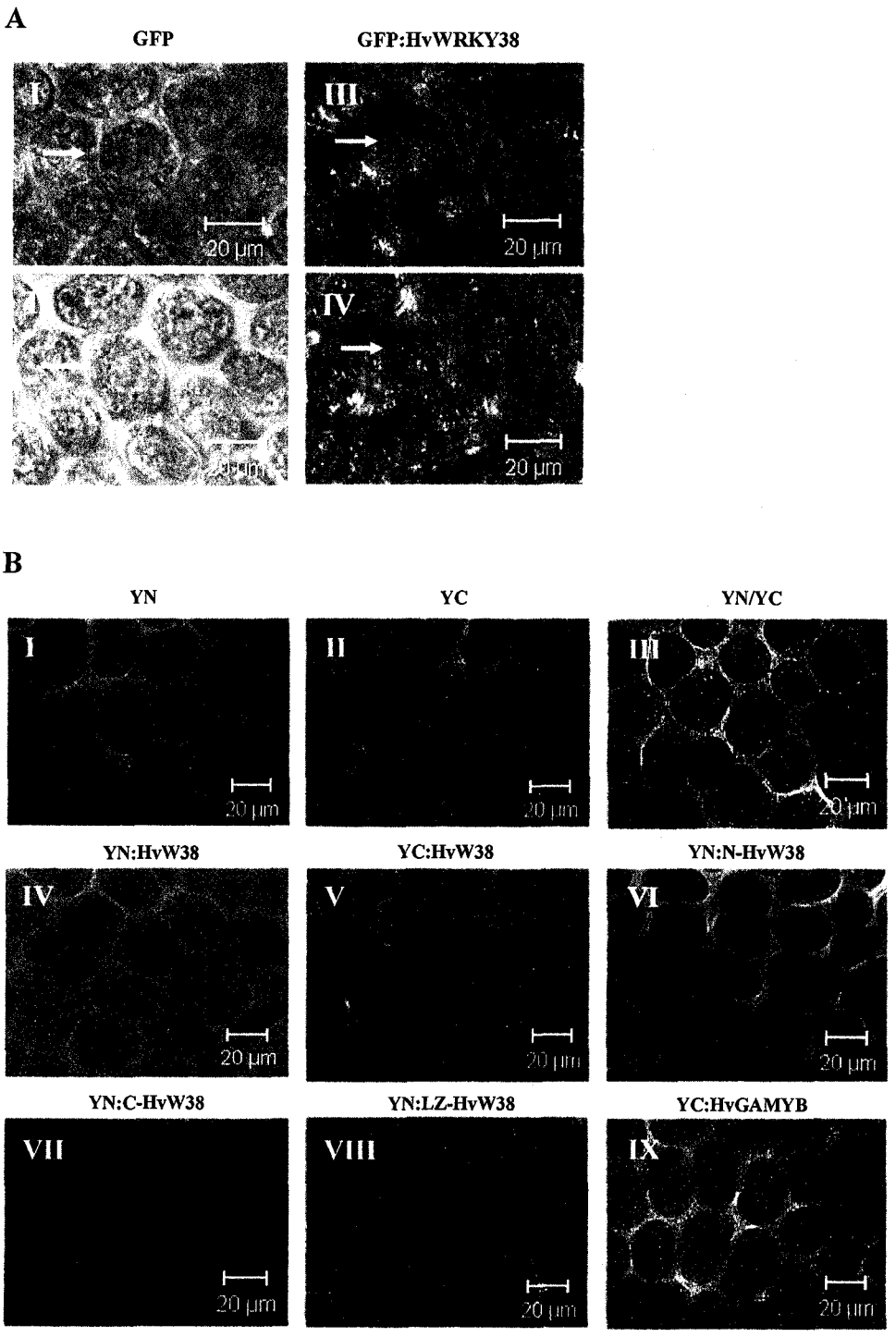

Figure 4-5. Visualization of interactions between transcription factors of GA signaling in aleurone cells by biomolecular fluorescence complementation.

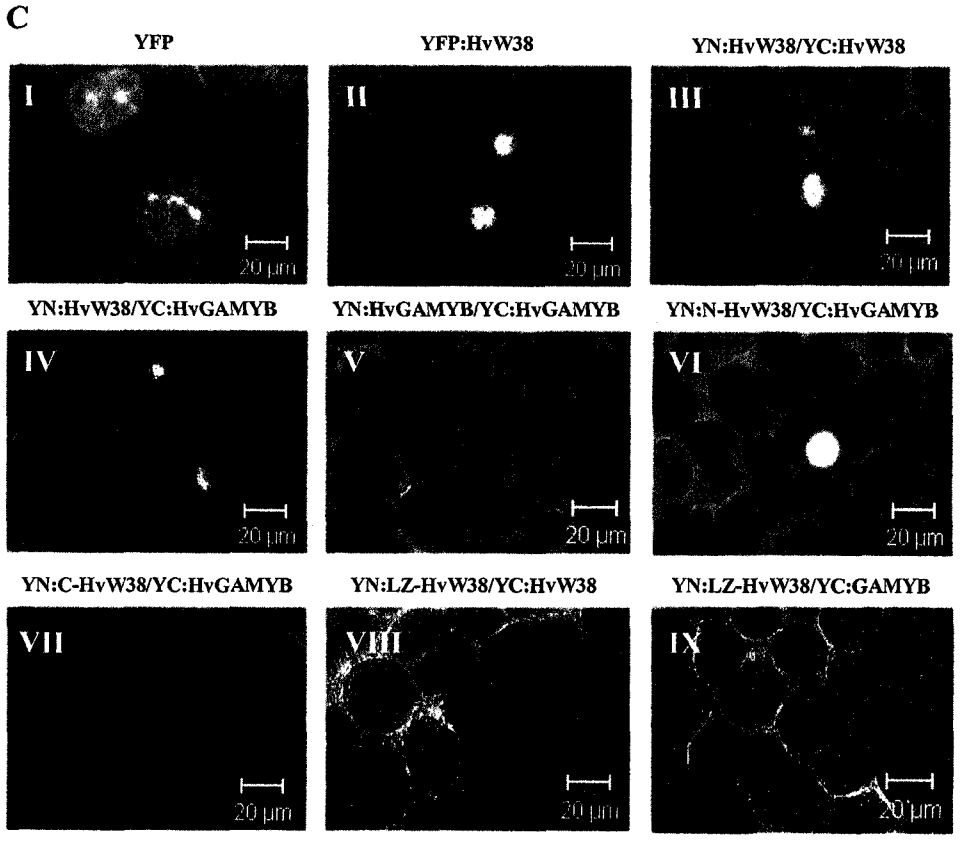

Figure 4-5. (continued)

A, Barley half-seeds were bombarded with either *UBI-GFP* (panels I and II) or *UBI-GFP:HvWRKY38* (panels III and IV). After incubation for 24 h, the aleurone cells were stained with SYTO17 to localize nuclei (panels II and IV), followed by examination of GFP fluorescence (panels I and III). Arrows point to the same cell. The bars represent 20 µm.

B, Barley aleurone cells were bombarded with *UBI-YN*, *UBI-YC*, a combination of *UBI-YN* and *UBI-YC*, *UBI-YN:HvWRKY38*, *UBI-YC:HvWRKY38*, *UBI-YN:N-HvWRKY38*, *UBI-YN:C-HvWRKY38*, *UBI-YN:LZ-HvWRKY38*, and *UBI-YC:HvGAMYB*. YN is the fragment containing amino acid residues 1-154 of YFP, and YC is the fragment containing amino acid residues 155-238 of YFP. After incubation at 24°C for 24 h, yellow fluorescence was observed through a confocal microscope.

C, Barley aleurone cells were bombarded with *UBI-YFP*, *UBI-YFP:HvWRKY38*, or a combination of constructs encoding the indicated fusion proteins. YN is the fragment containing amino acid residues 1-154 of YFP, and YC is the fragment containing amino acid residues 155-238 of YFP. After incubation at 24°C for 24 h, yellow fluorescence was observed through a confocal microscope.

fragment of YFP (YN) or the C-terminal fragment (YC), respectively. As expected, no fluorescence was detected when only one of the *UBI-YN* or *UBI-YC* constructs was introduced into aleurone cells (Figure 4-5B, panels I and II). Also, no fluorescence was detected when a combination of *UBI-YN-* and *UBI-YC-* constructs was introduced into aleurone cells (Figure 4-5B, panel III). The complementation between different combinations of fusion proteins was tested by introducing the corresponding constructs into barley aleurone cells. Confocal microscopic studies showed that YFP fluorescence was detected in the entire cells bombarded with the *UBI-YFP* construct (Figure 4-5C, panel I). Consistent with the nucleus localization data in Figure 4-5A, YFP fluorescence was only observed in the nuclei of the aleurone cells bombarded with *UBI-YFP:HvWRKY38* (Figure 4-5C, panel II). No fluorescence was detected when only one of the *YN-* or *YC-* fusion constructs was introduced into aleurone cells (Figure 4-5B, panels IV, V, and IX). However, yellow fluorescence was detected in the nuclei of aleurone cells bombarded with *UBI-YN:HvWRKY38* and *UBI-YC:HvWRKY38* constructs (Figure 4-5C, panel III), suggesting that HvWRKY38 proteins interact in the nuclei of aleurone cells. Surprisingly, yellow fluorescence was detected in the nuclei of aleurone cells bombarded with *UBI-YN:HvWRKY38* and *UBI-YC:HvGAMYB* (Figure 4-5C, panel IV), indicating that HvWRKY38 also interacts with HvGAMYB in the nuclei of aleurone cells. No fluorescence was observed when *YN:HvGAMYB* and *YC:HvGAMYB* constructs were introduced into barley aleurone cells, suggesting that HvGAMYB proteins do not form a dimer *in vivo* (Figure 4-5C, panel V).

To study which part of HvWRKY38 is essential for the interaction with HvGAMYB, the N-terminal and C-terminal regions of *HvWRKY38* were fused in-frame

with *UBI-YN* to produce *UBI-YN:N-HvWRKY38* and *UBI-YN:C-HvWRKY38*, respectively. Yellow fluorescence was detected in the nuclei of aleurone cells bombarded with *UBI-YN:N-HvWRKY38* and *UBI-YC:HvGAMYB* (Figure 4-5C, panel VI). However, no fluorescence was detected when *UBI-YN:C-HvWRKY38* and *UBI-YC:HvGAMYB* (Figure 4-5C, panel VII) were introduced into aleurone cells. These data indicate that the N-terminal region of HvWRKY38 is essential for the physical interaction with HvGAMYB in the nuclei of aleurone cells. Our data further suggest that the putative leucine zipper domain of HvWRKY38 is necessary for the dimerization (Figure 4-5C, panels VIII and IX).

HvWRKY38 interferes with the binding of HvGAMYB to the *Amy32b* promoter

Because HvWRKY38 and HvGAMYB physically interact in nuclei, it is of interest to study whether they bind to the promoter of *Amy32b* simultaneously or compete in binding to the promoter. Electrophoretic mobility-shift assays (EMSAs) were performed with glutathione-S-transferase (GST)-tagged HvWRKY38 and HvGAMYB. A DNA fragment containing the *Amy32b* promoter region from -176 to -111 was used as a probe (Figure 4-6A). No binding signal was detected in reactions without protein (Figure 4-6B, lane 1) or with GST only (lane 2). Both HvWRKY38 and HvGAMYB bound specifically to the *Amy32b* promoter (lanes 3 and 6), and excess amounts of unlabeled promoter fragment competed off the *in vitro* binding signal (lanes 4, 5, 7, and 8). It has been demonstrated that the dimerization of HvWRKY38 is essential for high-affinity binding to W-boxes in the *Amy32b* promoter (Mare et al., 2004). Consistent with their work, we observed that the GST-HvWRKY38 complex was shifted higher than that of

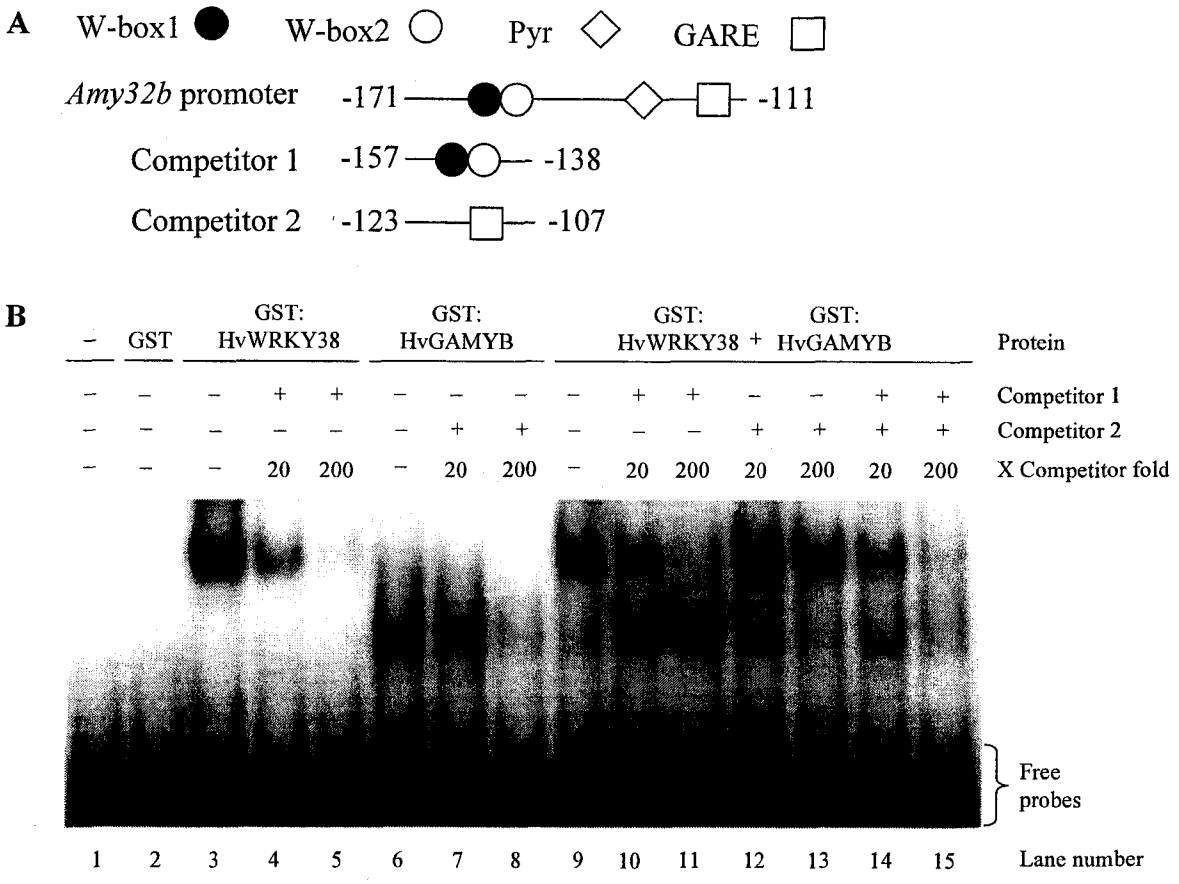

Figure 4-6. Electrophoretic mobility shift assays of GST:HvGAMYB and GST:Hv-WRKY38 fusion proteins.
A, The 61 bp probe (–171 to –111), and 20 bp or 28 bp synthetic oligonucleotide competitor containing W-boxes or GARE were used in EMSA. Circles denote W-boxes; a diamond represents the pyrimidine-box; and a rectangle represents GARE in the *Amy32b* promoter. The DNA probe was end-labeled with α-^{32}P-dATP.
B, EMSA with recombinant GST:HvWRKY38 or GST:HvGAMYB proteins without (-) or with excess amounts of competitors (20- or 200-fold).

GST-HvGAMYB although the size of the GST-HvGAMYB protein (~ 80 KD) is bigger than that of the GST-HvWRKY38 protein (~ 60 KD) (Figure 4-6B, compare Lane 3 with Lane 6). Interestingly, when both HvWRKY38 and HvGAMYB were present in the reactions, HvGAMYB binding to the *Amy32b* promoter was inhibited (lane 9). As HvWRKY38 binding was competed off by an excess amount of the competitor 1 which contains two W-boxes (the WRKY binding site), HvGAMYB was able to bind to the promoter (lanes 10 and 11). As expected, competitor 2, which contains GARE only (the HvGAMYB binding site), did not compete with the binding of HvWRKY38 (lanes 12 and 13). When both competitors 1 and 2 were added, the binding signals of both proteins were decreased (lanes 14 and 15).

HvWRKY38 and HvGAMYB functionally compete with each other to regulate the expression of *Amy32b*

EMSA data suggested that binding of HvWRKY38 to W-boxes prevented HvGAMYB from binding to the *Amy32b* promoter. Dosage experiments were performed to study the functional interactions of these two regulators. Varied amounts of *UBI-HvWRKY38* (from 0% to 100% relative to the reporter construct) were introduced into aleurone cells along with a constant amount of *UBI-HvGAMYB* (100%) and *Amy32b-GUS* (100%). The HvGAMYB induction of *Amy32b* was gradually suppressed by increasing amounts of *UBI-HvWRKY38* (Figure 4-7B). It also showed that 10% of UBI-HvWRKY38 is sufficient to repress the HvGAMYB transactivating activity on *Amy32* promoter (Figure 4-7B). We further performed a similar dosage experiment with a fixed amount of *UBI-HvWRKY38* (10%) and *Amy32b-GUS* (100%), and varied amounts of

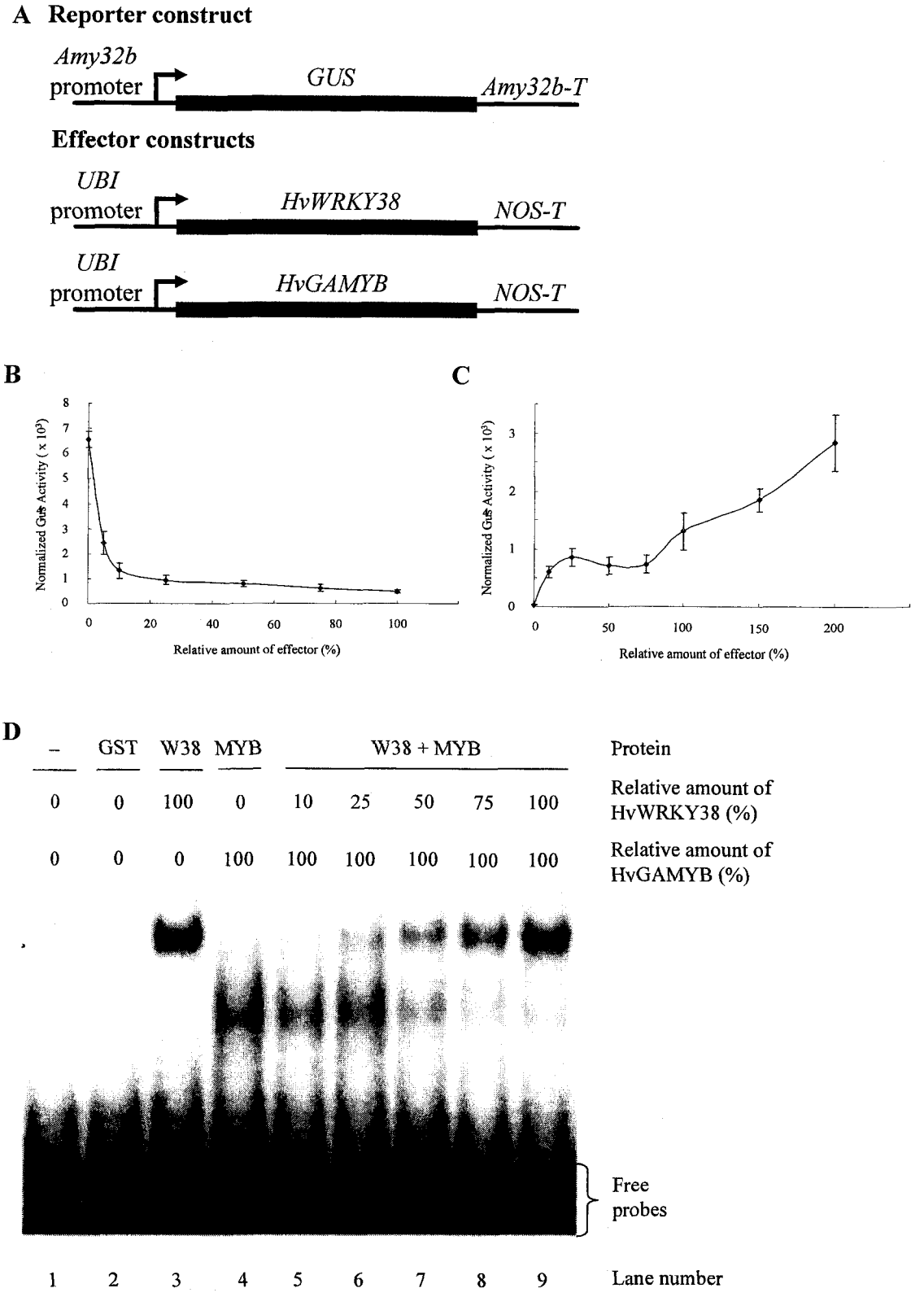

Figure 4-7. Physical and functional interactions between HvWRKY38 and HvGAMYB.

E

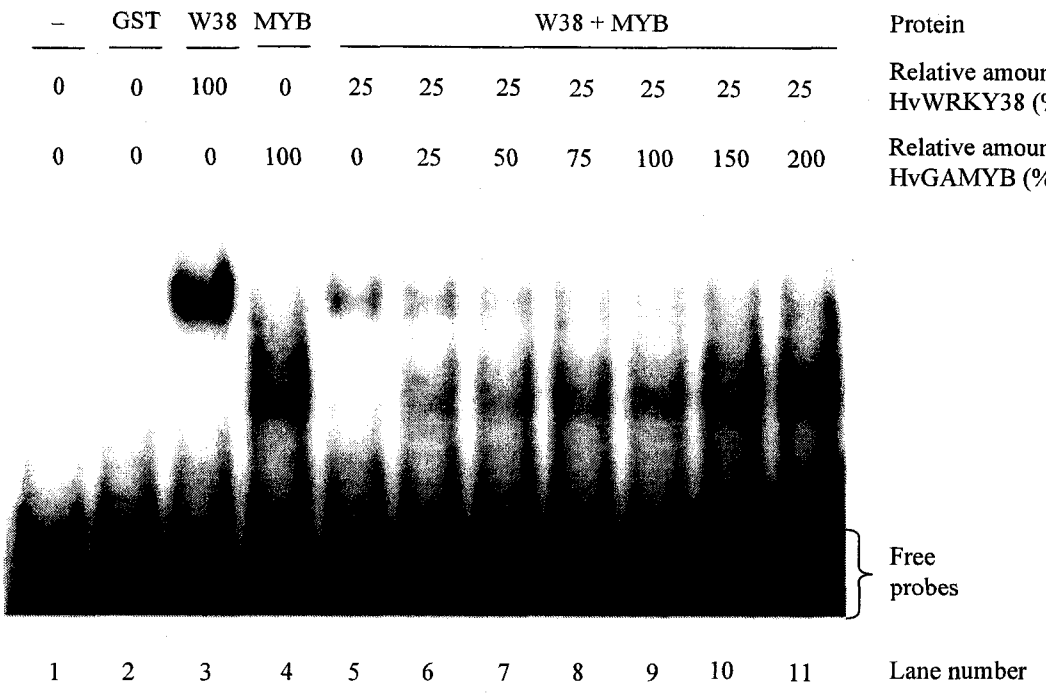

Figure 4-7. (continued)
A, schematic diagrams of reporter and effector constructs used in the co-bombardment experiment.
B, varied amounts of UBI-HvWRKY38 (from 0% to 100% relative to the reporter construct) were co-bombarded into barley aleurone cells with a constant amount of UBI-HvGAMYB (100%), Amy32b-GUS (100%), and the internal construct. The relative amount of UBI-HvWRKY38 is indicated as a percentage compared to the amount of reporter. Each data point indicates the mean ± SE of four replicates.
C, varied amounts of UBI-HvGAMYB (from 0% to 200% relative to the reporter construct) were co-bombarded into barley aleurone cells with a constant amount of UBI-HvWRKY38 (100%), Amy32b-GUS (100%), and the internal construct. The relative amount of UBI-HvGAMYB is indicated as a percentage compared to the amount of reporter. Each data point indicates the mean ± SE of four replicates.
D, EMSA with different amounts of recombinant GST:HvWRKY38 and GST:HvGAMYB proteins. The relative amount of GST:HvWRKY38 and GST:HvGAMYB is indicated as a percentage compared to the amount of GST:HvWRKY38 (100%) and GST:HvGAMYB (100%), respectively.
E, EMSA with different amounts of recombinant GST:HvWRKY38 and GST:HvGAMYB proteins. The relative amount of GST:HvWRKY38 and GST:HvGAMYB is indicated as a percentage compared to the amount of GST:HvWRKY38 (100%) and GST:HvGAMYB (100%), respectively.

UBI-HvGAMYB (from 0% to 200%). As shown in Figure 4-7C, the GUS activity was gradually increased as the amount of *UBI-HvGAMYB* augmented.

This dosage effect was also revealed by EMSAs. Varied amounts of GST-HvWRKY38 (from 0% to 100% relative to GST-HvGAMYB) with a fixed amount of GST-HvGAMYB (100%) were used in the competition study. Increasing amounts of HvWRKY38 protein gradually decreased the binding of HvGAMYB to the *Amy32b* promoter (Figure 4-6D, lanes 4, 5, 6, 7, 8, and 9). On the other hand, increasing amounts of HvGAMYB enhanced the binding signal of HvGAMYB to the *Amy32b* promoter (Figure 4-6E, lanes 6, 7, 8, 9, 10, and 11).

Interactions of HvWRKY38, BPBF, SAD, and HvGAMYB

on the expression of *Amy32b* promoter

SAD and HvGAMYB are transcriptional activators while BPBF is a transcriptional repressor of the *α-amylase* promoter (Gubler et al., 1995; Isabel-LaMoneda et al., 2003; Mena et al., 2002). SAD and BPBF interact with the same *cis*-acting element (the Pyrimidine box) while HvGAMYB interacts with GARE. Here, we further tested the interactions among HvWRKY38, BPBF, SAD, and HvGAMYB on regulating the expression of the *Amy32b* promoter. As expected, HvWRKY38 and BPBF had little effect on Amy32b-GUS in the absence of GA (Figur 4-8B, columns 3 and 4). In contrast, co-transformation of *UBI-SAD* or *UBI-HvGAMYB* alone resulted in 71-fold or 85-fold induction of the *Amy32b* promoter, respectively (columns 5 and 6). The inductive activity of SAD and HvGAMYB is repressed by HvWRKY38 (columns 7 and 8). Co-expression of *SAD* and *HvGAMYB* dramatically increased the expression of the *Amy32b* promoter to 201-fold induction (column 9). Interestingly, this combined effect of

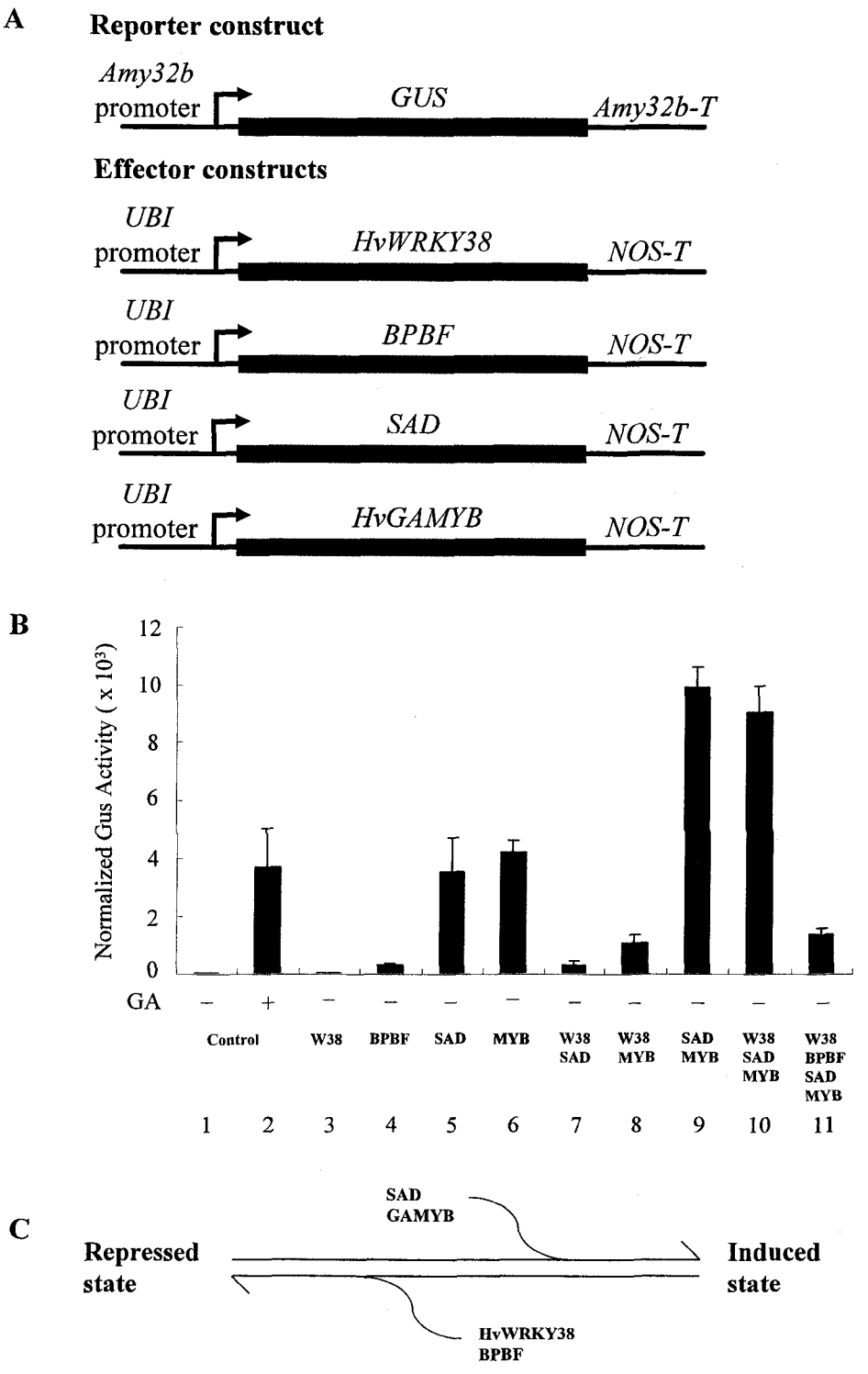

Figure 4-8. Interactions of HvWRKY38, BPBF, SAD, and HvGAMYB on the expression of *Amy32b* promoter.

Figure 4-8. (continued)
A, Schematic diagram of the reporter and effector constructs used in the cobombardment experiment.
B, The reporter construct, *Amy32b-GUS*, and the internal construct, *UBI-luciferase*, were co-bombarded into barley aleurone cells either with (+) or without (-) the effector construct by using the same molar amount of effector and reporter constructs. GUS activity was normalized in every independent transformation relative to the luciferase activity. Bars indicate GUS activities ± SE after 24 h of incubation of the bombarded aleurone cells with (+) or without (-) 1 μM GA. Data are means ± SE of four replicates.
C, Schematic diagram of transcriptional repressors and activators in mediating *Amy32b* gene expression.

SAD and HvGAMYB was not suppressed by HvWRKY38 (column 10). However, HvWRKY38 and BPBF together dramatically reduced the combined effect of SAD and HvGAMYB on the induction of the *Amy32b* promoter (column 11). Taken together, these suggest that the expression of *Amy32b* is governed by transcriptional repressors and activators (Figure 4-8C).

Discussion

In this work, we demonstrated that HvWRKY38, a WRKY transcription factor from barley, negatively regulates GA signaling in aleurone cells. Our results showed that HvWRKY38 specifically repressed the GA-induced *Amy32b* α-amylase promoter (Figure 4-1 and -2). The role of HvWRKY38 as a transcriptional repressor in GA response was further demonstrated by the dosage response, mutagenesis, and RNAi knock-out experiments (Figures 4-1, -3, and -4). We also found that HvWRKY38 repressed the transactivating activity of HvGAMYB by interacting and competing with HvGAMYB binding to the *Amy32b* α-amylase promoter (Figures 4-5, -6, and -7). This inhibitory effect of HvWRKY38 was overcome by the SAD and HvGAMYB complex (Figure 4-8). However, WRKY38 and BPBF together suppressed the combined effect of SAD and HvGAMYB on inducing the *Amy32b* promoter in the absence of GA (Figure 4-8).

HvWRKY38 is inducible by cold and drought treatments in barley leaves and roots (Mare et al., 2004). It is also induced by ABA and salicylic acid, but suppressed by GA in aleurone cells (Xie et al., 2007). HvWRKY38 represses GA induction of *Amy32b* (Figure 4-1, Xie et al., 2007). However, HvWRKY38 has no effect on two ABA-inducible promoters, *HVA22* and *HVA1* (Figure 4-2), indicating that the repression activity of

HvWRKY38 is specific for the GA pathway in aleurone cells. Consistent with its function as a repressor, co-expression of a *UBI-HvWRKY38* RNAi construct de-repressed the expression of the *Amy32b* promoter, leading to increased expression of *Amy32b* in the absence of GA (Figure 4-3). The effect of *UBI-HvWRKY38* RNAi was significant although it was lower than that of *UBI-HvSLN1*RNAi (Zentella et al., 2002). There are two possibilities accounting for the lower effect of *UBI-HvWRKY38* RNAi. The pre-existing HvWRKY38 protein might be more stable than the SLN1 protein; HvWRKY38 RNAi might only knock out *HvWRKY38*, but not other *WRKY* genes. The WRKY family has 74 members (Eulgem et al., 2000) in Arabidopsis and about 100 members in rice (Zhang and Wang, 2005; Ross, 2007). Several members could be involved in same processes. For example, Arabidopsis WRKY18, WRKY40, and WRKY60 have partially redundant roles in plant responses to the pathogens (Xu et al., 2006). Rice WRKY24, WRKY51, and WRKY71 blocked GA induction of the *Amy32b* promoter (Zhang et al., 2004; Xie et al., 2006; Zhang, Z.-L. and Shen, Q., unpublished results). In contrast, there appears to be only one copy of the *SLN1* gene in barley (Zentella et al., 2002). Hence, the *SLN1* RNAi effect is more potent in aleurone cells. It will be interesting to study the function of other barley WRKY family members in GA signaling.

HvWRKY38 contains a WRKY motif and a zinc finger motif at its C-terminal, and a putative leucine zipper domain and a nuclear localization sequence at its N-terminal (Mare et al., 2004). Consistent with its role as a transcription factor, the GFP-HvWRKY38 fusion protein was targeted to nuclei in aleurone cells (Figure 4-5A). Deletion studies of HvWRKY38 showed that the C-terminal region, which contains the WRKY domain and the zinc finger motif, was required for HvWRKY38 to repress the

expression of the *Amy32b* promoter (Figure 4-4). However, mutation in either the WRKY motif or in the conserved histidine residues of the zinc finger motif had little effect on its inhibitory activity on the GA induction of Amy32b-GUS even though these motifs of other WRKY proteins are required for their binding to the W-box sequences (Maeo et al., 2001; Yamasaki et al., 2005; Xie et al., 2006). It is possible that mutant proteins interact with endogenous HvWRKY38 and bind to the W-boxes of the *Amy32b* promoter because wild-type barley aleurone cells were used in the functional analyses throughout this study. Mare et al. (2004) have shown that dimerization of HvWRKY38 is essential for its high-affinity to the W-boxes in the *Amy32b* promoter. Dosage experiments also indicated that even a little amount of wild-type HvWRKY38 dramatically suppressed the GA induction of *Amy32b-GUS* (Figure 4-1). Therefore, it is feasible that a dimer consisting a wild type HvWRKY38 protein and a WRKY motif or zinc-finger motif mutant protein can still bind to the *Amy32b* promoter and suppress its transcription, as has been demonstrated for OsWRKY71 (Xie et al., 2006).

Mounting evidence suggest that each of the four *cis*-acting elements essential for GA induction of *Amy32b* can be bound by both transcriptional repressors and activators in barley aleurone cells. The Pyrimidine box can be bound by the SAD activator and the BPBF repressor; both are DOF proteins (Diaz et al., 2002; Isabel-LaMoneda et al., 2003; Diaz et al., 2005). In addition to the transcriptional activator GAMYB, the repressor HRT, a zinc finger protein, can also bind to GARE (Raventós et al., 1998). The Amy box can interact with the HvMCB1 repressor and the HvMYBS3 activator (Rubio-Somoza et al., 2006a; Rubio-Somoza et al., 2006b). We show here that HvWRKY38 interact with the W-boxes (Figure 4-6). An activator for this element has not been reported although

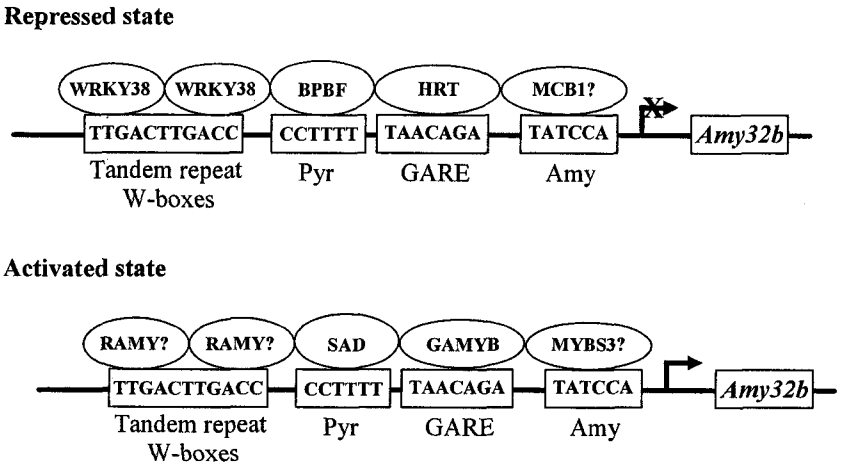

Figure 4-9. A hypothetical model for the control of the *Amy32b* α-amylase gene expression in aleurone cells.
A, Negative regulators (HvWRKY38, BPBF, HRT, and HvMCB1) bind to their corresponding *cis*-acting elements in the absence of GA.
B, Positive regulators (RAMY, SAD, HvGAMYB, and HvMYBS3) bind to corresponding *cis*-acting elements in the presence of GA. Question mark means the interaction of the transcription factor with the *cis*-acting element remains to be demonstrated.

RAMY, another zinc-finger protein also binds to this element (Peng et al., 2004). In light of these results, we propose a hypothetical model for the control of the *Amy32b* gene expression in aleurone cells (Figure 4-9). In the absence of GA, negative regulators such as HvWRKY38, BPBF, HRT, and HvMCB1 bind to corresponding *cis*-acting elements and form a "repressome", which diminishes the binding or transactivating activities of positive regulators to the promoter, thereby preventing *Amy32b* transcription. In the presence of GA, positive regulators such as RAMY, SAD, HvGAMYB, and HvMYBS3 bind to their respective DNA sequences and form an "activatesome", leading to a high level of *Amy32b* gene expression.

The intriguing question is how the "repressome" is replaced by the "activatesome" during the transition of *Amy32b* from the repressed state to the activated state or *vice versa*. Two possible mechanisms can be envisioned: 1) GA promotes the degradation of repressors, as reported for OsWRKY71 (Zhang et al., 2004), allowing transcriptional activators to occupy the *cis*-acting elements. 2) GA induces the expression of activators such as GAMYB (Gubler et al., 1995). These activators physically interact with repressors, eventually leading the dissociation of repressors from and association of activators to the corresponding *cis*-acting elements cooperatively. Interactions of repressors and activators have been observed in mammalian systems. For example, B-Myb, a cell-cycle-regulated transcriptional repressor, physically interacts with transcriptional activators Sp1 (specificity protein 1) and CBF (CCAAT-binding factor) and interferes with their binding to promoter (Cicchillitti et al., 2004). As a result, *COL1A1* (type I collagen) transcription in human scleroderma fibroblasts is decreased. The barley repressors BPBF, HvDOF17, and HvDOF19 physically interacts with

HvGAMYB, as demonstrated in the nucleus of onion cells using the BiFC approach (Diaz et al., 2005; Moreno-Risueno et al., 2007). HvDOF17 represses the expression of the *Al21*, a thiol-protease gene, probably by decreasing the binding affinity of HvGAMYB to GARE in the *Al21* promoter (Moreno-Risueno et al., 2007). Our data showed that HvWRKY38 physically interacted with HvGAMYB in aleurone cells (Figure 4-5). The binding of HvGAMYB to the *Amy32b* promoter was decreased in the presence of increasing amounts of HvWRKY38 (Figure 4-7), suggesting that HvWRKY38 interferes with the binding of HvGAMYB to the *Amy32b* promoter. Accordingly, the induction of *Amy32b* by HvGAMYB was reduced gradually with increasing amounts of HvWRKY38 (Figure 4-6). The repression effect of HvWRKY38 was overcome by co-expressing of two transcriptional activators, SAD and HvGAMYB. WRKY38 and BPBF together block the combined effect of SAD and HvGAMYB on inducing *Amy32b* promoter in the absence of GA (Figure 4-8). Therefore, ratios of repressors to activators and cooperative binding of repressors or activators to the *Amy32b* promoter determine level of *Amy32b* expression (Figures 4-8 and -9).

In summary, the data present in this study support the hypothesis that the GA induction of *Amy32b* is modulated by two protein complexes, one for activation and the other for repression. Further work is needed to isolate these endogenous complexes using immuno-precipitation with antibodies against different regulators or the technique that combines tandem affinity purification with mass spectrometry (TAP-MS) (Dziembowski and Séraphin, 2004; Leene et al., 2007). With these methods, we will be able to understand the interactions among these transcription factors in regulating the GA-controlled gene expression.

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CHAPTER 5

GENERAL DISCUSSION

Larrea tridentata (creosote bush) survives exceptionally well in the arid desert where rainfall events only occur a few times each year. As a most drought-tolerant evergreen C3 shrub that dominates in the North American warm deserts, *Larrea tridentata* has received a great deal of attention (Smith et al., 1997; Nowak et al., 2004). *Hordeum vulgare* L. (barley) is the fourth most important cereal crop in the world (Brown et al., 2001), which has a broad ecological adaptation and is mostly produced in regions climatically unfavorable for growing other major cereals such as wheat, maize, and rice. Understanding the molecular mechanism underlying their resistance to environmental stresses and their response to elevated [CO_2] is socially, biologically, and agriculturally important. For my dissertation research, I took an approach that integrates physiology, bioinformatics, and molecular biology to addressing the functions of WRKY transcription factors in ABA and GA signaling. These two hormones directly or indirectly mediate seed dormancy, germination, and plant responses to elevated [CO_2] and environmental stresses. Our long-term goal is to improve the yield and quality of crops by enhancing their abilities to tolerate stresses and to combat against global warming by engineering plants that can consume [CO_2] at a massively enhanced rate.

ABA in plant responses to elevated [CO_2] and stresses

The importance of ABA in plant environmental stress responses has long been studied (Zeevaart, 1999; Xiong and Zhu, 2003). In Chapter 2, I show for the first time that like abiotic stresses, elevated [CO_2] treatments increased ABA concentrations in the leaves of *Larrea tridentata* (Figure 2-1). This enhancement might be due to increased biosynthesis of ABA, or decreased catabolism of ABA, or both. Future studies are necessary to address the following questions: 1) Does elevated [CO_2]-induced calcium increase activate ABA biosynthesis genes? It has been shown that the expression of ABA biosynthetic genes, *ZEP*, *AAO3*, and *MCSU* are up-regulated by ABA, drought, and salt stresses (Audran et al., 1998; Seo et al., 2000; Iuchi et al., 2001; Xiong et al., 2001a; Xiong et al., 2001b; Xiong et al., 2002), likely through a Ca^{2+}-dependent protein phosphorylation and dephosphorylation cascade (Xiong and Zhu, 2003). Webb et al. (1996) found that elevated [CO_2] treatments increased cytosolic free calcium in guard cells. 2) Does high [CO_2] modulate ABA levels through sugar sensing and signaling (Rolland et al., 2006)? Several ABA biosynthetic genes were found to be up-regulated by glucose (Cheng et al., 2002). Certain ABA-deficient mutants are also altered in glucose sensitivity (Leon and Sheen, 2003). The *gin1* (*glucose insensitive 1*) mutant is known to be allelic to *aba2* (Laby et al., 2000; Rook et al., 2001), an Arabidopsis mutant that lacks the activity of short-chain dehydrogenase/reductase (SDR1) needed to convert xanthoxin to abscisic aldehyde (Schwartz et al., 1997; Rook et al., 2001; Cheng et al., 2002; Gonzalez-Guzman et al., 2002). Moreover, *gin5* is allelic to *aba3* (Arenas-Huertero et al., 2000a), an Arabidopsis mutant that lacks the activity of aldehyde oxidase. 3) Is hexokinase (HXK) involved in plant responses to elevated [CO_2] and stresses? Several

lines evidence showed that HXK functions as a sugar sensor in mediating photosynthetic gene expression at ambient and elevated [CO_2], and in controlling multiple plant hormone-signaling pathways (Jang et al., 1997; Rolland et al., 2002; Leon and Sheen, 2003; Rolland et al., 2006). For example, HXK may associate with protein kinase/phosphatases to initiate a signaling response, resulting in the repression of the promoter activities of *RBCS* (*Rubisco small subunit*) (Jang and Sheen, 1994; Jang et al., 1997; Moore et al., 1999). Also, several sugar insensitive mutants are allelic to the ABA insensitive mutant *abi4* (Arenas-Huertero et al., 2000b; Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001). It was found that the *ABI3* (B3-domain transcription factor) and *ABI5* (bZIP transcription factor) genes are induced by 6% glucose in an ABA-dependent manner (Arenas-Huertero et al., 2000b; Laby et al., 2000; Cheng et al., 2002). Further experiments are needed to detect the enzyme activities of ZEP, AAO3, MCSU, and HXK in response to elevated [CO_2] and stresses. Moreover, in many species, catabolic inactivation of ABA is mainly catalyzed by 8'-hydroxylase and a P450 mono-oxygenase (Cutler and Krochko, 1999; Marion-Poll A, 2006). The regulation of these two genes by high [CO_2] will be investigated.

Microarray technology has empowered researchers to approach gene expression analysis on a genomic scale. The ability to study changes in the expression of thousands of genes simultaneously has made it possible to attain a global view of a cell's transcriptional state and to associate genes with predictive functions or specific physiological conditions (Freeman et al., 2000). With the availability of 3000 cDNA clones and EST database from *Larrea tridentata* (Zou et al., 2004), transcriptome analyses using microarray technology will help us to better understand the mechanisms

underlying plant response to elevated [CO_2] and stresses (Gupta et al., 2005; Ainsworth et al., 2006). In brief, mRNAs from *Larrea tridentata* grown under ambient [CO_2] vs elevated [CO_2] and normal vs stress conditions will be purified, then used for preparation of fluorescent Cy3-labeled and Cy5-labeled cDNA probes via reverse transcription using Cy3-dUTP and Cy5-dUTP, respectively. These cDNA probes are mixed and hybridized with the cDNA microarray containing 3000 cDNA clones from *Larrea tridentata*. Ratio measurements can be determined via quantification of 532 nm (Cy3) and 635 nm (Cy5) emission values. Based on the ratios, we can identify up-regulated or down-regulated genes in response to elevated [CO_2] and stresses.

WRKY proteins in plant response to elevated [CO_2] and stresses

Bioinformatics is quickly emerging as a scientific discipline that uses computational algorithm to solve biological problems (Andrade and Sander, 1997). Using bioinformatics programs, we can compare genes of unknown function to homologue genes in other organisms to help decipher their functions. Because transcription factors are master switches of gene regulation, alternations in their expression levels, activities, and/or functions, as opposed to those of structural genes, are more likely to have broader impacts on the resistance of plants to environmental stresses and hence on the speciation of *Larrea tridentata*. Therefore, I decided to search for drought stress-induced transcription factors. Based on a publication (Seki et al., 2002), forty-three protein sequences of Arabidopsis stress-inducible genes were collected and BLAST was used to search against the *Larrea tridentata* EST database (Zou et al., 2004). Ten *Larrea tridentata* putative stress-inducible transcription factors belonging to different families

have been identified (Table 3-1). We focused on WRKY transcription factors because they regulate plant responses to various stresses. My study indicates that the expression of *LtWRKY21* is induced by elevated [CO_2], drought, high salinity, and wounding (Figure 2-3), LtWRKY21 enhances the expression of an ABA- and stress-inducible gene, *HVA1*, and inhibits that of an ABA- repressible and GA-inducible *α-amylase* gene (Figure 2-5). The role of LtWRKY21 in plant response to elevated [CO_2] and stresses is proposed (Figure 5-1). *LtWKY21* is induced by elevated [CO_2] and stresses. This transcription factor functions as a transcriptional activator on the ABA pathway and a transcriptional repressor on the GA pathway.

An intriguing question is how LtWRKY21 from *Larrea tridentata* mediates plant responses to elevated [CO_2] and stresses. Several *cis*-acting elements are involved in sugar responses. For example, a W-box is found in the promoters of wheat, barley, and wild oat α-amylase genes (Rushton et al., 1995). Another motif frequently found in sugar-regulated promoters is the G-box (Chattopadhyay et al., 1998; Martinez-Garcia et al., 2000), which is very similar to the ABA-responsive element (Pla et al., 1993). These data suggest that W-boxes and G-boxes are common elements in diverse promoters for different signaling in transcriptional control (Rolland et al., 2002). The W-boxes are bound by WRKY transcriptional factors (Eulgem et al., 2000). In barley, a *WRKY* gene, *SUSIBA2* (a sugar responsive element binding factor), is involved in sugar signaling (Sun et al., 2003). Plants have intricate and complex signaling webs for responses to diverse environments. Some transcription factors may act as integration nodes in the webs, receiving signals from multiple signals and generating integrated plant responses (Rolland et al., 2002). Our data also show that the expression of *LtWRKY21* is enhanced

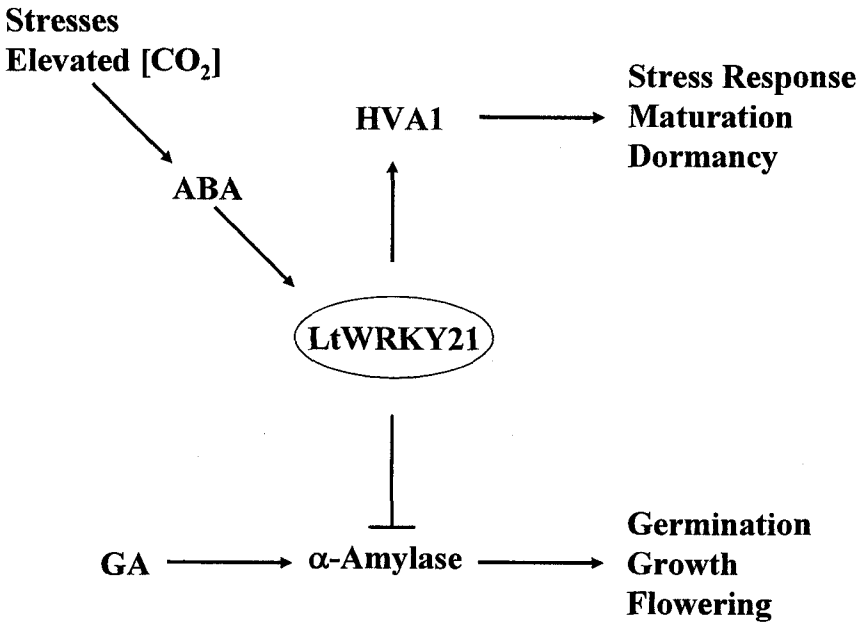

Figure 5-1. A proposed role of LtWRKY21 in response to elevated [CO_2] and stresses. LtWRKY21 is induced by elevated [CO_2] and stresses, and it functions as an activator on the ABA pathway and a repressor on the GA pathway.

by drought, high salinity, and wounding. However, cold and heat treatments promoted the degradation of wounding-induced *LtWRKY21* mRNA (Figure 2-3). Therefore, I propose that *LtWRKY21* mediates the plant response to elevated [CO_2] and stresses via an overlapping set of *cis*-acting elements and transcription factors. Recently, more stress-inducible genes were identified by using the Affimetrix 22K Gene Chip ATH1, and the data obtained are available from TAIR URL (http://www.arabidopsis.org/). A similar study could be carried out with plants treated with elevated [CO_2] and stresses. Functional analyses of these stress- and elevated [CO_2]-regulated genes using our transient expression system could be applied to further test this hypothesis.

Transgenic approaches provide powerful techniques to gain valuable information on the mechanisms that govern stress tolerance. They also open up new opportunities to improve stress tolerance by incorporating a gene involved in stress protection from any source into agriculturally or horticulturally important plants. Furthermore, transgenic approaches allow us to simplify studies on the mechanism governing stress tolerance by transferring a single gene into plants and observing the phenotypic, molecular, and biochemical changes before and after stress treatments (Bajaj et al., 1999; Zhang et al., 2004a). Because Arabidopsis has a small genome, a short life cycle, and is easy to be transformed, it has now been widely adopted as a model plant for biological research (Somerville and Koornneef, 2002). Over-expression of regulatory genes in transgenic plants can activate the expression of many stress tolerant genes simultaneously under stress conditions, hence enhancing plant tolerance to stresses. For example, Abe et al. (2003) transformed Arabidopsis with two transcriptional activators in ABA signaling, AtMYC2 and AtMYB2, driven by the constitutive cauliflower mosaic virus *35S* promoter.

Over-expression of these genes activated the expression of drought stress-tolerance genes, such as *rd29A*, and the transgenic plants were more tolerant to drought stress.

Our preliminary data indicate the Arabidopsis plants over-expressing the *LtWRKY21* gene survived the drought stress treatment much better than the control. In Figure 5-2, plants on the right carry *LtWRKY21* gene while those on the left do not. Over-expression of *HvWRKY21* did not appear to affect growth and development of the Arabidopsis plants (Panel A). Watering was withheld for one week from both plants (Panel B), all the wild-type plants died within a one-week period, whereas nearly all the transgenic plants survived this level of drought stress and continued to grow when rewatered (Panel C). We also found that over-expressing LtWRKY21 dramatically enhanced the germination rate of transgenic Arabidopsis seeds in the high NaCl (200 mM) medium. The transgenic plants also grew better than wild-type in the medium containing 100 mM NaCl (Figure 5-3). Further experiments are needed to confirm these results by using more *LtWRKY21* transgenic lines. Also, it will be extremely interesting to study the sensitivity of *35S-LtWRKY21* plants to [CO_2], in order to test the hypothesis that LtWRKY21 mediates plant responses to elevated [CO_2] and stresses. Furthermore, loss-of-function and complementation studies will help us to better understand the role of *WRKY* genes in plant responses to elevated [CO_2] and stresses. Because the life cycle of *Larrea tridentata* is very long and knock-out mutants are difficult to establish, loss-of-function study in *Larrea tridentata* is not applicable. Instead, we can knockout or knockdown the *LtWRKY21* gene by RNAi in suspension culture cells. Alternatively, we can further test the function of LtWRKY21 in Arabidopsis. LtWRKY21 is highly homologous to AtWRKY40, which is closely related to AtWRKY18 and AtWRKY60

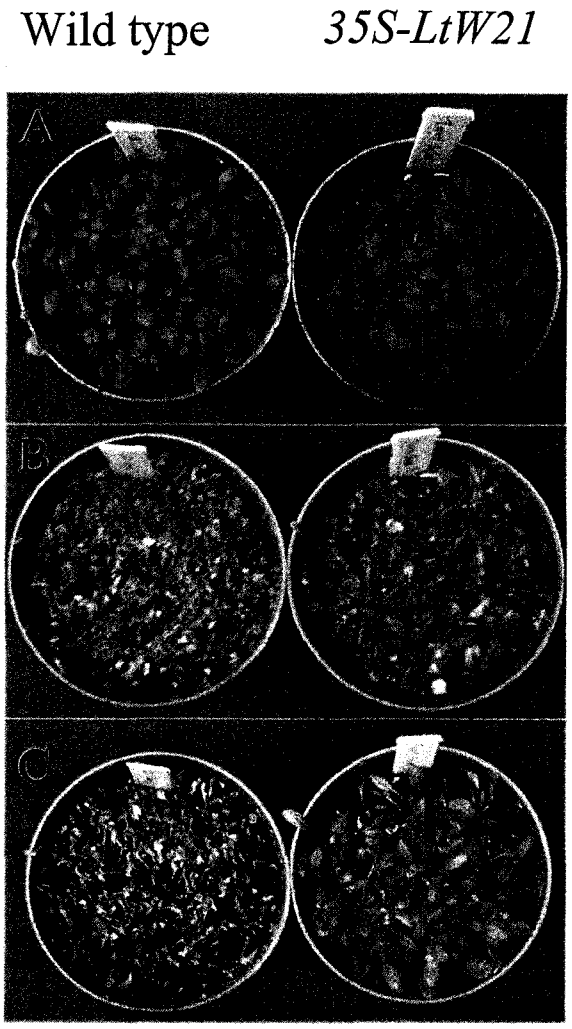

Figure 5-2. Over-expression of *LtWRKY21* enhances the drought tolerance of transgenic Arabidopsis plants.
(A), Transgenic and wild-type plants (n = 50 each) were grown on soil in the same tray for 2 weeks.
(B), Plant watering was withheld for 7 days.
(C), Plants were rewatered and the photographs were taken 2 hours after the rewatering.

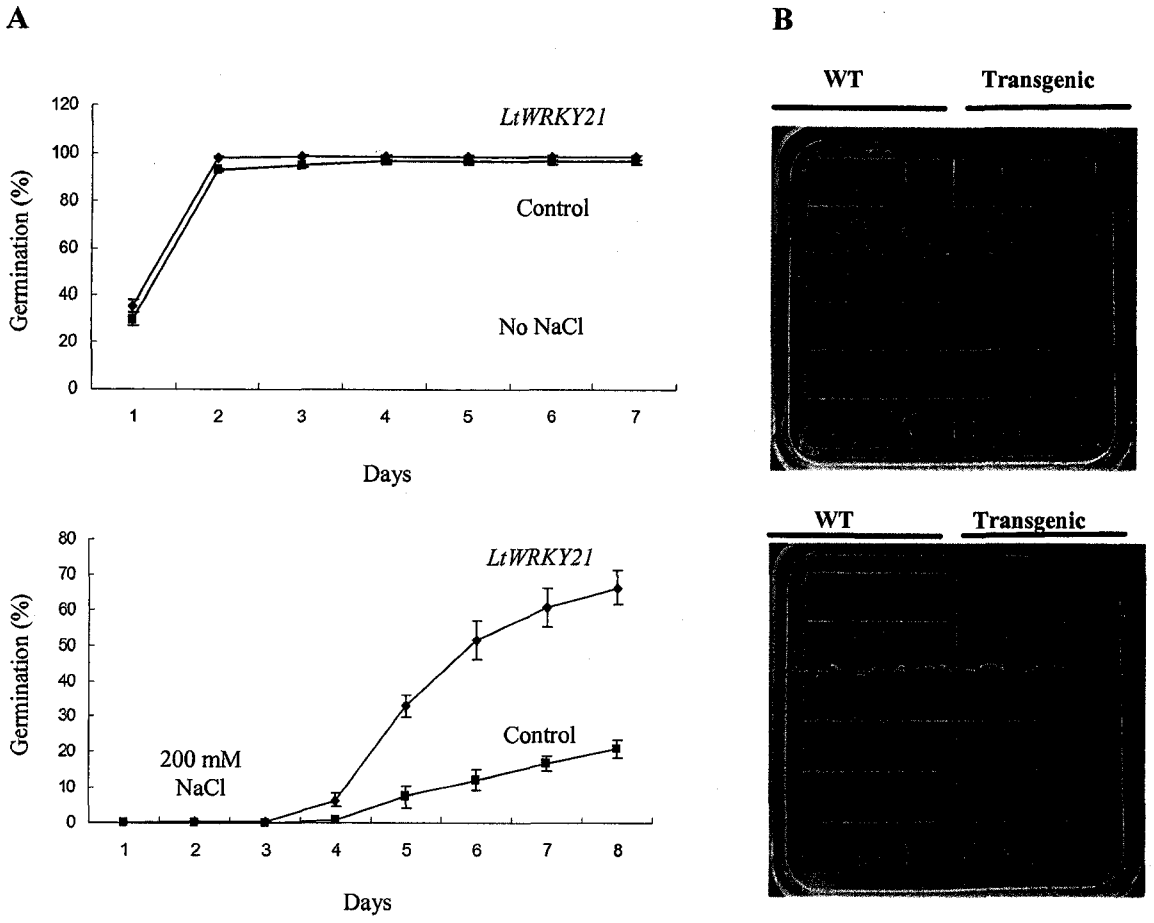

Figure 5-3. The germination rate and vegetative growth rate in the high NaCl medium are dramatically enhanced in Arabidopsis plants over-expressing *LtWRKY21*.
(A), Approximately 100 seeds each from the wild type (Columbia) and *LtWRKY21* transgenic lines were planted on ½ MS medium with 200 mM NaCl. Germination (emergence of radicles) was scored daily for 1 to 8 d.
(B), Wild-type and *LtWRKY21* transgenic seeds were planted on ½ MS medium with or without 100 mM NaCl. Photos were taken after 3 weeks.

(Xu et al., 2006). Our lab recently shows that the Arabidopsis *wrky18 wrky40 wrky60* triple mutant displays oversensitive phenotype to 100 mM NaCl (Shin and Shen, unpublished). It will be interesting to study whether elevated [CO_2] and drought have effects on this triple mutant. Furthermore, complementation study by transferring *LtWRKY21* gene into the *wrky18 wrky40 wrky60* triple mutant will allow us to test the hypothesis that the desert plant gene *LtWRKY21* functions better than its Arabidopsis homolog in protecting plants from stress-induced damages.

WRKY proteins in ABA signaling

In Chapter 3, I further dissect the function of LtWRKY21 as a positive regulator on the ABA pathway. Based on my finding, a hypothetical model of transcriptional control of *HVA22* expression is shown in Figure 5-4. ABA is perceived and transmitted through downstream components. ABA signaling is negatively regulated by a protein complex including protein phosphatases (ABI1 or ABI2) and positively regulated by transcription factors such as VP1/ABI3, ABI4, and ABI5. ABRC is composed of an ACGT-box ABRE and a coupling element CE1 in the *HVA22* promoter. Dimeric ABI5 binds to ABRE; ABI4 binds to CE1. LtWRKY21 cooperates with VP1/ABI3 and 14-3-3 to increase the binding affinity of ABI5 to ABRE and ABI4 to CE1, forming a transcriptional complex, to enhance *HVA22* expression.

One intriguing question is whether ABI4 and 14-3-3 are included in this complex with LtWRKY21, VP1/ABI3, and ABI5. Recently, the 14-3-3 proteins are shown to be important regulators in ABA signaling (van den Wijngaard et al., 2005; Schoonheim et al., 2007a; Schoonheim et al., 2007b;). In addition to interacting with VP1, 14-3-3 also

interacts with HvABI5 in mediating ABA signaling (Schoonheim et al., 2007a). VP1/ABI3 has been shown to potentiate ABA-inducible gene expression by forming a DNA-binding complex with bZIP, 14-3-3, zinc finger proteins, and the RNA polymerase II subunit RPB5 (Schultz et al., 1998; Hobo et al., 1999b; Jones et al., 2000; Kurup et al., 2000). Although VP1/ABI3 binds to the Sph/RY element (Suzuki et al., 1997) to activate the *C1* promoter in the absence of ABA (Kao et al., 1996), VP1/ABI3 also can enhance the transcription of the ABRC-containing promoters that lacks an Sph/RY element (Shen et al., 1996; Hobo et al., 1999a; Gampala et al., 2002; Casaretto and Ho, 2003). Our data suggest that LtWRKY21, VP1, and ABI5 may form a complex that functions downstream of ABI1 in ABA signaling. In addition, only two elements are present in the *HVA22* promoter: the ABRE and CE3 are bound by ABI5 or its related bZIP proteins (Guiltinan et al., 1990; Oeda et al., 1991; Kim et al., 1997; Choi et al., 2000; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Uno et al., 2000; Kang et al., 2002; Casaretto and Ho, 2003) and CE1 is bound by ABI4, an APETALA2 domain containing transcription factor (Finkelstein and Lynch, 2000; Niu et al., 2002). Therefore, it is unlikely LtWRKY21 can bind to the HVA22 promoter fragment. Further experiments are needed to address whether LtWRKY21 can physically interact with VP1, ABI5, ABI4, and 14-3-3 by using BiFC, yeast-two hybrid, and gel shift methods. Moreover, immuno-precipitation with antibodies against different co-regulators can be applied to further dissect protein complexes in regulating the expression of *HVA22*.

Another intriguing question is how to regulate the transactivating activity of LtWRKY21 protein. A striking feature of ABI3 and ABI5 is that they are subject to, at least in part, control by proteolysis. ABI3 is specifically targeted *in vivo* by the E3 ligase

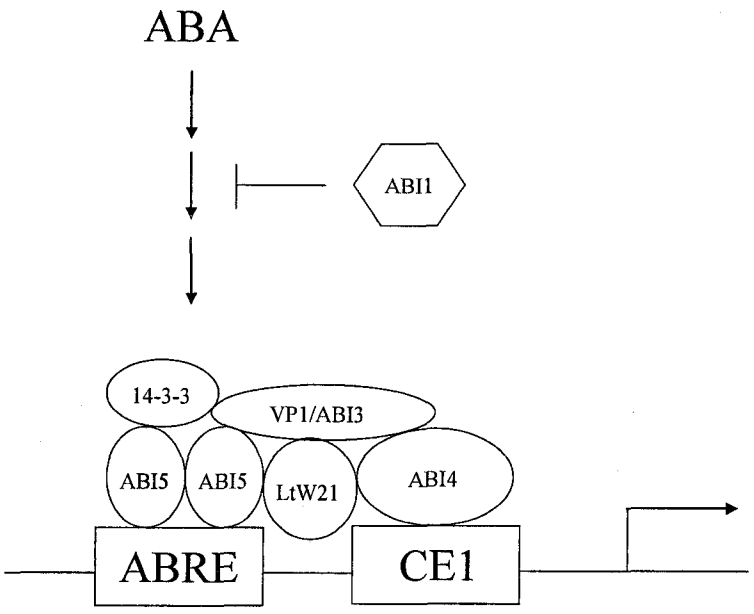

Figure 5-4. A hypothetical model of transcriptional control of *HVA22* expression by a protein complex including LtWRKY21.
ABA is perceived and transmitted through downstream components. ABA signaling is negatively regulated by a protein complex including protein phosphatases (ABI1 or ABI2), and positively regulated by transcription factors such as VP1/ABI3, ABI4, and ABI5. ABRC is composed of an ACGT-box ABRE and a coupling element CE1 in *HVA22* promoter. Dimeric ABI5 binds to ABRE; ABI4 binds to CE1. LtWRKY21 cooperates with VP1/ABI3 and 14-3-3 to increase the binding affinity of ABI5 to ABRE and ABI4 to CE1, forming a transcriptional complex, which enhances *HVA22* expression.

AIP2 (Zhang et al., 2005). ABI5 is targeted by AFP to proteasomes that also include a RING-type E3 ligase (Lopez-Molina et al., 2003; Stone et al., 2006). Conversely, ABI5 is stabilized by phosphorylation by an ABA-activated kinase that could be MAP kinase (MPK3) (Lu et al., 2002). In addition, sucrose nonfermenting1-related protein kinases (SnRKs) can function as activators of ABA signaling in rice (Kaneko et al., 2004), wheat (Johnson et al., 2002), and Arabidopsis (Fujii et al., 2007). It is possible that SnRKs activate ABRE-driven gene expression through the phosphorylation of ABFs (ABA response element binding factors) such as ABI5 (Fujii et al., 2007). Analysis of nuclear extracts from barley suspension culture cells demonstrated the existence of a preformed WRKY protein pool (Turck et al., 2004). Several WRKY proteins migrate as adjacent pearl strings, suggesting that the same WRKY proteins exist in different posttranslationally modified forms. The possible posttranslational modification and degradation of LtWRKY21 will be the focus of future work.

Deletion and substitution studies of LtWRKY21 show that the C-terminal region, which contains a WRKY domain (including a zinc-finger motif), is required for LtWRKY21 to transactivate the expression of the *HVA22* promoter. Interestingly, the EAR (ERF-associated amphiphilic repression) motif in LtWRKY21 is also essential for its transactivation activity (Figure 3-6). The EAR motif is necessary for the repression function of AtERF3, AtERF4, and their orthologues in ethylene signaling of Arabidopsis (Fujimoto et al., 2000), wheat, and petunia plants (Ohta et al., 2001). Mutant alleles of the ethylene signaling genes *EIN2* and *CTR1* have been identified as a suppressor and an enhancer of the ABA-insensitive *abil-1* allele, respectively (Ghassemian et al., 2000). These data illustrate the fact that ABA and ethylene antagonize each other during seed

germination and seedling development. Future studies are needed to address whether LtWRKY21 is involved in ethylene signaling.

WRKY proteins in GA signaling

My research also focuses on the understanding of how WRKY proteins cooperate with other transcription factors to control the expression of α-amylases in aleurone cells. In Chapter 4, we first show that HvWRKY38 represses the transactivating activity of HvGAMYB by interacting and competing with HvGAMYB binding to the *Amy32b* α-amylase promoter (Figures 4-5, -6, and -7), and this inhibitory effect of HvWRKY38 can be overcome by the SAD and HvGAMYB transactivating activators (Figure 4-8). However, co-expression of WRKY38 and BPBF blocks the combined effect of SAD and HvGAMYB on inducing the *Amy32b* promoter in the absence of GA (Figure 4-8).

Five *cis*-acting elements in the promoter of the *Amy32b* low pI α-amylase gene have been identified to be essential for its high-level expression. Four of these elements, O2S/W-box, pyrimidine (Pyr) box, GA response element (GARE), amylase box (Amy), and a down-stream amylase element (DAE), can be bound by one or more trans-activator(s) and trans-repressor(s) (Lanahan et al., 1992; Rogers and Rogers, 1992; Rogers et al., 1994; Gómez-Cadenas et al., 2001). Based on our and others' studies, a hypothetical model of transcriptional control of *Amy32b* expression is proposed and shown in Figure 4-8. In the absence of GA, negative regulators such as HvWRKY38, BPBF, HRT, and HvMCB1 bind to corresponding *cis*-acting elements and form a "repressome", which diminishes the binding or transactivating activities of positive regulators to the promoter, preventing transcription. Upon germination of barley seeds,

GA blocks the expression of the repressor genes, enhances the degradation of their mRNAs and protein products, and/or disrupts their binding to corresponding *cis*-acting elements. Simultaneously, GA-induced positive regulators such as RAMY, SAD, HvGAMYB, and HvMYBS3 bind to the same set of DNA sequences and form an "activatesome", leading to a high level of *α-amylase* gene expression. This model can be tested using tandem affinity purification combined with mass spectrometry (TAP-MS) (Figure 5-5), which allows the analysis of low abundant protein complexes under near-physiological conditions (Dziembowski and Seraphin, 2004; Van Leene et al., 2007). Matrix-associated laser desorption/ionization time-of-flight MS (MALDA-TOF-MS) is the most commonly used MS approach to identify proteins of interest excised from 2-D gels by generating peptide mass fingerprinting for the proteins (Landry et al., 2000; Aebersold and Mann, 2003). This method is based on two successive affinity chromatography steps. The tag fused to a target protein is composed of protein A that has a very high affinity for IgG, a TEV protease cleavage site, and a calmodulin binding peptide that has a high affinity for calmodulin. An extract containing the TAP-tagged protein is mixed with IgG beads, then the target protein is released by TEV protease cleavage. The eluate is further incubated with calmodulin resin in the presence of calcium that is essential for calmodulin binding. Finally, the binding complex is released by EGTA chelating calcium ion. The TAP-MS technique can be applied to study the "repressome" and "activatesome" controlling seed dormancy and germination (Figure 4-8).

In addition, future studies are needed to address the following questions: 1) Which domain of HvWRKY38 is necessary for its interaction with HvGAMYB? A

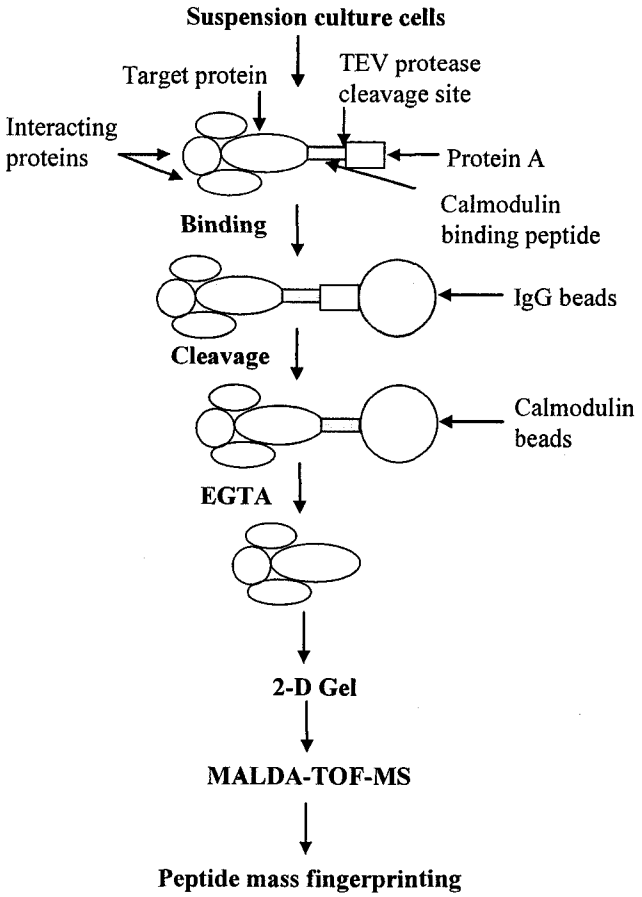

Figure 5-5. Strategy for identifying a protein complex.
The recombinant gene containing target protein and peptide tag coding sequences is introduced and expressed in the protoplasts. The tag fused to a target protein is composed of protein A that has a very high affinity for IgG, a TEV protease cleavage site, and a calmodulin binding peptide that has a high affinity for calmodulin. An extract containing the TAP-tagged protein is mixed with IgG beads, then the target protein is released by TEV protease cleavage. The eluate is further incubated with calmodulin resin in the presence of calcium which is essential for calmodulin binding. The binding complex is released by EGTA chelating calcium ion. The protein complex is separated by 2-D gel electrophoresis, and isolated subunits are identified by mass spectrometry.

putative leucine-zipper motif is present at the N-terminus of HvWRKY38. BiFC and EMSA experiments using mutants lacking the leucine-zipper motif might answer this question. 2) Are there any positive regulators binding to O2S/W-box of *Amy32b* promoter? Two negative regulators and two positive regulators have been identified to be involved in ABA signaling through functional analyses of rice WRKY transcription factors in aleurone cells (Xie et al., 2005). It is possible to find WRKY positive regulators on the GA pathway. Another possible candidate is RAMY, an unusual zinc finger protein, which has been shown to bind to the W-box/O2S *cis*-acting element (Peng et al., 2004). 3) Does HvWRKY38 physically and functionally interact with R1MYB transcription factors, such as HvMCB1 and HvMYBS3? HvMCB1 has been demonstrated to repress transcription of the *AMY6.4* promoter and reverse the HvGAMYB-mediated activation of this amylase promoter (Rubio-Somoza et al., 2006b). In contrast, HvMYBS3 transactivates the expression of *AMY6.4* (Rubio-Somoza et al., 2006a). 4) Does any regulator contact the DAE *cis*-acting element? DAE has been shown to be necessary for high level expression of α-amylase, but the mechanism in mediating GA signaling is still not clear (Gómez-Cadenas et al., 2001).

Another intriguing question is how GA controls the transcript level of HvWRKY38. The expression of HvWRKY38 is up-regulated by ABA and down-regulated by GA. Over-expression of an ABA-inducible *microRNA159* (*miR159*) suppresses the expression of two *MYB* genes, *MYB33* and *MYB101*, during Arabidopsis seed germination (Reyes and Chua, 2007). Also, OsGAMYB and OsGAMYB-like genes are negatively regulated by *miRNA159* in flowers (Tsuji et al., 2006). Is a microRNA involved in regulating HvWRKY38? Up to 46 miRNA families are found in Arabidopsis

and rice, encoding 117 and 178 miRNA genes, respectively (Bonnet et al., 2006). Bioinformatic prediction (http://sundarlab.uvdavis.edu/smrnas/), followed by wet-bench experiment could address whether microRNAs are involved in the regulation of *HvWRKY38* gene expression.

It is also necessary to address how GA removes the repression of the HvWRKY38 protein and other repressors to promote α-amylase expression and seed germination. We showed that GA promotes degradation of GFP:OsWRKY71 but not GFP: HvWRKY38 in barley aleurone cells by semi-quantitative confocol microscopy (Zhang et al., 2004b; Figure 5-6). It is necessary to confirm this result by studying the endogenous protein levels of HvWRKY38 in response to GA, ABA, and proteasome inhibitors. Recent discoveries showed that the activated GA receptor, GID1, triggers the degradation of the DELLA protein by an E3 ubiquitin ligase SCF complex through the ubiquitin-26S proteasome pathway (Itoh et al., 2003; Sun and Gubler, 2004; Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Nakajima et al., 2006; Willige et al., 2007). Future studies are needed to address whether GID1 interacts with HvWRKY38 and hence promotes the degradation of HvWRKY38. In addition, a yeast-two-hybrid assay can be applied to pull out potential HvWRKY38 regulators that are related to the ubiquitin pathway (Dreher and Callis, 2007). Furthermore, it has been demonstrated that SLN1, a barley DELLA protein, is necessary for repressing HvGAMYB (Gubler et al., 2002). However, very little is known about immediate downstream targets of SLN1 repression. SLN1 protein levels decline rapidly in response to GA before any increase in HvGAMYB levels, suggesting HvGAMYB may not be an immediate downstream target. It has been reported that SLR1, a DELLA protein in rice, may function as a transcrip-

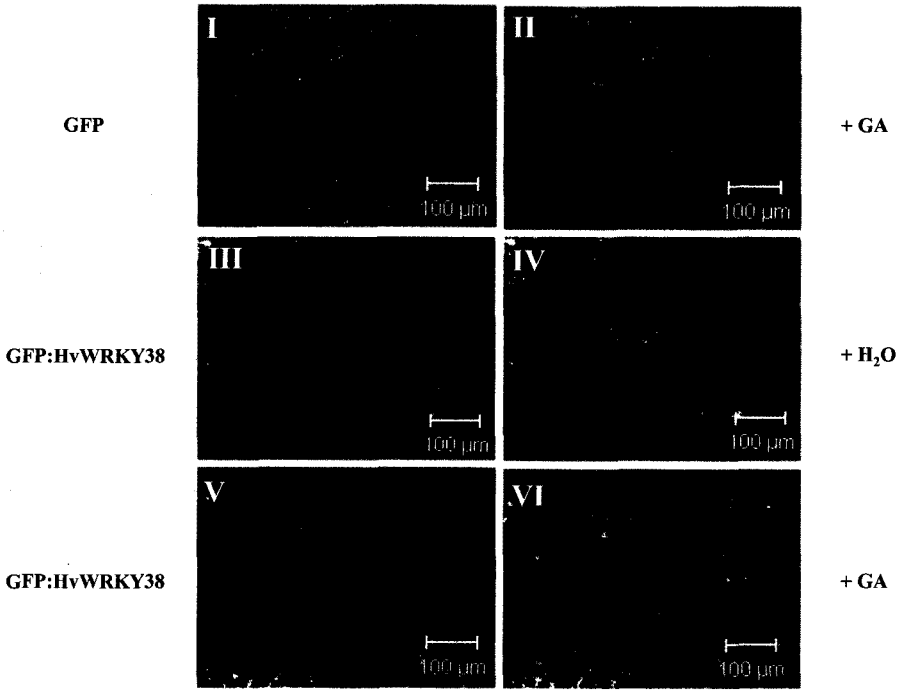

Figure 5-6. Exogenous GA treatment does not promote HvWRKY38 degradation in aleurone cells.

Embryoless half-seeds were transformed with *UBI-GFP* or *UBI-GFP:HvWRKY38*. After incubation for 24 h, the aleurone layers were treated with water or 100 µM GA for 12 h, followed by examination of GFP fluorescence under confocal microscope. Panels I, II and III, the green fluorescence from GFP control or GFP:HvWRKY38 fusion protein, respectively, before GA treatment. Other panels show the images of the same scopes after GA treatment (II and VI) or water-treated control (IV). The bars represent 100 µm.

tional activator (Ogawa et al., 2000). It is likely that SLN1 and other related protein may act as transcriptional activators of a repressor that inhibits the expression of HvGAMYB (Gubler et al., 2002). Future studies are needed to address whether SLN1 interacts with HvWRKY38 in regulating the expression of HvGAMYB. Yeast-two hybrid assay or the TAP-MS method described above can be applied to address these interactions.

In this dissertation study, I showed ABA levels in *Larrea tridentata* leaves are increased in response to water deficit and elevated [CO_2]. I then found a novel transcription factor, LtWRKY21, which functions as a key regulator in mediating plant response to elevated [CO_2] and stresses. Functional study of LtWRKY21 supports a novel model that LtWRKY21, VP1, and ABI5 form a complex that functions downstream of ABI1 on ABA signaling. Furthermore, I proposed a model for the crosstalk of ABA and GA in controlling seed germination and dormancy. Molecular and genomic studies have shown that plants have intricate and complex regulatory networks controlling gene expression (Rolland et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). New techniques developed in transcriptomics, proteomics, and interactomics, such as microarray, high-throughput yeast two-hybrid, and high-throughput TAP-MS, offer remarkable promise as tools to study and understand the mechanisms of the regulatory network in a genome-wide level (Fields, 2005; Singh and Nagaraj, 2006; Van Leene et al., 2007). The knowledge gained from this study will shed light on the mechanism controlling seed dormancy and germination as well as plant responses to elevated [CO_2] and environmental stresses. This research will eventually lead to improvement of crop yields and development of strategies to combat against global warming that is resulted

from elevated atmospheric [CO_2]. Moreover, it will contribute to sustainable urbangrowth by developing water-saving plants for landscaping.

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