Chapter 2
Cross-Talk Between Phytohormone Signaling Pathways Under Both Optimal and Stressful Environmental Conditions

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Abstract The perception of abiotic stress triggers the activation of signal transduction cascades that interact with the baseline pathways transduced by phytohormones. The convergence points among hormone signal transduction cascades are considered cross-talk, and together they form a signaling network. Through this mechanism, hormones interact by activating either a common second messenger or a phosphorylation cascade. This chapter reviews kinase cascades as cross-talk points in hormonal networks during abiotic stress conditions. These transduction cascades lead to the regulation of gene expression that directly affects the biosynthesis or action of other hormones. Examples of stress-related hormone transduction networks are provided for drought and wounding conditions. The expression of specific genes associated with drought and wounding stress will be compared with expression changes that occur during other abiotic stress conditions. This evaluation will be used to construct a model of abiotic stress signaling that incorporates the signaling components that are most common across all abiotic stress conditions and are, therefore, relevant to developing stress tolerance in crop plants.

Abbreviations

ABA	Abscisic acid
ABF	ABA-responsive element-binding factor
ABI	ABA insensitive
ABRE	ABA-responsive element
ACO	1-Aminocyclopropane-1-carboxylate oxidase

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ACS	1-Aminocyclopropane-1-carboxylic acid synthase
AGO1	Argonaute1
AP2C1	*Arabidopsis* Ser/Thr phosphatase of type 2C
AtHK1	*Arabidopsis* histidine kinase 1
CDPK	Calcium-dependent protein kinase
CKX	Cytokinin oxidase/dehydrogenase
CPK	Calcium-dependent protein kinase gene/protein abbreviation
EIN3	Ethylene insensitive
EREBP	Ethylene-responsive element-binding protein
ERF	Ethylene-response factor
GORK	Guard cell outward-rectifying K^+
GST	Glutathione-*S*-transferase 1
GUS	β-glucuronidase
HK	Histidine kinase
IP_3	Inositol trisphosphate
JA	Jasmonic acid
KAT	K+ channel in *Arabidopsis thaliana*
MAPK	Mitogen-activated protein kinase
MeJA	Methyl JA
miRNA	MicroRNA
MKK	MAPK kinase
MPK	MAPK gene/protein abbreviation
NO	Nitric oxide
OPR3	12-oxophytodienoat-10,11-reductase
PP2C	Protein phosphatase 2C
PYR	Pyrabactin (4-bromo-N-[pyridin-2-yl methyl] naphthalene-1-sulfonamide) resistance
RCAR	Regulatory component of ABA receptor
ROS	Reactive oxygen species
RSRE	Rapid stress response element
RWR	Rapid wound response
SEN1	Senescence-associated protein 1
SLAC1	Slow anion channel-associated 1
SnRK2	Sucrose nonfermenting 1-related protein kinase
TCH3	Touch-induced 3

2.1 Introduction

In plants, the perception of abiotic stress triggers the activation of signal transduction cascades that interact with the baseline pathways transduced by phytohormones. The convergence points among hormone signal transduction cascades are considered cross-talk, and together they form a signaling network. Through this mechanism, hormones interact by activating either a common second

messenger or a phosphorylation cascade. This chapter reviews kinase cascades as cross-talk points in hormonal networks during abiotic stress conditions. These transduction cascades lead to the regulation of gene expression that directly affects the biosynthesis or action of other hormones, a process that represents an additional layer of hormonal cross-talk also addressed here.

Examples of stress-related hormone transduction networks are provided for drought and wounding conditions. The expression of specific genes associated with drought and wounding stress will be reviewed and compared with expression changes that occur during other abiotic stress conditions. This evaluation will be used to construct a basic model of abiotic stress signaling that incorporates the signaling components that are most common across all abiotic stress conditions and are, therefore, relevant to developing stress tolerance in crop plants.

2.2 Drought Stress

Information regarding the regulation of drought stress comes from experiments that directly examine either drought (induced by dehydration causing loss of turgor) or osmotic stress (induced by increasing extracellular solute concentration, which also results in turgor loss), and from inferences based on experiments involving the functions of hormones and other signals. In a meta-analysis of more than 450 papers concerning the role of drought in photosynthesis, the authors conclude that experimental data are often disjointed or not comparable, making it difficult to discern general trends (Pinheiro and Chaves 2011). However, changes in gene expression induced by drought stress certainly overlap with hormone-regulated gene expression. Therefore, this discussion will present data from studies of hormone action as well as from those that focus on events that occur during drought stress.

Stomatal closure is a rapid response to drought stress and is regulated by a complex network of signaling pathways. During drought stress, abscisic acid (ABA) is considered to be the primary phytohormone that triggers short-term responses such as stomatal closure (Zhang et al. 2006). ABA controls longer-term growth responses through the regulation of gene expression that favors maintenance of root growth, which optimizes water uptake (Zhang et al. 2006). As part of the regulation of drought stress responses, ABA may interact with jasmonic acid (JA) and nitric oxide (NO) to stimulate stomatal closure, while its regulation of gene expression includes the induction of genes associated with response to ethylene, cytokinin, or auxin. ABA-mediated regulation of signal transduction pathways during drought stress is presented here as an example to illustrate the signal transduction elements that may play roles in cross-talk associated with the response to drought stress.

2.2.1 Stomatal Closure in Response to Drought Stress

ABA undergoes a dramatic increase in concentration after drought stress (Hoad 1975), and studies indicate that ABA is the major regulatory hormone that controls drought stress-induced stomatal closure (Finkelstein and Rock 2002).

The increase in ABA concentration in guard cells is trigged by the reduction in the amount of water around the roots. During drought conditions, dehydration of root tissue is sensed by a drought-specific histidine kinase (HK) osmoreceptor. In *Arabidopsis* plants grown under normal conditions, ATHK1, a transmembrane, two-component histidine kinase, is expressed at a higher level in the roots than in the stems and leaves. Loss-of-function *athk1* mutants produced lower levels of endogenous ABA, indicating that the increase in ABA synthesis during osmotic stress is dependent upon sensing of drought conditions by ATHK1 (Wohlbach et al. 2008). *ATHK1* expression greatly increases, and its protein accumulates to a high level, in root tissue experiencing drought stress conditions (Urao et al. 1999). ATHK1 is a positive regulator of the drought stress response and activates many downstream targets, including ABA biosynthetic enzymes (Wohlbach et al. 2008) and ABA-responsive transcription factors (Tran et al. 2007).

2.2.1.1 ABA Accumulation in Guard Cells Regulates Stomatal Closure

Changes in ABA concentration in response to drought stress vary dramatically from tissue to tissue. Using an ABA-dependent reporter construct, Christmann et al. (2005) demonstrated a basic time course for drought-induced ABA accumulation in tissues of *Arabidopsis* seedlings whose roots were subjected to a −1.0 MPa water stress treatment. An increase in ABA concentration in the vascular tissue of the cotyledons was observed by 4 h after treatment (Christmann et al. 2005). After 4 h, ABA was relatively uniformly distributed in the leaf tissue, but by 8 h posttreatment, a higher concentration of ABA was present in guard cells than in other leaf tissue. However, within this 8-h time frame, virtually no change in ABA concentration was detected in the roots. Therefore, the activation of ATHK1 in root tissue may trigger a hydraulic signaling system, proposed by Christmann et al. (2007), that acts as a long-distance signal to stimulate ABA production in the vascular parenchyma and guard cells. Other studies confirm that vascular parenchyma cells contain the enzymes associated with ABA synthesis, which thus leads to the high concentration of ABA in the vascular tissue after drought stress (Koiwai et al. 2004; Endo et al. 2008).

From the vascular tissue, a specific type of ATP-binding cassette transporter exports ABA (Kuromori et al. 2010), while another transporter imports ABA into leaf tissue, including guard cells (Umezawa et al. 2010). In addition, the increase in ABA concentration may in part originate from the release of active ABA from its ABA–glucose ester conjugate, which is stored in the vacuoles of leaf cells and can also circulate in the plant (Wasilewska et al. 2008; Seiler et al. 2011).

2.2.1.2 ABA Regulates the Activity of Ion Channels

ABA regulates several types of ion channels, causing anion efflux, K^+ efflux, and the inhibition of K^+ import (Fig. 2.1). One mechanism of ABA action begins when ABA binds to receptors belonging to a protein family known as PYR/RCARs, the pyrabactin (4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide) resistance (PYR)/regulatory component of ABA receptor (RCAR) (Hubbard et al. 2010). In *Arabidopsis*, 14 highly conserved PYR/RCARs have been identified. Upon binding to ABA, PYR/RCARs inhibit the activity of specific protein phosphatase 2Cs (PP2Cs), which are negative regulators of ABA signaling (Hubbard et al. 2010). The inactivation of PP2C allows for the phosphorylation and activation of three sucrose nonfermenting 1-related protein kinase 2s (SnRK2s), which belong

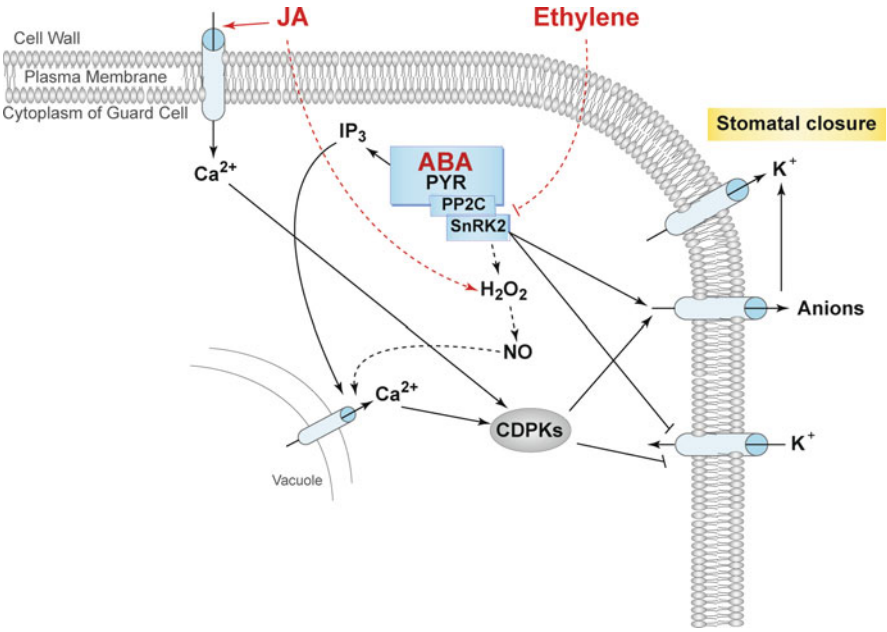

Fig. 2.1 This model illustrates the interactions between phytohormone signal pathways during ABA-induced stomatal closure as a result of drought stress. Drought triggers an increase in ABA concentration in guard cells, where it becomes bound to its receptor complex, in which SnRK2 is activated. The SnRK2 phosphorylates and inactivates a potassium import channel and activates an anion efflux channel, which in turn simulates K^+ efflux. SnRK2 also activates enzymes involved in H_2O_2 production; the resultant H_2O_2 activates NO that then triggers the influx of Ca^{2+} from the vacuole into the cytoplasm. The Ca^{2+} activates CDPKs, which then stimulate anion efflux and inhibit K^+ influx. The resulting ion loss causes water efflux, loss of turgor, and stomatal closure. In addition, Ca^{2+} influx from the vacuole into the cytoplasm is induced by ABA-mediated production of IP_3. Stress-induced JA production interacts with ABA-mediated stomatal closure by stimulating the influx of extracellular Ca^{2+} and/or by activating H_2O_2/NO signaling. Ethylene acts as a negative regulator of ABA, and thus, of this pathway. Steps inferred from experiments exploring ABA function using turgid cells are indicted with *dashed lines*

to the SnRK2 subfamily, whose members are associated with the regulation of abiotic stress (Fujita et al. 2009). While the precise mechanism of SnRK2 activation is unknown, it has been shown to autophosphorylate. SnRK2 acts as a positive regulator of several targets on the plasma membrane, including the anion exporter slow anion channel-associated 1 (SLAC1) and the K^+ channel in *Arabidopsis thaliana* (KAT1) in guard cells (Hubbard et al. 2010). SnRK2 activates SLAC1 through phosphorylation, while phosphorylation of KAT1 by SnRK2 inhibits its function, thus decreasing the influx of K^+ into the cell (Fig. 2.1). Increased SLAC1 activity causes an efflux of anions, which depolarizes the membrane and results in the loss of K^+ through the depolarization-activated K^+ efflux channel called guard cell outward-rectifying K^+ (GORK) (Jeanguenin et al. 2008) (Fig. 2.1). The collective loss of anions and K^+ ions from the guard cells causes water to move out of these cells, which results in the reduction in turgor that triggers stomatal closure in response to ABA.

2.2.1.3 ABA Activates Ca^{2+} Signaling Pathways

Ca^{2+} influx into the cytoplasm of guard cells has been observed within minutes after ABA treatment (Schroeder and Hagiwara 1990). This influx may occur through the release of the second messenger inositol 1, 4, 5-trisphosphate (IP_3), which activates Ca^{2+} channels located in the vacuole and endoplasmic reticulum (Krinke et al. 2007; Kwak et al. 2008) (Fig. 2.1). The influx of Ca^{2+} into the cytoplasm initiates a number of Ca^{2+}-dependent events associated with ABA signal transduction.

Numerous Ca^{2+}-dependent protein kinases (CDPKs) are activated during drought stress conditions and control stomatal closure through the regulation of ion channels (Fig. 2.1). In ABA-associated regulation of SLAC1, SnRK2 inhibits the phosphatase ABA insensitive 1 (ABI1), a negative regulator of CPK21. Therefore, ABI1 inactivation allows the activation of CPK21, which phosphorylates SLAC1, and thus activates anion efflux (Geiger et al. 2010). In *Arabidopsis* leaves, the concentration of another CDPK, CPK10, increases within 30 min after drought stress begins and causes the inhibition of inward K^+ currents (Zou et al. 2010). These results indicate that an increase in the cytoplasmic concentration of Ca^{2+} stimulates Ca^{2+}-dependent pathways that inhibit K^+ import while activating SLAC1, triggering the membrane depolarization that activates K^+ efflux (Fig. 2.1). CDPK pathways thus contribute to the loss of ions from guard cells, which in turn results in the loss of turgor, and ultimately to stomatal closure.

2.2.1.4 H_2O_2 and NO Are Associated with ABA in the Regulation of Stomatal Closure

An increase in oxidative stress is a common result of most abiotic stress conditions, including drought (Jaspers and Kangasjärvi 2010), and is often associated with an increase in NO production (Neill et al. 2008). While there is considerable evidence

for the roles of H_2O_2 and NO in ABA-mediated stimulation of stomatal closure, their roles in signaling associated with drought stress remains unclear (Neill et al. 2008; Sirichandra et al. 2009). Studies of ABA action demonstrate that ABA-mediated regulation of stomatal closure requires NO and H_2O_2 (Bright et al. 2006). In these studies, both H_2O_2 and ABA treatments stimulated NO synthesis within 25 min after drought induction and resulted in stomatal closure in leaf tissue within 2.5 h (Bright et al. 2006). ABA-stimulated stomatal closure was reduced when NO was removed, and in mutants with impaired NO biosynthesis. Likewise, mutants that lack a key enzyme for H_2O_2 production demonstrated reduced NO production and reduced stomatal closure. In addition, an ABA-induced SnRK2 activates a guard cell NADPH oxidase that releases H_2O_2 (Sirichandra et al. 2009; Hubbard et al. 2010). These results demonstrate both the complex interactions between these molecules and that ABA regulation requires the production of H_2O_2 to stimulate NO production (Fig. 2.1).

2.2.1.5 Cross-Talk Between ABA and Ethylene Involves H_2O_2 and NO

An active interaction between ethylene and ABA has been shown to control the regulation of stomatal closure. For instance, an elevated ethylene concentration in leaves inhibits ABA-induced stomatal closure (Tanaka et al. 2005). In drought-stressed *Arabidopsis ethylene overproducer 1* mutants, stomata closed more slowly and were less sensitive to ABA; in addition, ethylene applied to ABA-treated epidermal peels from wild-type *Arabidopsis* leaves inhibited stomatal closure (Tanaka et al. 2005). When ethylene was applied independently of ABA, it induced H_2O_2 synthesis within 30 min after treatment (Desikan et al. 2006). Using ethylene-response mutants, Desikan et al. (2006) provided evidence that stomatal closure by ethylene is regulated through its signal transduction pathway, which both stimulates production and requires H_2O_2 synthesis.

Direct evidence that ethylene production increases as a result of drought stress has been inconsistent, with reports of both increased ethylene production and ethylene inhibition after drought or osmotic treatment (Abeles et al. 1992). However, most studies that report increased ethylene production use detached leaves, and thus may not reflect the response in intact plants under drought conditions (Morgan et al. 1990). In studies using intact cotton plants, ethylene production was consistently lower in drought-exposed plants than in plants that were watered daily (Morgan et al. 1990). In addition, ABA appears to inhibit ethylene production, and ABA-deficient maize seedlings have greatly increased ethylene production (Sharp 2002). Therefore, the dramatic increase in ABA concentration that occurs under drought stress probably causes a reduction in ethylene production. Conversely, ethylene concentration may increase in response to other stress conditions, which could interfere with ABA-regulated stomatal closure. Wilkinson and Davies (2009) propose that the impact of the interaction between ethylene and ABA is dependent upon the oxidative stress load of the plant. In their model, ethylene and ABA can act independently to induce the formation of reactive oxygen species (ROS), such as

H_2O_2, which then stimulate NO production and stomatal closure. However, when ethylene is present in combination with high ABA levels, ABA-regulated stomatal closure is inhibited (Fig. 2.1). Therefore, as part of the stress response, ethylene may act as a feedback mechanism to allow the influx of some CO_2 for photosynthesis, even during extreme stress conditions (Tanaka et al. 2005; Neill et al. 2008).

2.2.1.6 JA Cross-Talk Involves Protein Kinase Phosphorylation Cascades

JA biosynthesis is induced by stress conditions such as wounding and herbivory (Wasternack 2007), and many JA-associated signaling genes are regulated by drought stress (Huang et al. 2008). JA interacts with ABA-regulated stomatal closure by increasing Ca^{2+} influx, which stimulates CDPK production and the resultant signal cascade. Treatment of turgid, excised *Arabidopsis* leaves with either ABA or methyl JA (MeJA) results in stomatal aperture reduction within 10 min (Munemasa et al. 2007). Inhibition of ABA biosynthesis by using chemical inhibitors or ABA-deficient mutants suppresses MeJA-induced Ca^{2+} oscillations in guard cells and also impairs stomatal closure (Hossain et al. 2011). Therefore, MeJA-mediated regulation of stomatal closure interacts with ABA-mediated regulation of Ca^{2+} signal transduction pathways (Fig. 2.1).

Studies involving the interactions of ABA with MeJA in guard cells reveal that both induce the formation of ROS and NO, and that both are present at reduced concentrations in MeJA-insensitive plants (Munemasa et al. 2007). Munemasa et al. (2011) demonstrated that CPK6 acts downstream of NO and ROS signaling, and therefore may be a target of NO-stimulated Ca^{2+} influx into the cytoplasm. CPK6 is also required for ABA activation of cytoplasmic Ca^{2+} channels (Ca^{2+}-permeable cation channels) (Munemasa et al. 2011) and slow-type anion currents in guard cells (Munemasa et al. 2007). Therefore, JA-induced Ca^{2+} influx into the cytoplasm initiates CPK6 activation, which in turn activates slow-type anion channels, therefore interacting with ABA-induced stomatal closure NO, ROS, ethylene and JA (Munemasa et al. 2011) (Fig. 2.1).

2.2.1.7 A Comparison of ABA- and Drought-Mediated Stomatal Closure

Studies of ABA- and drought-associated regulation of stomatal closure demonstrate the complexity of overlapping transduction pathways. As indicated by Neill et al. (2008), signaling mechanisms may not necessarily be the same under both optimum and environmentally stressful conditions. Many of the studies that investigate the regulation of stomatal aperture rely on the responses displayed by turgid cells (Bright et al. 2006; Desikan et al. 2006; Hossain et al. 2011; Munemasa et al. 2007, 2011; Tanaka et al. 2005). These studies present strong evidence to support a role for NO in ABA-mediated regulation of stomatal closure (Bright et al. 2006). However, the use of turgid cells in studies that explore the interactions of JA and ethylene with ABA relative to the regulation of stomatal closure may mean that

those results do not translate to situations where physiological stress is occurring. Interestingly, Garcia-Mata and Lamattina (2001) showed that an increase in NO concentration improves drought tolerance in wheat.

2.2.2 ABA-Mediated Regulation of Gene Expression

A continually elevated ABA concentration initiates changes in gene expression that inhibit shoot growth and maintain root growth, thus increasing the root-to-shoot ratio. In a whole-genome microarray study using *Arabidopsis*, more than 1,900 genes were found to be drought-responsive, 1,300 of which are also regulated by ABA (Huang et al. 2008). Expression of these drought-responsive genes overlapped with expression profiles of genes regulated by hormones such as JA, auxin, cytokinin, or ethylene (Huang et al. 2008). In addition, numerous microRNAs (miRNAs) are upregulated in response to osmotic stress (Liu et al. 2008).

2.2.2.1 ABA-Dependent Gene Expression During Drought Stress

Longer-term physiological responses to abiotic stress conditions are caused by changes in gene regulation through ABA-mediated regulation of transcription factors that bind to ABA-responsive elements (ABREs) on ABA-regulated genes (Fig. 2.2). In addition to signaling stomatal closure, phosphorylation cascades also lead to changes in ABA-regulated transcription factors. For example, the ABA-responsive transcription factors (ABFs) ABF1 and ABF4 are activated when they are phosphorylated by CPK4 or CPK11 (which are ABA-inducible kinases) (Zhu et al. 2007).

Zhu et al. (2007) also demonstrated that other kinases can phosphorylate ABF1 and ABF4. For example, ABF1 was reported to be a target of some SnRK2 members (Fujita et al. 2009), and CPK32 phosphorylates ABF4 (Choi et al. 2005). Therefore, these transcription factors are probably affected by several signals and pathways (Zhu et al. 2007) and are thus important cross-talk points.

2.2.2.2 MAPK-Directed Phosphorylation Cascades Also Act as Cross-Talk Points

Osmosensing and subsequent signal transduction require phosphorylation of key intermediates through the activity of mitogen-activated protein kinases (MAPKs) (Boudsocq and Laurière 2005). Current models indicate that NO is a central signaling molecule that triggers phosphorylation events through MAPKs as an early response to drought stress (Courtois et al. 2008; Neill et al. 2008) (Fig. 2.2). In drought-stressed wheat seedlings, elevated NO concentrations enhance the transcription of specific drought-induced genes (Garcia-Mata and Lamattina 2001).

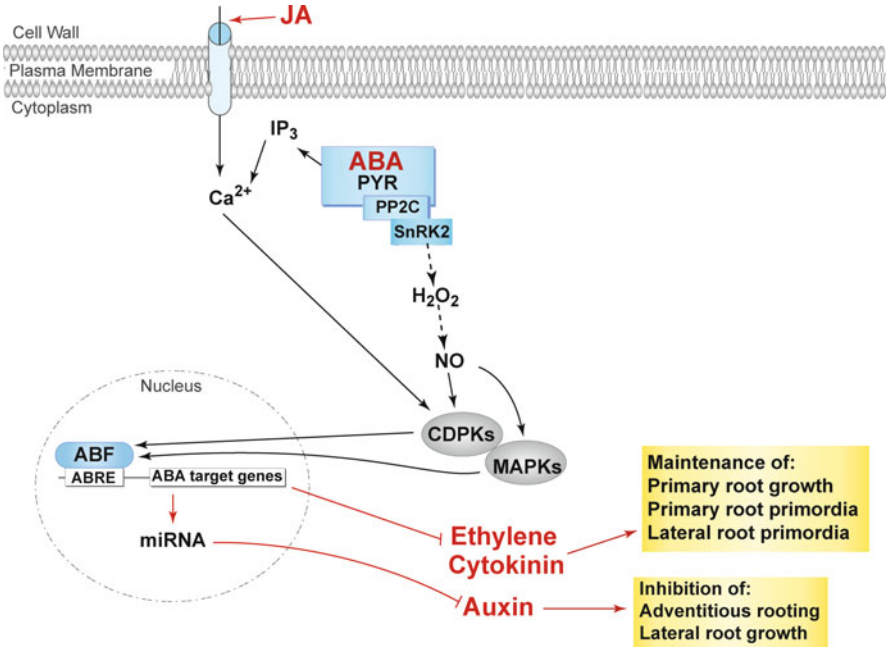

Fig. 2.2 This model illustrates drought-induced gene regulation that controls root growth and architecture. Elevated ABA concentration activates H_2O_2, NO, and Ca^{2+} signaling as discussed in Fig. 2.1. As shown, specific CDPKs and MAPKs activate ABA-regulated transcription factors, which control the expression of ABA-responsive genes. These genes inhibit the ethylene response via the downregulation of several ethylene-responsive and ethylene biosynthetic genes; they also reduce cytokinin levels by triggering an increase in the expression of enzymes that oxidize cytokinin. An increase in the expression of miRNAs associated with auxin signaling downregulates auxin responses. Collectively, these interactions favor the maintenance of the root primordia of primary roots and existing lateral roots while inhibiting lateral root elongation and adventitious root formation. Steps inferred from experiments exploring ABA function using turgid cells are indicted with *dashed lines*

In *Arabidopsis*, MPK6 and its associated MAPK cascade components are activated by drought stress (Boudsocq and Laurière 2005). MPK6 targets two ethylene biosynthetic enzymes, 1-aminocyclopropane-1-carboxylic acid [ACC] synthase 2 (ACS2) and ACS6 (Liu and Zhang 2004; Jaspers and Kangasjärvi 2010), and is also involved in ethylene signaling that leads to the regulation of ethylene-responsive genes (Hahn and Harter 2009). In addition, both *ACS2* and *ACS6* contain ABRE on their promoters (PlantCARE, Lescot et al. 2002). Expression profiles from the roots of 18-day-old wild-type *Arabidopsis* plants show that *ACS2* expression increases within 30 min of 300 mM mannitol treatment to the roots (*Arabidopsis* eFP Browser, Winter et al. 2007). *ACS2* is highly induced by osmotic stress by 3 h of treatment and remains elevated for 24 h. However, when osmotic treatment is compared to drought caused by exposing roots to a 15-min

stream of air, causing the plant a water loss that totals 10% of the fresh weight, a different *ACS2* expression pattern emerges. Under drought conditions, *ACS2* expression is transiently upregulated in the roots for 6–12 h after dehydration, and shoots displayed no expression change from the control level (eFP Browser, Winter et al. 2007). In addition, evaluation of the promoter regions of other ethylene biosynthetic genes shows that in addition to ACS2 and ACS6, ACS7, ACS11, and ACC oxidase 4 (ACO4) have at least one ABRE on their promoters and therefore are candidates for ABA-mediated regulation (PlantCARE, Lescot et al. 2002). Thus, ethylene production may be differentially regulated by various stress conditions, and the responses to water loss and osmotic stress are likely to have differing regulatory components. While drought conditions may transiently upregulate specific ACS genes in root tissue, genes involved in ethylene biosynthesis and/or the response to ethylene tend to be downregulated in response to drought stress (Huang et al. 2008). For example, Huang et al. (2008) noted that ACC oxidase (ACO), which regulates the final step in ethylene biosynthesis, is downregulated by dehydration; its expression increased after rehydration. Therefore, these studies support a reduced, but perhaps transient, expression of ethylene during drought stress (Fig. 2.2).

The negative regulation of the MAPKs may serve as an essential feedback component of ABA-mediated signaling. Two forms of MAP kinase phosphatase, PP2C5 and each its related homolog, *Arabidopsis* Ser/Thr phosphatase of type 2C (AP2C1), each acts as negative regulators of MPK3 and MPK6 by dephosphorylation and each is upregulated by treatment with ABA (Schweighofer et al. 2007; Brock et al. 2010). Interestingly, Brock et al. (2010) provide evidence that these phosphatases are positive regulators of ABA-mediated signaling. Loss-of-function mutants (*ap2c1* or *pp2c5*) displayed reduced expression of selected ABA-responsive genes. Huang et al. (2008) also reported the increased expression of several PP2Cs during drought stress. Thus, increased dephosphorylation of MAPKs may provide a feedback mechanism for multiple regulatory pathways associated with drought responses.

2.2.2.3 Maintenance of Primary Root Growth During Water Stress

When *Arabidopsis* plants are exposed to osmotic (100–400 mM sorbitol) stress, mutants deficient in the osmotic sensor ATHK1 have shorter primary roots compared to wild-type plants, indicating that root growth regulation involves perception by an osmoreceptor (Wohlbach et al. 2008). This hypothesis is supported by studies in which mutants that overexpress *ATHK1* maintain root elongation under osmotic conditions (Wohlbach et al. 2008). Under drought conditions, the region near the root tip continues to grow, while regions away from the tip are inhibited or cease to grow (Sharp 2002). The tip region of the primary root is also the area where ABA and ROS accumulate and where a decrease in ethylene production is noted 3.5 h after drought stress (Yamaguchi and Sharp 2010). Therefore, during drought stress,

an increase in ABA concentration inhibits ethylene production and downregulates genes that respond to the presence of ethylene, thus maintaining root growth (Sharp 2002) (Fig. 2.2).

Cytokinin is considered to be a negative regulator of root growth and branching, and root-specific degradation of cytokinin may also contribute to the primary root growth and branching induced by drought stress (Werner et al. 2010). Using *Arabidopsis* plants that display increased expression of cytokinin oxidase/dehydrogenase (CKX) genes under the control of a root-specific reporter, Werner et al. (2010) demonstrated that an increase in cytokinin degradation in the roots results in an increase in both primary root length and lateral root formation during drought conditions. An analysis of the *Arabidopsis* CKX promoter indicates that the region contains three ABREs (PlantCARE, Lescot et al. 2002), which implies potential ABA-based regulation of cytokinin degradation in the roots (Fig. 2.2). This activity would stimulate root growth and lateral root production; thus, increasing the root-to-shoot is associated with drought conditions and with increased drought tolerance (Werner et al. 2010).

2.2.2.4 Regulation of Lateral and Adventitious Root Development During Water Stress

While auxin is considered to be the primary hormone involved in the initiation and growth of lateral and adventitious roots, ABA may play a role in regulating lateral root growth under stress conditions (De Smet et al. 2006). During drought conditions, *Arabidopsis* produces specialized, short lateral roots that remain in a dormant or nongrowing condition while the plant is under stress (Wasilewska et al. 2008). These roots replace dehydrated lateral roots once drought conditions are relieved. Therefore, for *Arabidopsis*, the additional ABA produced in response to drought stress inhibits the outgrowth of lateral roots from existing meristems (De Smet et al. 2006).

Several miRNAs that are associated with auxin signaling are upregulated by drought or osmotic stress and may play important roles in the regulation of root architecture. For example, in *Arabidopsis*, both *miR167* and *miR168* are involved in the regulation of auxin response factors and both contain ABREs, indicating their own regulation by ABA signaling (Liu et al. 2008). *miR167* targets auxin response factors *ARF6* and *ARF8*, which act redundantly to regulate responses to auxin, and are positive regulators of adventitious root development from shoot tissue (Gutierrez et al. 2009). *miR168* targets *ARGONAUTE1* (*AGO1*), which encodes a RNA Slicer enzyme that downregulates the expression of *ARF17*, which is a negative regulator of root formation. *Arabidopsis* plants that overproduce ARF17 or are deficient in ARF6 or ARF8 have fewer adventitious roots than wild-type plants (Gutierrez et al. 2009). Interestingly, *miR167* and *miR168* are differentially regulated after osmotic stress treatment (Liu et al. 2008). *miR168* expression is upregulated in *Arabidopsis* seedlings 2–6 h after stress induced by 200 mM mannitol, while *miR167* expression increases 6–24 h after this treatment (Liu et al. 2008).

Data from the *Arabidopsis* eFP Browser indicate that osmotic (300 mM mannitol) treatment to roots downregulates *ARF8* at 3, 12, and 24 h after treatment (Winter et al. 2007). A microarray analysis supports the overall downregulation of genes involved in auxin-regulated responses as a consequence of root dehydration (Huang et al. 2008).

Gutierrez et al. (2009) proposed a model in which miRNAs fine-tune auxin-mediated regulation of adventitious rooting by controlling ARF transcription. In this case, during drought stress, ABA-induced production of *miR167* (which targets *ARF6* and *ARF8*) would inhibit adventitious root growth, and increased expression of *miR168* reduces *AGO1* expression, which would result in an increase in *ARF17* concentration, again leading to a decrease in adventitious root production. Considering that these miRNA genes appear to be differentially controlled, the level of auxin production could be nimbly up- or downregulated in response to changing stress conditions, particularly through the regulation of these transcription factors. Such a system would allow a dynamic response, where, for instance, under drought conditions, it would transiently favor decreased adventitious rooting while also increasing overall primary root growth.

In contrast to the adjustment in adventitious rooting, there were no changes in either the length or number of lateral roots for either loss-of-function mutants or overexpressers of *ARF6*, *ARF8*, or *ARF17* (Gutierrez et al. 2009). In a separate study, *miR167* was found to regulate the elongation, but not the initiation, of lateral roots in *Arabidopsis* (Gifford et al. 2008). Current models support the differential regulation of lateral and adventitious roots by miRNAs and their associated targets (Meng et al. 2010).

2.2.2.5 A Summary of Drought Regulation of Root Growth and Development

Drought-associated changes in root architecture are directed by cross-talk between ABA with ethylene, auxin, and cytokinin (Fig. 2.2). When *Arabidopsis* is exposed to drought stress, an increase in ABA in the roots inhibits ethylene production and stimulates cytokinin degradation, which together result in the maintenance of primary root growth. ABA-controlled regulation of genes (such as those that encode ARFs and miRNAs) associated with auxin-regulated root production inhibits the formation of adventitious roots and the outgrowth of lateral roots from existing primordia. However, research on other species demonstrates that the regulation of lateral root formation is highly adaptive, and in rice, for instance, ABA stimulates lateral root formation (Chen et al. 2006). Thus, the complex balance between stimulation and feedback inhibition produced by interacting hormone signaling pathways may result in different scenarios that are fine-tuned by the specific stress conditions and plant species.

2.3 Hormonal Cross-Talk Associated with Wounding

Mechanical wounding of leaf tissue is associated with increases in ethylene, JA, and ABA, which collectively trigger cellular senescence at the wounding site. Cross-talk points that affect gene regulation after wounding also trigger defense mechanisms associated with biotic signaling.

2.3.1 Ethylene Production Increases After Mechanical Wounding

Mechanical wounding causes a transient increase in ethylene production above the normally low, basal level in most plant tissues (Saltveit and Dilley 1978). While there is considerable variation between the levels of basal and peak wound-induced ethylene production based on species and tissue types, the timing of the induction and peak rate of wound-induced ethylene production remain consistent within tissues of a given species (Saltveit and Dilley 1978). For example, in etiolated corn seedlings, wound-induced ethylene increases after a lag of 20 min, with peak production occurring at 58 min, while light-grown plants such as *Forsythia* had a 30-min lag and peak ethylene production at 133 min (Saltveit and Dilley 1978). This transient increase in ethylene production is considered to be a short-term response to wounding that leads to subsequent long-term growth and developmental responses (León et al. 2001; Telewski 2006).

Many ethylene biosynthetic enzymes, including ACS and ACO, are wound inducible (Reymond et al. 2000; Tsuchisaka and Theologis 2004). Analysis of the *Arabidopsis* ACS isoforms composed of ACS promoters fused to a reporter gene demonstrates that *ACS2*, *ACS4*, *ACS6*, *ACS7*, and *ACS8* are each locally upregulated in tissue around the site of wounding (Tsuchisaka and Theologis 2004) (Fig. 2.3), and an increase in phosphorylation of both ACS2 and ACS6 by MPK6 has been measured within 15 min after wounding of *Arabidopsis* seedlings (Liu and Zhang 2004). This indicates that in addition to stimulating expression of *ACS* genes, wounding activates a MAPK kinase cascade, which activates and stabilizes ACS2 and ACS6, resulting in an increase in ethylene production (Liu and Zhang 2004).

2.3.2 Rapid Signaling Events Associated with Mechanical Wounding

The most rapid cellular change associated with wounding or mechanical stimulation (e.g., by touch) is the increase in concentration of cytoplasmic Ca^{2+} that occurs within seconds of stimulation (Haley et al. 1995; Monshausen et al. 2009).

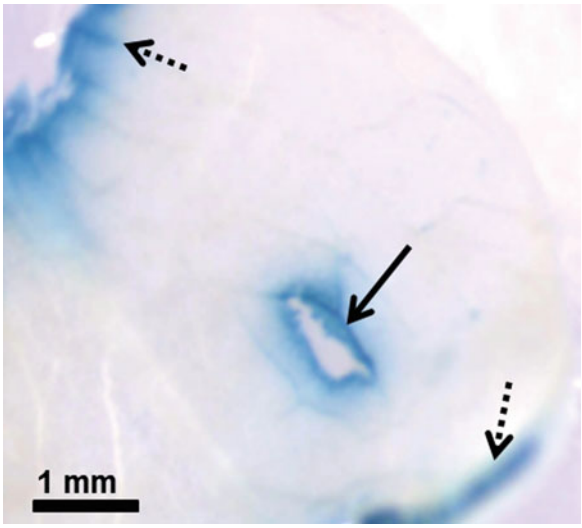

Fig. 2.3 This image illustrates an example of a localized gene expression pattern associated with wound-inducible *ACS* genes. Reporter expression in a transgenic *Arabidopsis* leaf carrying *ACS6 promoter::GUS* constructs occurs around the area punctured with a dissecting needle (*arrow*). GUS staining also occurs at the cut edge where the leaf was excised from the plant (*dashed arrows*)

This cytosolic Ca^{2+} interacts with Ca^{2+}-binding or Ca^{2+}-responsive proteins whose expression is also rapidly upregulated after wounding (Walley et al. 2007).

An increase in ROS production occurs within 2 min of wounding of *Arabidopsis* leaf tissue (Miller et al. 2009). ROS production rapidly increases after treatment with Ca^{2+} ionophores that release Ca^{2+} ions, and is inhibited by Ca^{2+}-transport inhibitors (Monshausen et al. 2009). Monshausen et al. (2009) propose that the influx of Ca^{2+} into the cytoplasm stimulates NADPH oxidation to form superoxide in the cell wall. Superoxide is rapidly converted to H_2O_2, which can easily enter the cell and trigger the regulation of gene expression (Monshausen et al. 2009) (Fig. 2.4).

Microarray studies have revealed that numerous genes are expressed within minutes of wounding; for instance, Walley et al. (2007) found that 162 genes were induced within 5 min of wounding of *Arabidopsis* rosette leaves. The promoters of 47 of these rapid wound-responsive (RWR) genes contain a rapid stress response element (RSRE) (Walley et al. 2007). In transgenic *Arabidopsis* plants containing four RSREs fused to a reporter, wound-induced reporter expression occurred within 5 min in an area local to the wound site, indicating that the response element is capable of detecting wounding stimulus in a manner similar to that observed for the RWR genes (Walley et al. 2007). Ca^{2+} and ROS are both considered candidates for the regulation of RSRE-containing genes, but currently there is no direct evidence confirming that they regulate proteins that bind to RSREs (Walley and Dehesh 2010) (Fig. 2.4). However, a number of RWRs, such as calmodulin-related protein and touch-induced 3 (TCH3), are Ca^{2+}-activated genes (Walley et al. 2007), and the RSRE sequence is similar to one which binds a calmodulin-binding transcription activator, thus supporting a role for Ca^{2+} in the regulation of RWR genes (Walley and Dehesh 2010).

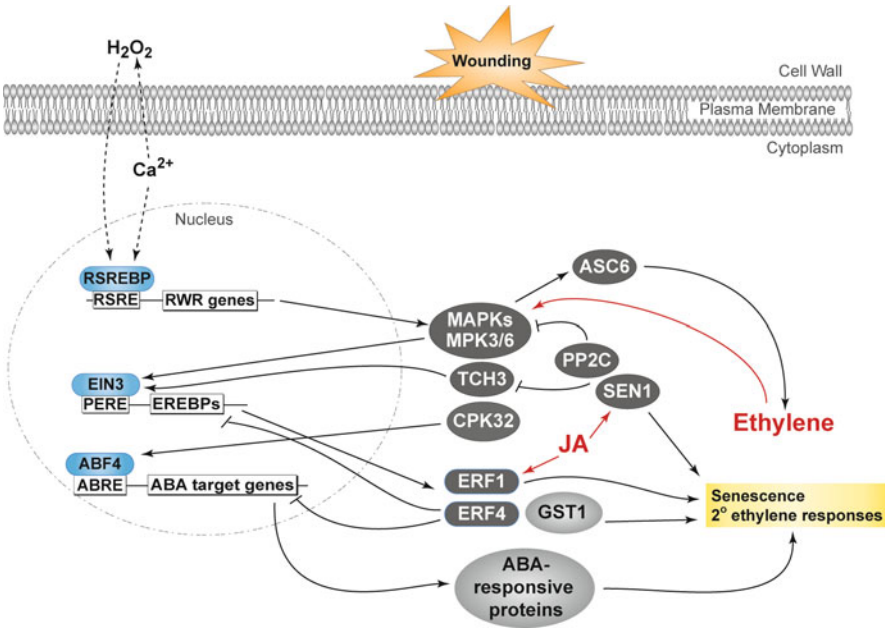

Fig. 2.4 This model illustrates wound-induced changes in gene expression that result in increased leaf senescence. Wounding causes rapid Ca^{2+} oscillations in the cell, which stimulate H_2O_2 production. Ca^{2+} and H_2O_2 regulate the expression of rapid wound-responsive (RWR) genes through the activation of transcription factors (RSRE binding proteins, RSREBPs), which include MAPKs associated with the activation of the ethylene signaling component, EIN3. EIN3 regulates the expression of ethylene-responsive element-binding proteins (EREBPs), including ERF1 and GST1, which trigger ethylene-mediated stimulation of senescence. ERF4, which is also an EREBP, is a negative regulator of both ABA- and ethylene-mediated responses. Other RWRs include the ethylene biosynthetic enzyme ACS6, senescence-associated gene SEN1, and CPK32. CPK32 activates the ABA transcription factor ABF4 to initiate ABA-mediated responses. Another RWR, the phosphatase PP2C, acts to dephosphorylate and inactivate other RWRs such as MAPKs and TCH3. Wound-induced JA synergistically interacts through its activation of ERF1 and SEN1. Rapid wound-responsive genes products are indicated by *shaded symbols with white lettering*

Early signaling events include rapid upregulation of components of the ethylene-response pathway. Transcription profiles show rapid upregulation of kinase cascade components, including the MAPK kinase, MKK9, and its target MPK3, supporting an important role for increased phosphorylation in the early responses to wounding (An et al. 2010). MPK3 phosphorylation targets transcription activator ethylene insensitive 3 (EIN3) (An et al. 2010). EIN3 binds to promoter regions of ethylene-responsive element-binding proteins (EREBPs) and regulates transcription of both the senescence-associated gene glutathione-S-transferase 1(GST1) and ethylene-response factor 1 (ERF1), a RWR that regulates the secondary gene targets of ethylene signaling such as those involved in the defense response and cellular inhibition (Schaller and Kieber 2002). Therefore, the phosphorylation and stimulation of EIN3 activity would explain the rapid increase in the expression of primary

ethylene-responsive genes, such as ERF1 and ERF4 (Fig. 2.4). In addition, MPK3 also phosphorylates ACS6 (a RWR), which stimulates ethylene biosynthesis (An et al. 2010) (Fig. 2.4). Therefore, the expression of many of the RWR genes serves to initiate early tissue senescence at the wound site.

Interestingly, PP2C-type phosphatases (including AP2C1) that dephosphorylate MAPKs are also identified as RWRs (Walley et al. 2007). Plants carrying the AP2C1 promoter fused to a GUS (β-glucuronidase) reporter gene demonstrate an increase in AP2C1 expression localized around the site of wounding (Schweighofer et al. 2007). Transgenic *Arabidopsis* plants that overexpress AP2C1 display both greatly reduced MPK6 activity and reduced ethylene production after wounding (Schweighofer et al. 2007). In addition, TCH3 is also negatively regulated by dephosphorylation, and may be a target of the RWR PP2C-type phosphatases (Wright et al. 2002). Therefore, feedback regulation by dephosphorylation is also a rapid response to wounding (Fig. 2.4)

2.3.3 Gene Expression Patterns Associated with Mechanical Wounding

Expression studies of wound-induced genes in *Arabidopsis* demonstrated the upregulation of many genes associated with JA and with ethylene biosynthesis and response (Cheong et al. 2002). Microarray studies that profiled gene expression in wounded *Arabidopsis* leaves revealed that many genes have a similar expression pattern, in which induction occurs by 15 min and expression peaks at 1–2 h after wounding; this pattern is similar to the ethylene profile described above (Reymond et al. 2000). Genes that display this type of expression pattern include JA- and ethylene-associated genes as well as several RWRs, including calmodulin (*TCH1*), calmodulin-related proteins (*TCH2* and *TCH3*), *MPK3*, and *GST1* (Reymond et al. 2000).

ABA also accumulates in the tissue around a wound site, probably in response to dehydration associated with the damage caused by wounding (León et al. 2001). The expression of CPK32, which phosphorylates (and thus activates) the ABA-responsive transcription factor ABF4 (Choi et al. 2005), greatly increases from 5 to 30 min after wounding (Chotikacharoensuk et al. 2006). ABA is known to promote leaf senescence (Finkelstein and Rock 2002), and thus, the increase in ABA-responsive transcription factors suggests that ABA may contribute to the stimulation of senescence around a wound area.

JA production is also wound inducible and stimulates leaf senescence in plant tissues (Wasternack 2007). JA synthesis follows a similar pattern of transient increase as that of ethylene and wound-induced gene expression (Chotikacharoensuk et al. 2006; Schweighofer et al. 2007). JA is activated independently from ethylene, but JA treatment upregulates numerous *ERF* genes (McGrath et al. 2005). These include the transcriptional activator ERF1 and transcriptional repressor ERF4, both

of which are RWR proteins (Walley et al. 2007). Interestingly, ERF4 is a negative regulator of ABA and ethylene responses (Yang et al. 2005) (Fig. 2.4). Senescence-associated protein 1 (SEN1) is also potentially regulated by JA (The Arabidopsis Information Resource, Swarbreck et al. 2008) (Fig. 2.4).

2.3.4 *Hormonal Responses to Mechanical Wounding Are Rapid and May Also Be Involved in Biotic Stress Response*

Mechanical wounding stimulates rapidly induced events that involve increases in both Ca^{2+} and ROS, which then activate the expression of MAPK and CDPK cascade components. These protein kinases activate transcription factors and proteins that are part of response pathways that involve the phytohormones ethylene, JA, and ABA (Fig. 2.4). Thus, the response pathways can be stimulated downstream of the point where an increase in the hormone itself would be necessary for inciting a response. Subsequent increases in hormone production may act to sustain the wound-induced response.

Many RWR genes are known to be upregulated under both abiotic and biotic stress conditions (Walley et al. 2007). Walley et al. (2007) examined both abiotically induced RWR expression as well as profiles for various biotic stresses and found considerable overlap in the expression of genes associated with early responses to pathogenic infections, indicating that RWR genes may participate in the regulation of the initial defense mechanism. JA is a key regulatory signal associated with both the local and systemic response to pathogen attack (Wasternack 2007). It is known that mechanical wounding stimulates JA biosynthesis (León et al. 2001), and it has been proposed that JA also contributes to the systemic response that stimulates immunity to subsequent herbivory (Wasternack 2007).

2.4 Signaling During Abiotic Stress Conditions

Several signaling components discussed earlier are common across many abiotic stress conditions. ABA is considered the primary hormone involved in the signaling of many abiotic stresses, including drought, salt, and cold. Therefore, the stress conditions that result in elevated ABA concentrations may be part of a general stress-regulation network that shares many of the same signaling components described for the regulation of drought stress. Likewise, mechanical stresses would stimulate the production of RWRs and follow a common mechanosensory-regulated response mechanism. RWR genes are also associated with several abiotic responses. Therefore, rapid gene expression changes may be characteristic to most abiotic stress responses. In addition, wounding damage to tissue causes

dehydration around the wound, so some aspects of that pathway will converge with the drought-stress response.

While many components of the pathways act in a similar manner, and many of the same genes are induced by several abiotic stresses, it should be noted that the expression pattern of any specific gene varies in its timing and level of expression within specific tissues and under different experimental conditions. For example, MKK9, which is upregulated by osmotic stress, dehydration, salt, wounding, and cold treatments (Ma and Bohnert 2007), is highly upregulated in roots from 1 to 24 h when salt-stressed but during this same time period is expressed most strongly in the shoots during osmotic stress (eFP Browser, Winter et al. 2007). While the variation may occur because the treatments vary in their severity and duration, the hormonal status of the plants under the different stress conditions may also affect cross-talk and the complex feedback regulatory mechanisms associated with the interacting pathways.

2.4.1 Common Regulatory Elements of Abiotic Stress Responses

Many of the genes that are affected by wounding are associated with several abiotic stressors, such as cold, drought, heat, salt, and ABA treatment (Cheong et al. 2002). For example, several RWR genes (e.g., *ACS6*, *ERF1*, and several other *ERFs*, Ca^{2+}-signaling components, *TCH3*, and *MPK3*) are rapidly induced in roots not only by wounding but also by salt stress (Ma and Bohnert 2007) (Fig. 2.5). In addition, 47% of the RWR genes are also activated by cold stress, and other genes associated with several abiotic responses are RWRs, providing evidence that the rapid regulation of these genes is a common component of abiotic stresses (Walley et al. 2007) (Fig. 2.5).

Cellular dehydration is a result of many abiotic stress conditions, especially drought, salt, and cold stress, but also occurs in wounded tissue. Microarray studies have provided substantial evidence of overlapping gene expression associated with abiotic stress conditions that involve dehydration. For example, a comparison of changes in gene expression caused by drought or high salt conditions found that 154 genes were upregulated and 104 were downregulated, indicating a high correlation with gene regulation during the same two stress conditions in *Brassica rapa* (Chen et al. 2010). In another study, 197 genes were identified as being induced by several different stress conditions (wounding, cold, salt, osmotic, biotic, and chemical) (Ma and Bohnert 2007). The functions of the gene products include signaling components that are involved in ROS metabolism, Ca^{2+} signaling (such as CPK32, which regulates ABF4), ethylene signaling (including ACS6 and numerous ERFs), and MAPK cascade components (e.g., MKK9, which leads to ACS6 activation). Therefore, Ma and Bohnert (2007) concluded that both ROS and ethylene signaling pathways are central to the coordination of stress signals while ABF4 binds to promoter regions of ABA-responsive genes, thereby regulating them (Zhu et al. 2007).

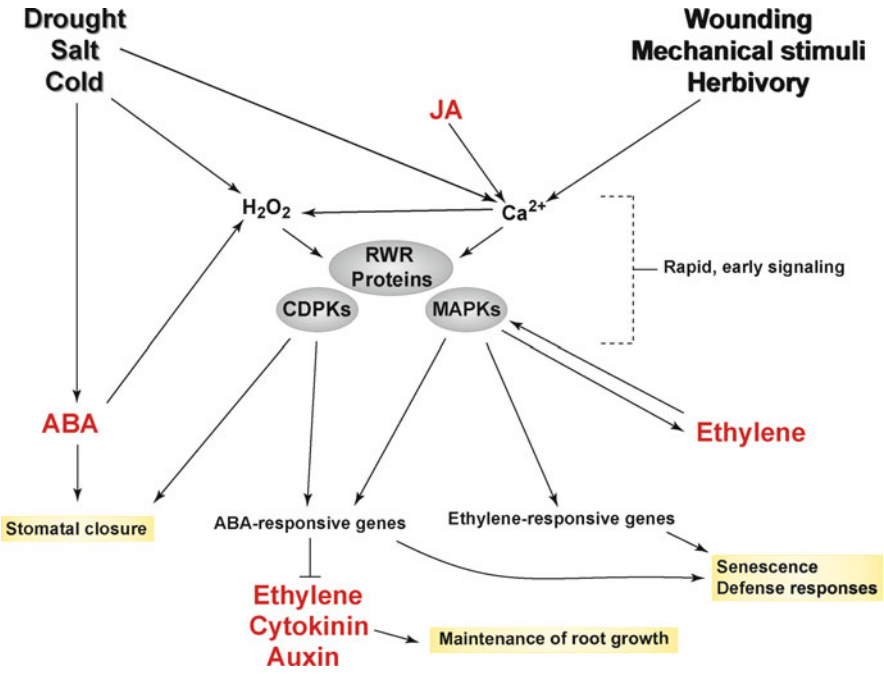

Fig. 2.5 This model of hormonal cross-talk integrates elements involved in the responses to numerous abiotic and biotic stress conditions. Negative regulators and regulatory feedback steps have been omitted for clarity

Kinase cascades are associated with several abiotic stress conditions, and certain components may serve as hubs. In *Arabidopsis*, MPK4 and MPK6 (which activate ACS2 and ACS6) are induced by cold, salt, and osmotic stress conditions, indicating that these MAPK forms are central hubs in abiotic stress conditions (Boudsocq and Laurière 2005). Jaspers and Kangasjärvi (2010) note that conditions (drought, cold, salt, temperature extremes, etc.) that lead to oxidative stress and subsequent H_2O_2 production activate a key MAP kinase cascade whose targets include transcription factors involved in ABA and ethylene signaling. Therefore, ROS such as H_2O_2 convey information from a variety of upstream signals and transduce the information to targeted genes to generate a response.

Stress conditions often increase the expression of certain miRNAs, including *miR167*, *miR168*, and *miR393*, which are involved in auxin regulation. *miR168* is upregulated during cold, osmotic, and salt stresses, and *miR167* is upregulated by cold and salt (Liu et al. 2008), while *miR393* expression increases during cold, drought, salt, and UV irradiation conditions (Martin et al. 2010). The promoters of both *miR167* and *miR168* contain response elements for both auxin and ABA (Liu et al. 2008). As discussed previously, *miR167* and *miR168* have been shown to control the regulation of auxin-mediated responses.

The *miR393* gene contains an ABRE (Liu et al. 2008) and regulates the expression of the auxin-binding protein transport inhibitor response 1 (*TIR1*), which

enables the expression of auxin-regulated genes. Increased levels of TIR1 favor lateral root formation (Pérez-Torres et al. 2008); therefore, the degradation of *TIR1* transcripts would result in less lateral root formation under abiotic stress conditions (Martin et al. 2010). This is consistent with a general downregulation of auxin-responsive genes found in microarray studies of plants experiencing abiotic stress (Cheong et al. 2002). In addition to the ABRE, *miR393*'s promoter also contains response elements for ethylene, auxin, and JA, indicating potential regulation by several hormones (Liu et al. 2008).

As with the histidine kinase osmoreceptor, AHK1, the cytokinin receptors AHK2 and AHK3 were induced within 10 min of stress treatment in *Arabidopsis* plants (Tran et al. 2007). AHK2 was upregulated after dehydration, and AHK3 production was stimulated by multiple abiotic stresses (dehydration, salt, and cold, as well as by ABA treatment) (Tran et al. 2007). Loss-of-function mutants *ahk2* and *ahk3* exhibit an increased tolerance to drought and salt, which indicates that AHK2 and AHK3 act as negative regulators of osmotic stress responses (Tran et al. 2007).

As previously discussed, JA biosynthesis is induced by wounding (Figs. 2.1 and 2.2). In addition, the expression of a JA biosynthetic enzyme, 12-oxophytodienoat-10,11-reductase (OPR3), increased by 3 h after treatment with salt or Ca^{2+} (Chotikacharoensuk et al. 2006). Ludwig et al. (2005) found that abiotic stress conditions stimulated Ca^{2+} signaling and CDPK2 activity resulting in elevated levels of OPR3 and JA. In addition, the expression of 197 genes was found to be common to both drought and JA-associated signaling (Huang et al. 2008). An analysis of transcription regulation sites determined that several genes involved in the JA biosynthetic pathway are upregulated after NO treatment, indicating a potential regulation of JA signaling through the ROS/NO pathway (Palmieri et al. 2008). However, the role of NO in the signaling of abiotic stresses remains unclear. While NO may play a role in ABA regulation of stomatal closure during non-stressed conditions, and may be involved in JA signaling, abiotic stress conditions may preferentially trigger a Ca^{2+} pulse and ROS burst that both activate RWRs, inducing the rapid stress-responsive pathway (Fig. 2.5).

2.4.2 Cross-Talk Between Abiotic and Biotic Stresses

A model by Ludwig et al. (2005) indicates that both abiotic and biotic stress conditions stimulate CDPK and MAPK pathways and that ethylene acts as a critical cross-talk point between the two pathways. Many of the RWRs are also associated with biotic stress conditions and may also be considered part of the pathogen defense response, especially in association with tissue wounding and through the activation of ethylene biosynthetic and signaling components (Walley et al. 2007) (Fig. 2.5). In addition, JA synthesis results from both wounding and biotic stresses (León et al. 2001), thus indicating its involvement in ethylene regulation and the regulation of stress responses such as senescence (Fig. 2.5).

2.4.3 Stress Tolerance

The breeding of plants to increase their tolerance to abiotic stress is an ongoing and important agricultural effort. Plants tolerant to abiotic stress conditions would possess such desirable traits as an increase in sensitivity to ABA, which would result in drought-tolerant traits such as increases in stomatal closure, root growth, and lateral branching of roots. Current laboratory methods used to produce transgenic plants allow researchers to manipulate genes much more precisely than by traditional breeding methods. However, the complexity of native stress regulation, which involves numerous proteins and cross-talk with phytohormone pathways, makes precise regulation difficult. Understanding the hormonal cross-talk that occurs during the regulation of stress conditions may provide insight into useful gene targets that would allow the production of stress-tolerant plants. Unfortunately, current public and political opinion of transgenic plants blunts the effectiveness of this approach. Nonetheless, specific genes could be targeted using strategies that involve miRNAs or transcription factors, which might be more acceptable to the general public. The combination of traditional breeding with modern genetic approaches could be used to improve screening for stress-tolerant cultivars.

2.4.3.1 Gene Targets

Evaluation of loss-of-function and gain-of-function mutants has identified numerous genes that confer stress tolerance to several abiotic conditions. Loss-of-function mutants associated with components of ABA signaling generally result in plants with increased sensitivity to abiotic stress conditions. Overexpression of these genes often results in tolerance. For example, plants that overexpress the osmoreceptor ATHK1 demonstrate increased tolerance to drought (Tran et al. 2007). Specific types of CDPKs (e.g., CPK10 in *Arabidopsis* and OsCDPK7 in *Oryza*) have been found to increase stress tolerance in plants in which they are overexpressed (Saijo et al. 2001; Zou et al. 2010). Conversely, loss-of-function mutants of the negative regulators of ABA signaling tend to result in increased stress tolerance. For example, loss-of-function mutants for the cytokinin receptors AHK2 and AKH3 display increased tolerance to salt and drought (Tran et al. 2007). Mutants of miRNA genes have also been shown to alter the regulation of transcription factors as well as other signaling components, making them potential targets for engineering stress-tolerant crops.

2.4.3.2 Strategies for Developing Stress Tolerance in Plants

The major cross-talk points that involve both ABA and ethylene signaling pathway components have been considered as primary targets for manipulation to improve the response of crops to multiple stress conditions. Other important targets include

the gene products that protect plants from the increased oxidative stress created by many abiotic stressors (Neill et al. 2008). However, given the complexity of interactions associated with abiotic and biotic stress responses, a targeted strategy is preferred.

One strategy that provides a more targeted approach involves manipulating the expression of transcription factors, which would only affect a subset of stress-responsive genes (Umezawa et al. 2006). For example, the overexpression of an ethylene-responsive element-binding protein (EREBP) increases ROS detoxification and ROS-induced cell death (Ogawa et al. 2005). In a shared response to various stresses, many RWR genes that are transcription factors are upregulated early and in a transient manner; this feature may be useful in identifying plants resistant to multiple stress conditions. Walley et al. (2007) suggested that using RSREs to drive expression of specific genes could be a useful tool in engineering stress tolerance in plants. Interestingly, Capiati et al. (2006) demonstrated that wounding induces salt tolerance in tomatoes, indicating that focusing on the rapid responses to wounding that show significant cross-talk with other abiotic stress conditions could be a useful approach. The regulation of specific miRNAs may also provide a method for more precisely modulating the stress response through the regulation of specific transcription factors. However, transcription factors and miRNAs have multiple targets, and altered regulation that improves certain aspects of plant growth may also result in unfavorable traits.

Another approach to improve the targeting of specific regulatory components is to direct expression through the use of tissue-specific or stress-inducible promoter constructs (Hardy 2010). For example, the overexpression of OsCDPK7 in rice is localized to the root primordial and vascular tissues and may protect those tissues during abiotic stress conditions (Saijo et al. 2001). Another target could be the root-specific reduction of cytokinin levels through overexpression of cytokinin oxidase/dehydrogenase, which increases stress tolerance while maintaining adequate shoot growth and development in both *Arabidopsis* and tobacco (Werner et al. 2010). The use of stress-inducible promoters allows for normal plant growth and development since the gene(s) will only be expressed under stress conditions (Hardy 2010).

Gene expression profiles of *Arabidopsis* transcripts have led to a wealth of knowledge concerning the genes associated with that plant's response to abiotic stress. Increased computational capability, such as the clustering methods used by Ma and Bohnert (2007) have allowed more sophisticated data analysis, including an improved ability to sort genes into functional groups. Data from *Arabidopsis* is useful for identifying key components but is not readily transferable to crops, especially the cereal species (Tester and Bacic 2005). As pointed out by Langridge et al. (2006), a functional genomic approach can help improve our knowledge of stress tolerance in cereal species, but the application of this information to field use will require additional time. As more crop genomes are sequenced, it will be determined whether the approaches used for *Arabidopsis* may be effectively applied to crop species. Identification of the key regulatory components involved in tissue specificity and in the responses to various stressors could provide a mechanism for identifying important genes and critical components of stress-related

signaling pathways. Such information could be used to develop transgenic plants or to identify natural variants, perhaps by using improved molecular-based screening methods (Tester and Bacic 2005).

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