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Crosstalk Between Gibberellin and Abscisic Acid in Cereal Aleurone

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Abstract

The antagonism between gibberellins (GA) and abscisic acid (ABA) is an important factor regulating the developmental transition from embryogenesis to seed germination. In cereal aleurone layers, the expression of genes encoding hydrolytic enzymes needed for seedling growth, such as α-amylases and proteases, is induced by GA but suppressed by ABA. In addition, ABA induces the expression of genes that may play a role in the establishment of stress tolerance. Because of these well-defined biochemical and molecular markers, the cereal aleurone layers have been used as a convenient system for studying GA/ABA actions. Both gain- and loss-of-function approaches have been followed by the constitutive or the RNAi-mediated knockdown expression of specific regulatory molecules. The GA signaling pathway is anchored by the transcription factor GAMyb, which interacts with a specific region in the promoter of GA up-regulated genes, and an upstream regulatory molecule SLN1 (SLR1) that appears to be a functional homolog of the *Arabidopsis* GAI/RGA regulatory proteins. It is established that the ABA induction and suppression of gene expression follow two distinct signaling pathways, with the former requiring a transcription factor ABI5, but inhibited by a protein phosphatase 2C, and the latter mediated by a protein kinase PKABA1 and an unknown factor. The ABA suppression action has been pinpointed to be upstream from the formation of functional GAMyb but downstream from the site of action of SLN1 (SLR1).

Key words: Abscisic acid; Cereal aleurone; Gene expression; Gibberellins; RNAi; Signal transduction

Introduction

Hormonal antagonism is an important factor regulating crucial biological processes. The blood sugar level in mammals is carefully monitored and regulated by two classes of antagonizing hormones. Epinephrine or glucagon trigger a fast breakdown of glycogen to increase the sugar level in blood in response to shock or other demands. On the other hand, insulin promotes glycogen biosynthesis as a means to reduce unnecessary free sugar in circulation (for review see Roach and others 1991). The larva-to-pupa transition in insect development is regulated by the balance between juvenile hormone and ecdysone (Gilbert and others 1996). In plants, it is well known that the ratio of indoleacetic acid (IAA) and cytokinins is involved in apical dominance and shoot/root differentiation (for review see Srivastava 2002).

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The crosstalk between gibberellins (GA) and abscisic acid (ABA) is a major factor in regulating the developmental transition from seed formation to germination. The level of ABA increases during mid and late stages of seed formation, correlating with the deposition of storage nutrients and onset of seed dormancy. Break of seed dormancy is often associated with the decrease of ABA level. Treatments with ABA biosynthesis inhibitors also lead to loss of seed dormancy (for review see Zeevaart and Creelmann 1988). Genetic mutants deficient in ABA biosynthesis or with reduced ABA sensitivity usually germinate precociously (McCarty 1995; Nambara and others 2000). On the other hand, applications of exogenous GA often promote seed germination. Treatments promoting seed germination, such as cold and light, are often correlated with an increase in endogenous GA (Yamaguchi and others 1998).

Recently, it has been shown that a mutation of the *Arabidopsis RGL2* gene, which is involved in GA signaling, affects seed dormancy (Lee and others 2002). Due to the complexity of the process, most of the studies on the action of GA and ABA during seed germination or seedling growth are largely at the physiological level. To support post-germination seedling growth, mobilization of storage nutrients in cereal endosperm is also regulated by GA and ABA.

These clearly defined hormone-mediated biochemical processes in this particular tissue have allowed researchers to unravel molecular mechanisms underlying the interactions between these two hormones (for recent reviews see Bethke and Jones 1998; Lovegrove and Hooley 2000; Olszewski and others 2002; Ritchie and Gilroy 1998).

Cereal Aleurone Tissue as a Convenient System for Studies of GA and ABA Action

Since the early 1960's, the cereal aleurone tissue has been used as a convenient system to study the GA mode of action (for review see Fincher 1989). This tissue consists of one to several layers of cells surrounding the starchy endosperm where the bulk of seed reserve nutrients are stored. The aleurone cells, as the rest of the endosperm cells, are triploid and the progeny of the fertilized polar nuclei generated during the double fertilization process. However, during seed development the aleurone cells undergo a unique differentiation path and remain alive even after seeds are mature (Olsen 2001). During seed germination, the aleurone cells respond to GA released from the embryo by synthesizing a group of hydrolytic enzymes, such as α-amylases, proteases, and nucleases, which are then secreted for the mobilization of endosperm nutrients. Abscisic acid, whose level remains high in dormant seeds, inhibits the action of GA in aleurone cells. At least 40% of the newly synthesized proteins in GA-treated aleurone layers are α-amylases, thus this group of enzymes and their genes has been used as a convenient marker for studies of GA action. There are about a dozen α-amylase genes in rice and barley, and many of them are induced by GA. The *cis*-acting elements involved in the GA induction of barley α-amylase genes have been delimited, consisting of at least three components: a pyrimidine box, a GARE (GA response element), and an amylase box (also termed box 1 or TATCCA box) (Lanahan and others 1992; Gubler and Jacobsen 1992). Reporter constructs, consisting of an α-amylase promoter linked to the β-glucuronidase (GUS) coding region, can be introduced into aleurone cells via particle bombardment. Transient expression studies with this type of reporter constructs indicate that these reporter genes can be induced by GA following the same kinetics as the resident α-amylase genes. In barley aleurone layers, treatment with 1 μM GA_3 (gibberellic acid) for 24 h leads to 50–100-fold induction of α-amylase::GUS reporter constructs. Thus, the GA response can be precisely quantified by assaying for GUS activities. In addition, effector gene constructs consisting of a constitutive promoter linked to the coding region of regulatory molecules (for example, kinases, transcription factors, and so on) can be co-bombarded with the α-amylase::GUS reporter construct into the same aleurone cells. This approach permits the studies of effect of potential regulatory molecules (Shen and others 1996; Gomez-Cadenas and others 2001).

Recently, it has been demonstrated that transiently expressed dsRNA-based interference (RNAi) can also be used in the aleurone tissue to knock down expression of target genes (Zentella and others 2002). An effector construct consisting of a constitutive promoter and a transcribed region with inverted repeats is co-bombarded into the same cells as the reporter construct. This RNAi knock-down mechanism takes place very quickly in the bombarded cells, and expression of the target gene is almost totally suppressed by 4 h after the effector construct is introduced (Zentella and others 2002). Therefore, both gain- and loss-of-function approaches can be followed in elucidating the role of signaling molecules in aleurone tissues.

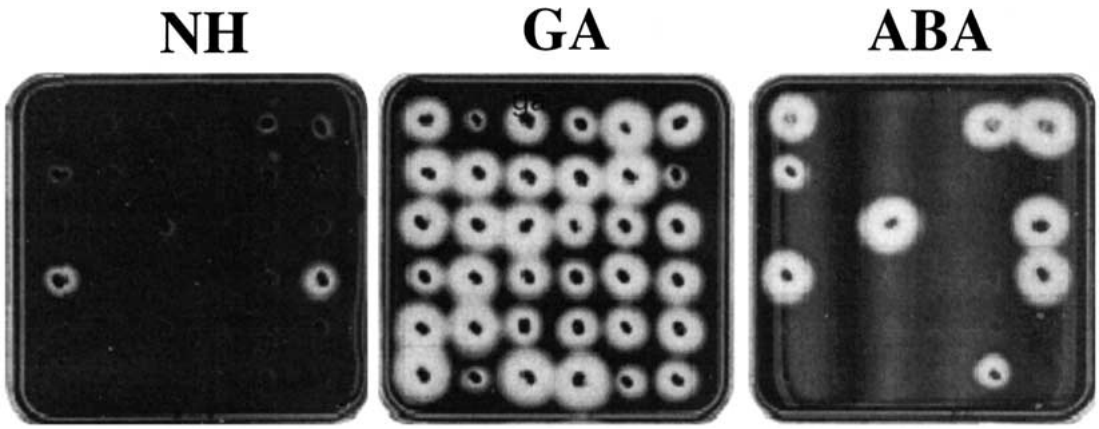

Figure 1. ABA inhibits the constitutive α-amylase expression in *sln1* mutant barley seeds. Progeny seeds from selfed heterozygous barley plants carrying the slender gene (*SLN1*/*sln1*) were de-embryonated and cut transversely into three pieces of approximately the same size. A slice from each seed was put into the same quadrant of starch plates containing ABA (20 μM), GA_3 (1 μM), or no hormone (NH). The plates were incubated for 2 d and then flooded with iodine solution. The GA plate shows GA-induced α-amylase synthesis and secretion from a heterogeneous population of seeds. Most of the seeds, regardless of their genetic background, generate a clear halo due to the hydrolysis of starch by the secreted α-amylase. The NH plate depicts GA-independent synthesis and secretion of α-amylase by about 25% of seeds tested, which are the homozygous *sln1*/*sln1* mutant seeds. The ABA plate demonstrates the ability of ABA to inhibit the GA-independent α-amylase synthesis and secretion from *slender* (*sln1*/*sln1*) seeds. From Lanahan and Ho (1988).

Isolation of hormone response mutants by directly monitoring the hormone response in aleurone tissue has been attempted, but no specific mutants have been reported (Ho 1980). However, there are many GA-sensitive and insensitive dwarf mutants in rice and other cereals, and several of them have been studied and led to the isolation of intriguing regulatory genes for GA signaling or biosynthesis (for examples, see Ueguchi-Tanaka and others 2000; Ikeda and others 2001; Sasaki and others 2002). It has been shown that these regulatory genes also affect GA responses in the aleurone tissue. The combination of molecular and genetic approaches in investigating hormone responses in this tissue has contributed significantly to our current knowledge in the mode of action of GA (Olszewski and others 2002).

Mode of Action of GA and ABA in Cereal Aleurone

Three key regulatory molecules have been identified in the GA signaling pathway in cereal aleurone tissue: a heterotrimeric G protein, a GRAS family regulatory protein SLENDER (S1N1/SLR1), and a transcription factor GAMyb. Jones and others (1998) have used Mas7, a cationic amphiphilic tetradecapeptide that stimulates GDP/GTP exchange by heterotrimeric G proteins, to specifically induce α-amylase gene expression in wild oat aleurone protoplasts. Furthermore, the rice *d1* dwarf mutant is defective in the single copy gene of α subunit of heterotrimeric G protein, and this mutation affects both shoot elongation and the GA induction of α-amylase in the aleurone tissue (Fugisawa and others 1999; Ueguchi-Tanaka and others 2000). *Slender* mutants exist in both rice and barley with GA-independent shoot elongation and α-amylase synthesis in the aleurone tissue (Ikeda and others 2001; Chandler 1998; Lanahan and Ho 1998; also see Figure 1). Both rice *SLR1* and barley *SLN1* genes have been isolated and characterized, and they appear to be functional homologs of the *Arabidopsis GAI*/*RGA*/*RGL* genes (Ikeda and others 2001; Chandler and others 2002; Peng and others 2002; Silverstone and others 1998). Although there are multiple genes in *Arabidopsis* for *GAI*/*RGA*/*RGL*, it is intriguing to note that there appears to be a single copy of *SLR1* gene and *SLN1* gene in rice and barley, respectively.

The transcription factor GAMyb has been shown to bind to GARE on α-amylase promoters, and it is necessary and sufficient for GA induction of α-amylase (Gubler and others 1995; Zentella and others 2002). GAMyb has a similar effect on GA-induced proteases, nucleases, and cell-wall degrading enzymes (Gubler and others 1999; Cercos and others 1999). Cereal aleurone consists of terminally differentiated cells that, upon GA treatment undergo programmed cell death involving the formation of large vacuoles (Bethke and others 1999).

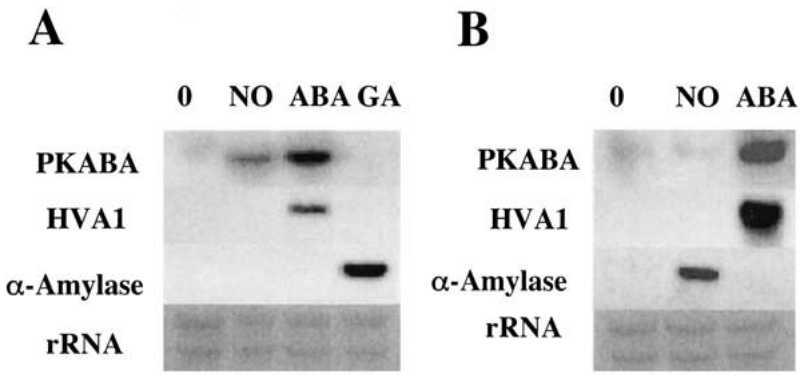

Figure 2. ABA induces the expression of protein kinase PKABA1. RNA samples were prepared from barley aleurone layers (**A**) and embryos (**B**), separated on a 1.4% agarose gel (10 μg/lane), and transferred onto a nylon membrane. Hybridization was carried out with HvPKABA1 cDNA (PKABA1), Hva1 cDNA (HVA1) and α-amylase cDNA (Amylase) as probes. (0), RNA from aleurone layer before incubation; (No), incubation for 24 h without phytohormone; (ABA), incubation for 24 h with 20 μM ABA; (GA), incubation for 24 h with 1 μM GA_3. Gels stained with 0.04% methylene blue in 0.5 M sodium acetate buffer (pH 5.2) are shown at the bottom of each blot (rRNA). From Yamauchi and others (2002).

Over-expression of GAMyb is also sufficient to trigger this process in the absence of GA (R. Zentella and T.-H.D. Ho unpublished observations). Furthermore, the expression of GAMyb itself is up-regulated by GA and down-regulated by ABA (Gomez-Cadenas and others 2001). Therefore, it appears that GAMyb is a central regulatory molecule responsible for triggering diverse downstream GA-mediated events in aleurone tissue. Although a GAMyb-like protein is probably involved in inflorescence formation in *Lolium temulentum* induction, its role is far from being clear. To date, there is no compelling evidence to suggest that GAMyb-like proteins are involved in GA-mediated shoot elongation.

Therefore, it is conceivable that heterotrimeric G protein and SLR1/SLN1 are signaling molecules shared by both GA-mediated shoot elongation and α-amylase expression in the aleurone tissue because mutations in either of these signaling molecules affect both processes. It is well documented that GAMyb is responsible for α-amylase induction and related processes in the aleurone tissue. This regulatory molecule is probably also involved in reproductive development because it is strongly expressed in barley anthers, and over-expression of GAMyb reduces male sterility (Woodger and others 2003). Furthermore, loss-of-function mutants of GAMyb in rice also affect reproductive development (M. Matsuoka personal communication). However, GAMyb does not seem to have a clear role in stem elongation. Thus, somewhere between SLR1/SLN1 and GAMyb, the GA signaling pathway branches out to modulate different sets of responses in shoot elongation, reproductive development and seed germination.

The inhibitory role of ABA on GA induction of α-amylase was recognized soon after ABA was first isolated as a plant growth regulator (Chrispeels and Varner 1965). However, it took more than 20 years to observe that ABA also induces a large number of genes, most of them related to stress responses (Skriver and Mundy 1990). In the cereal aleurone tissue, ABA induces the synthesis of late embryogenesis-abundant (LEA) proteins that are likely to be related to the high level of stress tolerance of this tissue (Dure III 1993; Hong and others 1988). Among the few non-LEA proteins induced by ABA, there is a SNF1-related protein kinase, PKABA1 (Anderberg and Walker-Simmons 1992; Yamauchi and others 2002). As shown in Figure 2, the PKABA1 mRNA is readily detectable in aleurone tissue treated with no hormone although its level is further enhanced by ABA treatment and suppressed by GA treatment (Yamauchi and others 2002). It has been shown that phospholipase D is likely involved in early events of the ABA signaling pathway (Ritchie and Gilroy 1998). However, the early ABA signaling appears to branch out into two separate pathways: one involved in the induction of *LEA* genes and the other one in the suppression of α-amylase.

It has been determined that ABA induction of gene expression requires cis-acting elements called ABA response promoter complexes, consisting of either two copies of a specialized G-box element (also called ABA response element: ABRE) or one copy of a G-box (ABRE) plus a copy of a coupling element (two have been determined: CE1 and CE3) (Hobo and others 1999a; Hobo and others 1999b; Shen and Ho 1995; Shen and others 1996). Fur-

thermore, the ABA induction of *LEA* genes requires either a particular class of bZIP transcription factors, including ABI5, which interacts with the ABA response element (ABRE) and coupling element 3 (CE3) (Finkelstein and Lynch 2000; Casaretto and Ho 2003), and/or an AP2 domain transcription factor ABI4, which interacts with the coupling element 1(CE1) (Finkelstein and others 1998; Söderman and others 2001; Niu and others 2002). It has been shown that ABA induces the synthesis as well as activation of ABI5 via phosphorylation (Casaretto and Ho 2003; Kagaya and others 2002). The stability of ABI5 is also somehow affected by ABA (Lopez-Molina and others 2001).

A third factor, VP1/ABI3 also appears to be needed for ABA induction of gene expression (Hattori and others 1992; Hobo and others 1999; Nakamura and others 2001; Casaretto and Ho 2003). However, although VP1/ABI3 has a transactivation domain, it does not bind directly to any components of the ABA response promoter complex. Thus, it probably interacts with ABI5 to activate gene transcription. The ABA suppression of α-amylase expression appears to be quite different from the induction pathway as evidenced by several observations. First, the protein kinase PKABA1 mimics ABA action in suppressing α-amylase gene expression, yet it has no significant effect on ABA induction of *LEA* genes (Figure 3) (Gomez-Cadenas and others 1999). Second, the transcription factor ABI5 is required for ABA induction of *LEA* genes, but does not play a role in ABA suppression of α-amylase expression (Casaretto and Ho 2003). Third, the expression of a heterologous *Arabidopsis* mutant gene, *abi1*, in barley aleurone tissue blocks ABA induction of *LEA* genes, yet has no effect on ABA suppression of α-amylase expression (Shen and others 2001).

Crosstalk Between GA and ABA in Cereal Aleurone Tissue

In their classical work published more than 30 years ago, Chrispeels and Varner (1965) studied α-amylase production as affected by various concentrations of GA and ABA. Their kinetic analysis indicated that GA and ABA do not compete for a common site, yet little is known about the antagonism between these two hormones. Although the GA receptor remains unidentified, circumstantial evidence suggests that it is likely localized on the external face of plasma membrane (Gilroy and Jones 1994). It is conceivable that the interactions between GA and its putative membrane receptor activate the heterotrimeric G protein and eventually

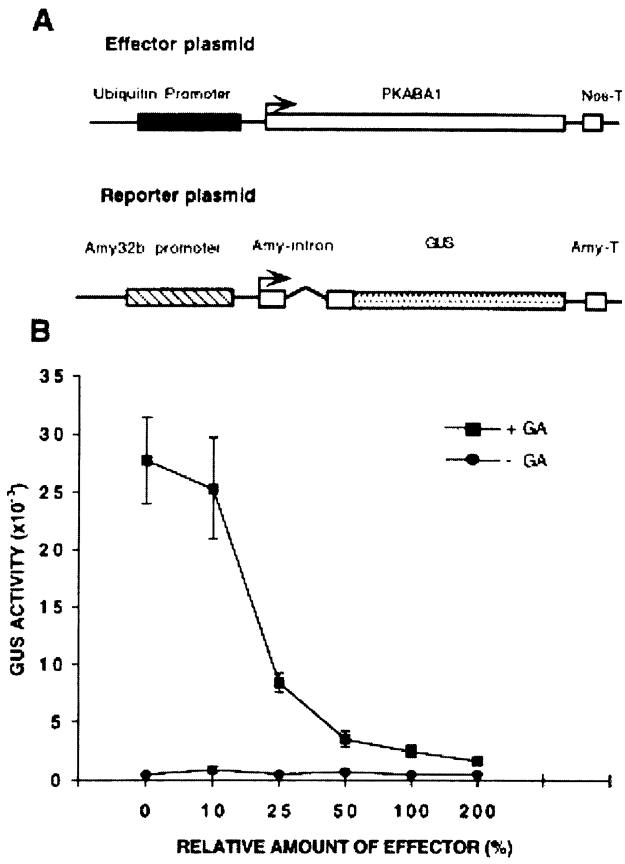

Figure 3. The protein kinase PKABA1 suppresses the GA3-induced expression of a low-pI α-amylase gene. (**A**) Schematic diagram of the effector and reporter constructs used in cobombardment experiments. *NOS-T*, terminator of nopaline synthetase gene. (**B**) The effector construct, UBI-PKABA1, was cobombarded into barley embryoless half-seeds (with aleurone layers still attached to the starchy endosperm) along with the reporter construct (*α-amylase–GUS*) and the internal control construct (UBI-luciferase). The amount of reporter and control plasmid DNA was always constant, whereas that of effector varied with respect to the reporter as shown in the *x*-axis. Transfected half-seeds were incubated for 24 h with (■) or without (●) 1 μM GA_3. GUS activity was normalized in every independent transformation relative to the luciferase activity. From Gomez-Cadenas and others (2001).

lead to the destabilization of SLR1/SLN1 (Fu and others 2002; Itoh and others 2002), allowing the expression of GAMyb and other downstream events to take place.

In theory, ABA could block any of these steps along the way. The results shown in Figure 1 strongly suggest that the site of action of ABA has to be downstream from where SLN1 acts. About a quarter of the progeny seeds from the selfing of a heterozygous *SLN1/sln1* barley plant synthesize and secrete α-amylase from its aleurone tissue in the

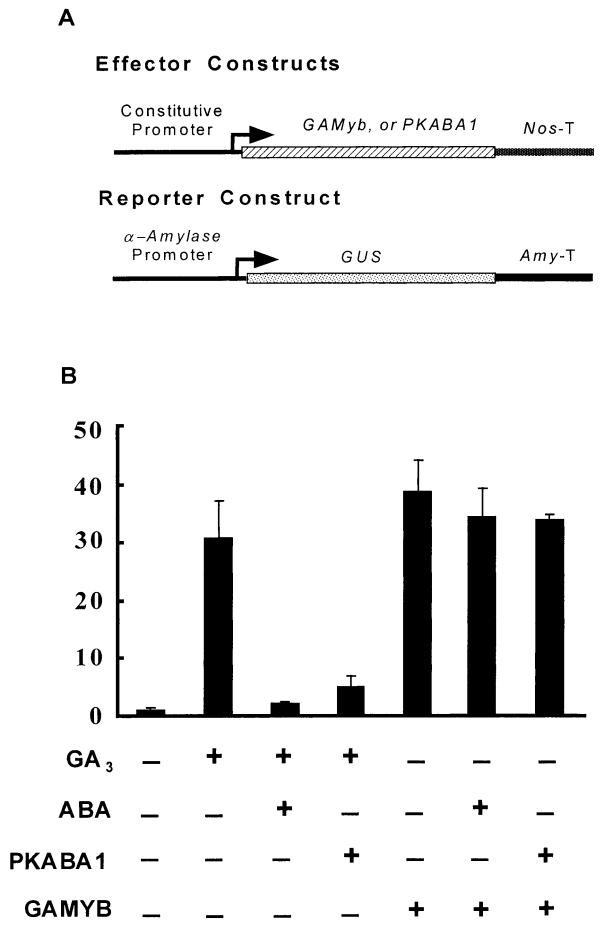

Figure 4. The GAMyb transactivation of the α-amylase promoter is not suppressed by ABA or the protein kinase PKABA1. (**A**) Schematic diagram of the effector and reporter constructs used in the cobombardment experiments. *NOS-T*, terminator of nopaline synthetase gene. (**B**) The reporter construct, *α-amylase–GUS*, and the internal control construct, *UBI–luciferase*, were cobombarded into wild-type embryoless half-seeds with (+) or without (−) the effector constructs (*UBI–GAMyb* and *UBI–PKABA1*) by using the same amount of effector and reporter constructs. Bars indicate GUS activities ±SE after 24 h of incubation of the bombarded half-seeds with no hormones or different combinations of 1 μM GA_3 and 20 μM ABA. From Gomez-Cadenas and others (2001).

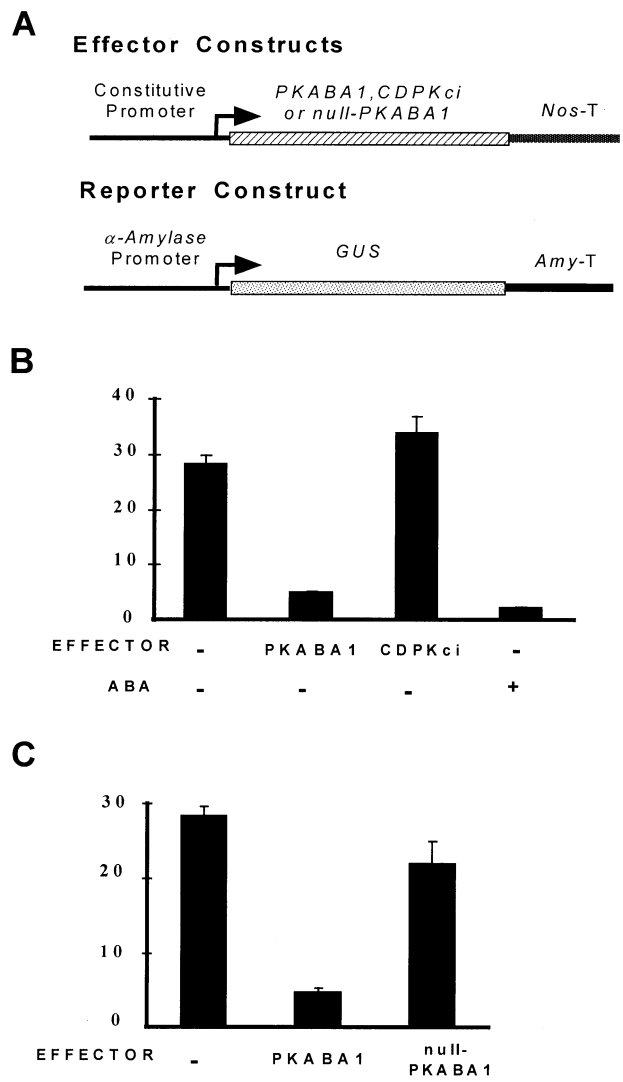

Figure 5. The protein kinase PKABA1 specifically represses the constitutive expression of α-amylase in the *slender* mutant. (**A**) Schematic diagram of the effector and reporter constructs used in the cobombardment experiments. *NOS-T*, terminator of nopaline synthetase gene. (**B**) The reporter construct, *α-amylase–GUS*, and the internal control construct, *UBI–luciferase*, were cobombarded into *slender* mutant embryoless half-seeds with (+) or without (−) the effector constructs (*35S-PKABA1* or *35S–CDPKci* [a Ca^{+2} insensitive mutant of a calcium-dependent protein kinase) by using the same amount of effector and reporter constructs. Bars indicate GUS activities ±SE after 24 h of incubation of the bombarded half-seeds with (+) or without (−) 20 μM ABA. (**C**) The experimental conditions are the same as in (**B**), except that the effector constructs used were *UBI–PKABA1* and *UBI–null-PKABA1*. Bars indicate GUS activities ±SE after 24 h of incubation of the bombarded half-seeds without hormone treatment. From Gomez-Cadenas and others (2001).

absence of GA due to the absence of functional repressor SLN1 (the genotype is *sln1/sln1/sln1*). Yet, this GA-independent synthesis of α-amylase can still be suppressed by ABA, indicating that ABA must work downstream from where SLN1 exerts its regulatory role. Because the particular *sln1* mutation is in the coding region of *SLN1* gene, it is not likely that the effect of ABA is to enhance the activity level of activity of SLN1.

Ectopic expression of GAMyb via the introduction of a transgene containing the coding reading of GAMyb driven by a constitutive promoter is able to transactivate α-amylase expression in a GA-

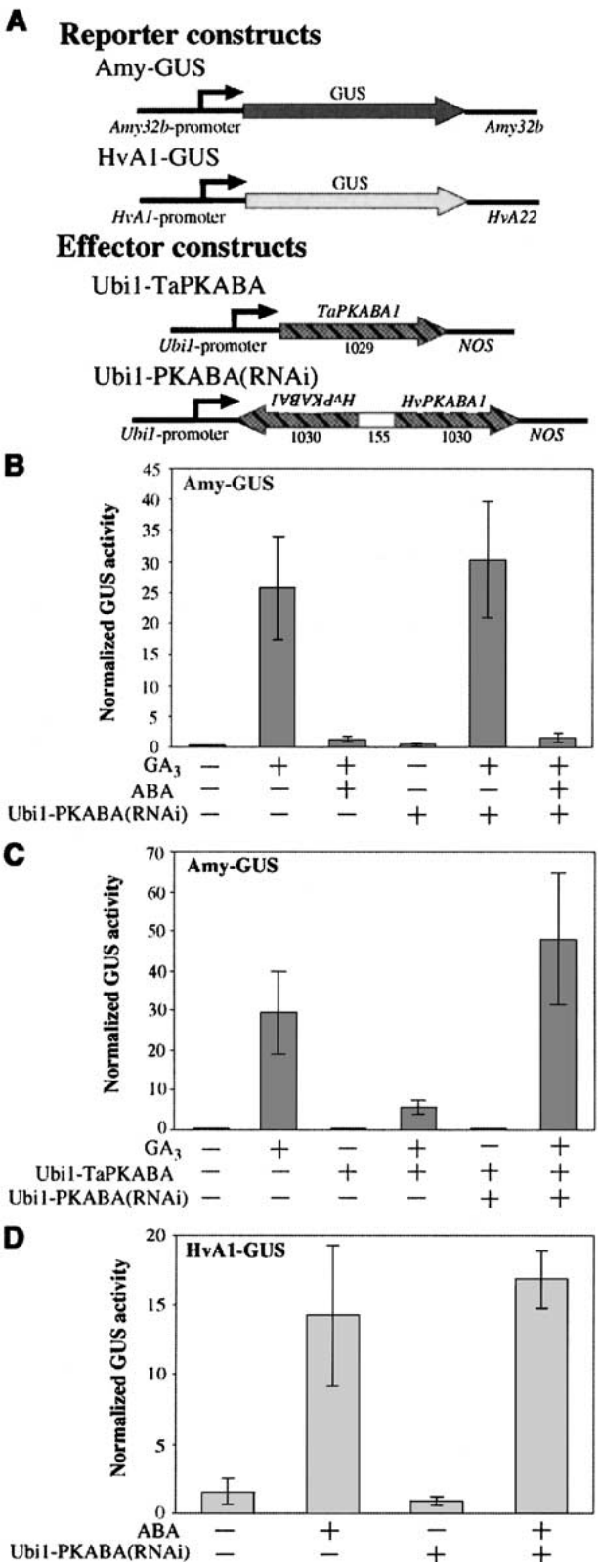

Figure 6. *PKABA1* RNAi does not affect the antagonistic effect of ABA on the GA-mediated induction of α-amylase. (**A**) Schemes of gene constructs.The effector construct shares 100% sequence identity with the endogenous gene *HvPKABA1* throughout the entire inverted repeats and shares 94% identity with the wheat *TaPKABA1*. Arrowheads indicate the orientation of the gene or gene fragment. Numbers below the effector constructs represent the size (in bp) of every segment or the entire ORF (not drawn to scale). (**B**) The reporter construct Amy-GUS was cobombarded with either the Ubi1-Empty vector (−) or the effector construct Ubil-PKABA(RNAi) at a 1:1 ratio. Embryoless half-seeds then were incubated with (+) or without (−) 1 μM GA_3 and/or 20 μM ABA for 24 h. (**C**) The GA-inducible reporter construct Amy-GUS was cotransformed with the Ubi1-TaPKABA and/or Ubi1-PKABA(RNAi) effector constructs. The Ubi1-Empty vector (−) replaced either or both effector constructs to account for a 1:1:1 ratio. Embryoless half-seeds were incubated for 24 h with (+) or without (−) 1 μM GA_3. (**D**) The ABA-inducible reporter construct HvA1-GUS was cotransformed with the Ubi1-Empty vector (−) or the effector construct Ubi1-PKABA(RNAi) at a 1:1 ratio. Embryoless half-seeds then were incubated for 24 h with (+) or without (−) 20 μMABA. For (**B**) to (**D**), bars represent normalized GUS activities ± SE (n = 4). From Zentella and others (2002).

independent manner (Gubler and others 1995; Gomez-Cadenas and others 2001). Although ABA is very effective in blocking the GA-induced α-amylase expression, it has no effect on the GAMyb-transactivated α-amylase expression (Figure 4). Furthermore, the GA induction of GAMyb expression can still be repressed by ABA, and the same results are observed if a reporter gene, GAMyb promoter::GUS, is used (Gomez-Cadenas and others 2001; Gubler and others 2002). Taken together, these observations indicate that at least the primary effect of ABA is to block the GA-induction of GAMyb expression at the transcriptional level (Gomez-Cadenas and others 2001; Gubler and others 2002).

Therefore, the action of ABA is to intersect the GA signaling pathway somewhere between SLR1/SLN1 and GAMyb. However, it should be pointed out that very little is known about the GA signaling pathway between these two points. SLR1/SLN1 is likely to be a nuclear-localized transcription factor, yet nothing is known about its target site (Chandler and others 2002; Itoh and others 2002). Although GA can induce GAMyb at the transcriptional level, the level of this induction (3–5-fold) is much lower than the GA induction of α-amylase gene expression (50–100-fold). Thus, it is likely that other levels of regulation of GAMyb by GA also exist, and more investigations in this area are definitely needed.

The ABA-induced PKABA1 is a member of the SnRK2 subfamily of SNF-1-related protein kinase

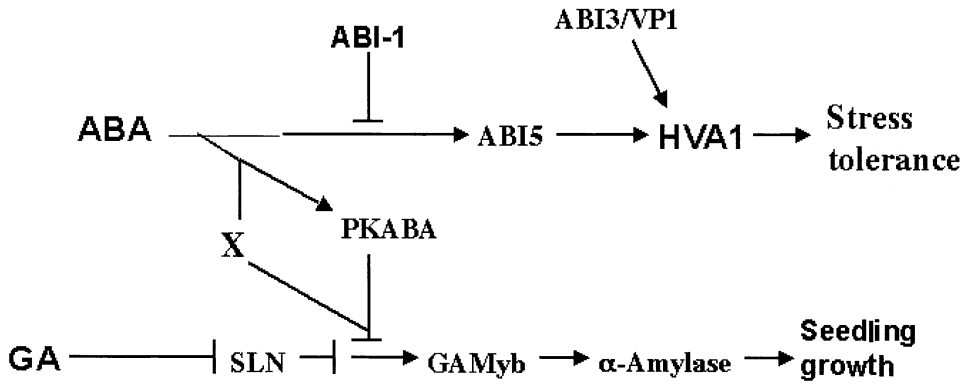

Figure 7. Important steps involved in the GA/ABA crosstalk in regulating α-amylase expression and related events in cereal aleurone cells. A represser, SLN1, and an activator, GAMyb, are essential components in the GA signaling pathway leading to the induction of α-amylase. An alternative route from ABA to the site of interaction with the GA signaling cascade (represented by X) is proposed to indicate that the transcriptional induction of *PKABA1* is sufficient, but not necessary, to block α-amylase expression. Two transcription factors, ABI5 and VP1 are required for the ABA induction of LEA proteins, such as HVA1.

(Yamauchi and others 2002). Unlike the *LEA* genes, the ABA induction of PKABA1 can be reversed by GA, an indication that PKABA1 could be involved in GA/ABA antagonism (D. Yamauchi unpublished). When PKABA1 is over-expressed, it mimics the ABA effect on the suppression of α-amylase in a dosage-dependent manner (Figure 3). Similar to ABA, over-expression of PKABA1 in *sln1* mutant seeds can also block the GA-independent α-amylase expression (Figure 5). This effect of PKABA1 appears to be quite specific: 1) another protein kinase, CDPK, is totally ineffective in suppressing α-amylase expression (Figure 5B) (Gomez-Cadenas and others 1999), and 2) mutation in the ATP binding site of the coding region of PKABA1 renders it ineffective (Figure 5C) (Gomez-Cadenas and others 1999). Like ABA, over-expression of PKABA1 has no effect on GAMyb transactivated α-amylase expression (Figure 4). Therefore, the site of action of PKABA1 relative to GA signaling molecules appears to be the same as that for ABA.

Although the ABA-induced PKABA1 can replace ABA in suppressing GA induction of GAMyb and α-amylase, it is not absolutely required for the action of endogenous ABA. As shown in Figure 6, synthesis of an RNAi molecule designed specifically for knocking down PKABA1 expression can effectively eliminate the effect of constitutive expression of PKABA1 on GA induction of α-amylase expression (Figure 6C), that is, the effect of a constitutively expressed PKABA1 fails to suppress α-amylase expression in the presence of PKABA1-RNAi (Zentella and others 2002). However, RNAi for PKABA1 does not affect either the ABA suppression of α-amylase expression (Figure 6B) or the ABA induction of a *LEA* gene, *HvA1* (Figure 6D). Taken together, these data demonstrate that although PKABA1-RNAi is effective in knocking down PKABA1 expression, it has no significant effect on ABA regulated gene expression, indicating that there could be another protein kinase sharing similar function as PKABA1. Knocking down PKABA1 expression by its specific RNAi would still allow this unknown factor to carry out the ABA suppressive effect on α-amylase expression. However, it is possible that PKABA1 is a very stable enzyme, and a certain level of PKABA1 has already existed in the tissue prior to ABA treatment. If ABA somehow activates the preexisting enzyme, simply knocking down the synthesis of new PKABA1 molecules would have little effect on ABA suppression of α-amylase expression. More investigations to clarify this possibility seem to be warranted.

Recently, it has been shown that an ABI5-like protein in wheat is the potential substrate for PKABA1 (Johnson and others 2002). Since ABI5 has been shown to be phosphorylated and required for ABA induction of *LEA* genes, does this observation suggest that PKABA1 still has a role in this process? If so, it is puzzling why neither over-expression of PKABA1 nor knocking down PKABA1 expression by RNAi has any significant effect on the ABA induction of *LEA* genes.

Perspectives

The crosstalk between GA and ABA in regulating the developmental transition from seed formation to germination is a challenging research topic as well as an agriculturally important problem. The cereal aleurone tissue appears to be a convenient

system in elucidating the molecular mechanisms underlying this process. The existence of well-characterized downstream hormone effects in terms of GA-induced and ABA-suppressed hydrolytic enzyme synthesis and ABA induction of *LEA* genes has allowed researchers to decipher three distinct signaling pathways: 1) GA signaling involving activation of heterotrimeric G protein, destabilization of the represser SLR1/SLN1 and induction of the transcription factor GAMyb, 2) ABA gene induction pathway characterized by the activation and induction of the bZIP transcription factor ABI5, and 3) ABA gene suppression pathway intersecting the GA signaling pathway between SLR1/SLN1 and GAMyb (Figure 7). With the rice genome project finished, the existence of many GA mutants, and the current functional genomics effort in generating more targeted mutants, the cereal aleurone system would be ideal for an integrated approach combining physiology, biochemistry, molecular biology, cell biology and genetics to further investigate these signaling pathways and crosstalk between GA and ABA.

Several outstanding questions await more research efforts. First, what is the relationship between SLR1/SLN1 destabilization and GAMyb induction, and how does ABA affect this process? Second, what is the unknown factor that may share functional redundancy with PKABA1 in suppressing GA-induced α-amylase expression? Third, how do early ABA signaling events lead to the divergence between the induction and suppression pathways? Fourth, is the information learned from studying the aleurone system useful in understanding GA/ABA interactions in other tissues?

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