An Abscisic Acid-Induced Protein, HVA22, Inhibits Gibberellin-Mediated Programmed Cell Death in Cereal Aleurone Cells

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Plant HVA22 is a unique abscisic acid (ABA)/stress-induced protein first isolated from barley (Hordeum vulgare) aleurone cells. Its yeast homolog, Yop1p, functions in vesicular trafficking and in the endoplasmic reticulum (ER) network in vivo. To examine the roles of plant HVA22, barley HVA22 was ectopically expressed in barley aleurone cells. Overexpression of HVA22 proteins inhibited gibberellin (GA)-induced formation of large digestive vacuoles, which is an important aspect of GA-induced programmed cell death in aleurone cells. The effect of HVA22 was specific, because overexpression of green fluorescent protein or another ABA-induced protein, HVA1, did not lead to the same effect. HVA22 acts downstream of the transcription factor GAMyb, which activates programmed cell death and other GA-mediated processes. Moreover, expression of HVA22: green fluorescent protein fusion proteins showed network and punctate fluorescence patterns, which were colocalized with an ER marker, BiP:RFP, and a Golgi marker, ST:mRFP, respectively. In particular, the transmembrane domain 2 was critical for protein localization and stability. Ectopic expression of the most phylogenetically similar Arabidopsis (Arabidopsis thaliana) homolog, AtHVA22D, also resulted in the inhibition of vacuolation to a similar level as HVA22, indicating function conservation between barley HVA22 and some Arabidopsis homologs. Taken together, we show that HVA22 is an ER- and Golgi-localized protein capable of negatively regulating GA-mediated vacuolation/programmed cell death in barley aleurone cells. We propose that ABA induces the accumulation of HVA22 proteins to inhibit vesicular trafficking involved in nutrient mobilization to delay coalescence of protein storage vacuoles as part of its role in regulating seed germination and seedling growth.

Abscisic acid (ABA) mediates various important plant developmental and physiological processes and also plant responses to stress conditions (Zeevaart and Creelman, 1988; Leung and Giraudat, 1998). In particular, ABA is required for seed maturation programs and the maintenance of seed dormancy (Nambara and Marion-Poll, 2003; Gubler et al., 2005). The unique feature of de novo synthesis of hydrolytic enzymes in response to gibberellin (GA) and ABA has made the cereal aleurone cells an ideal system for studying hormone signaling and action during seed germination and seedling growth. In barley (Hordeum vulgare), more than a dozen ABA-induced proteins have been isolated from aleurone layers as part of the effort to study ABA action in seed physiology (Shen et al., 1993). Among those, LEA (for late embryogenesis abundant) proteins have been proposed to provide cell tolerance for seed desiccation and environmental stresses (Bartels et al., 1991; Xu et al., 1996; Sivamani et al., 2000). However, little is known about how these proteins function during seed development and germination.

The barley HVA22 gene encodes one of these ABA-induced LEA proteins isolated from the aleurone tissue (Shen et al., 1993). Bioinformatic analysis has revealed that 354 HVA22 homologs are present in diverse eukaryotic organisms, including plants, mosses, yeast, and mammals (Supplemental Fig. S1). These homologs share high amino acid sequence similarity in a conserved TB2/DP1 domain. Interestingly, no homologs were found in prokaryotes to date (Supplemental Fig. S1), suggesting that HVA22 is likely involved in eukaryote-specific functions.

The regulation of HVA22 expression during plant development is well characterized. In both barley and Arabidopsis (Arabidopsis thaliana), transcripts of HVA22 homologs in leaves are highly induced by ABA, drought, cold, and salt stresses (Shen et al., 1993, 2001; Chen et al., 2002). The ABA response complex located in the barley HVA22 gene promoter is necessary and sufficient in mediating the induction of this gene by ABA (Shen and Ho, 1995). Moreover, expression of the barley HVA22 gene is correlated with seed dormancy status (Shen et al., 2001). HVA22 mRNA gradually accumulates in the aleurone layer during the late stage of seed maturation (Shen et al., 2001), when high levels of endogenous ABA biosynthesis occur to maintain seed dormancy (Karssen et al., 1983). In nondormant seeds, transcripts of barley HVA22 in...
In contrast, the HVA22 transcript level remains high in dormant grains, in which GA treatment is able to break dormancy with a concomitant decline of HVA22 transcripts. These results suggest that turnover of HVA22 gene products in aleurone layers controls seed dormancy and germination (Shen et al., 1993). However, how plant HVA22 proteins function at the molecular and cellular levels in regulating seed germination is unknown.

Studies with Saccharomyces cerevisiae (Yop1p) and Xenopus (DP1) homologs of HVA22 reveal their potential roles in vesicular trafficking. Yeast Yop1p is able to physically interact with several Rab GTPases (Ypt1, Ypt6, Ypt7, and YIF1) and YIP1, which are involved in endoplasmic reticulum (ER)-to-Golgi transport in yeast (Yang et al., 1998; Calero et al., 2001; De Antoni et al., 2002). Deletion or overexpression of Yop1p resulted in abnormal vesicle accumulation in yeast cells (Calero et al., 2001; Brands and Ho, 2002). Furthermore, Yop1p/DP1 proteins also interact with ER reticulum proteins (Rtn4/NogoA) to shape the ER network in vivo (Voeltz et al., 2006; Hu et al., 2008). The simultaneous absence of Rtn4a and Yop1p resulted in disrupted tubular peripheral ER. Amino acid sequence alignment between Yop1p and HVA22 homologs from barley and Arabidopsis indicated that plant HVA22 proteins contain structures similar to yeast Yop1p, a short hydrophilic loop flanked by two hydrophobic stretches (Brands and Ho, 2002). It is plausible that plant HVA22 proteins also play a role in vesicle trafficking processes in plant cells.

How is vesicle trafficking related to seed germination? Since HVA22 was first cloned from barley aleurone cells, we focused our initial studies on the role of HVA22 in this particular tissue. The cereal aleurone layer is a metabolically active tissue surrounding the nutrient-rich starchy endosperm. Upon germination, GA produced by the growing embryo induces the production and secretion of hydrolytic enzymes from the aleurone to the starchy endosperm (Fincher, 1989). These enzymes liberate sugars and amino acids needed for the growing embryo and seedlings (Jones and Jacobsen, 1991). ABA blocks this process. Therefore, the numbers and sizes of PSVs in the aleurone cells have been used as semiquantitative markers of PCD (Bethke et al., 1999, 2007). To conveniently observe changes of PSVs in living cells, a red fluorescent protein, DesRed, was transiently expressed in barley aleurone cells driven by the constitutive maize (Zea mays) ubiquitin promoter (Fig. 1A). To eliminate the interference from endogenous GA or ABA synthesized by embryo, barley half-seeds without embryos were used in this study. Transformed aleurone tissues were incubated in buffer containing no hormone or 1 μM GA for 48 h, then the transformed aleurone cells were observed with a confocal microscope. The DesRed fluorescence disappeared in the PSVs due to pH and proteolytic activity (Zentella et al., 2002; Fluckiger et al., 2003; Fig. 1B). Thus, PSVs appeared as dark intracellular bodies whose sizes and numbers could be easily examined.

Many small PSVs were observed in the aleurone cells when incubated in the hormone-free control buffer (Fig. 1B, control). However, these vacuoles coalesced and became one large vacuole after 48 h of treatment with 1 μM GA (Fig. 1B, GA). The enlargement of storage vacuoles was significantly inhibited when 20 μM ABA was added with GA at the same time (Fig. 1B, GA + ABA). To provide a quantitative analysis, cells containing only one to three large vacuoles (as shown in Fig. 1B, GA) were classified as vacuolated cells. The percentage of vacuolated cells was calculated relative to total observed transformed cells. More than 80% of observed cells became distinctly vacuolated after 48 h of GA treatment, and ABA significantly inhibited the vacuolation to the control level (i.e. 20%–30%; Fig. 1C). These results demonstrate that transient expression of a fluorescent marker, DesRed, provides a simple but efficient method to monitor the extent of PCD-associated vacuolation of PSVs in barley aleurone cells, and the antagonistic effect of GA and ABA on this process can be quantified.
Overexpression of HVA22 Inhibits GA-Induced PCD

To examine if plant HVA22 affects GA-mediated PCD, an effector construct to overexpress barley HVA22 in aleurone cells was cobsombombed with the reporter construct for DesRed expression (Fig. 2A). The expression of HVA22 open reading frame (ORF) is driven by the maize ubiquitin promoter. Transformed barley half-seeds were first incubated in hormone-free control buffer for 24 h to allow the expression of HVA22 protein. These samples were then transferred to a new buffer with or without 1 μM GA for another 48 h before observation by confocal microscopy. Low and high magnifications of transformed aleurone tissues. Aleurone tissues were bombarded as described for B. Cells vacuolated were scored, and the percentage of vacuolated cells was calculated relative to total cells observed (see “Materials and Methods”). The results represent averages ± se (n = 6).

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To exclude the possibility that the inhibition of vacuolation by HVA22 is due to a general effect of overexpressing a toxic protein or any ABA-induced protein, constructs to overexpress GFP or barley HVA1 were also cotransformed with DesRed (Fig. 2A). Barley HVA1 was also isolated from ABA-induced cDNA clones of aleurone tissues and has been proposed to protect cells from dehydration during seed development (Hong et al., 1988; Xu et al., 1996). However, overexpression of HVA1 or GFP resulted in a similar level of vacuolated cells to those transformed with vector only under no-hormone (control) or GA-treated conditions (Fig. 2D). These results indicate that HVA22 specifically inhibits the GA-induced PCD-associated vacuolation of PSVs.

HVA22 Functions Downstream of GAMyb

In the GA signaling pathway in cereal aleurone cells, SLN1 is an upstream repressor of the GAMyb transcription factor, which is a crucial positive regulator of GA induction events (see Fig. 10 below; Gubler et al., 2002; Zentella et al., 2002; Kaneko et al., 2004). It has been reported that SLN1 also negatively regulates GA-induced PCD in aleurone cells (Zentella et al., 2002).Transient expression of SLN1RNAi was able to trigger GA signaling to induce PSV vacuolation more than the vector-alone control by 35%. Whether PCD of aleurone cells is also regulated by GAMyb transcription has not been shown. To further determine where in the GA signaling pathway HVA22 interferes with the GA-mediated PCD, we analyzed if GAMyb was also required for GA-induced PCD and how overexpressing HVA22 affects GAMyb action.

To express GAMyb transiently, the ORF for GAMyb was cloned into the same vector as HVA22, where its expression was under the control of the maize ubiquitin promoter Ubi1 (Fig. 3A). Without GA treatment, overexpression of GAMyb resulted in approximately 80% vacuolated cells (Fig. 3B), which is similar to the vacuolation level induced by GA treatment (Fig. 1C). Overexpression of HVA22 together with GAMyb could still inhibit GAMyb-induced vacuolation by 40%. These results suggest that GAMyb is also involved in GA-mediated PCD and HVA22 function downstream of GAMyb action.

Whether GAMyb is absolutely necessary to induce PCD in aleurone cells was further resolved via the RNA interference (RNAi) approach. Introduction of an RNAi-generating construct with particle bombard-
ment has been shown to be an effective method to perform transient loss-of-function studies of target genes in the aleurone tissue (Zentella et al., 2002). Thus, an RNAi effector construct, GAMybRNAi, was generated using a region of the GAMyb gene (351 bp) that encodes the transactivation domain of this transcription factor (Fig. 3A). When GAMybRNAi was cobombarded with GAMyb into the aleurone cells, expression of GAMybRNAi reduced GAMyb-mediated PSV vacuolation by 40% under control conditions (without GA treatment) after 48 h of transformation (Fig. 3B). Consistently, in aleurone cells treated with 1 μM GA for 48 h, GAMybRNAi inhibited GA-induced vacuolation by 35% compared with the vector-alone control (Fig. 3C). Cotransformation of GAMybRNAi with HVA22 could only lower another 15% of vacuolation cells compared with GAMybRNAi only, similar to the level seen with HVA22 alone. This result indicates that little additive effect exists between HVA22 and GAMybRNAi and that the actions of HVA22 and GAMyb on PCD are likely in the same regulatory pathway in aleurone cells.

Since the GAMybRNAi construct was not able to completely block GAMyb- or GA-induced PCD-associated PSV vacuolation, we wondered whether the GAMybRNAi construct was sufficiently effective at inactivating GA signaling in vivo. A previous study has shown that GAMyb is not only sufficient but also necessary for GA-upregulated α-amylase transcription (Zentella et al., 2002). We further examined the effectiveness of GAMybRNAi on the GA induction of α-amylase expression. The reporter construct used would express GUS driven by the α-amylase32 promoter (Amy::GUS; Fig. 4A; Lanahan et al., 1992). Another reporter construct, which expressed luciferase (Ubi::LUC) under the control of a constitutive maize Ubi1 promoter, was used to provide an internal control of transformation. The embryoless barley half-seeds were cobombarded with the effector constructs together with both of these reporter constructs. The relative GUS activity in vivo was analyzed after 24 h of incubation. Without GA treatment, expression of GAMyb significantly induced GUS activity (97-fold compared with vector alone) and GAMybRNAi almost completely blocked GAMyb-induced GUS activity (Fig. 4B). When treated with 1 μM GA for 24 h, expression of the Amy::GUS reporter construct was induced 27-fold compared with that in the no-GA control (Fig. 4C). However, expression of GAMybRNAi dramatically diminished the expression of GUS to the level seen in samples treated without GA (Fig. 4C). These observations indicated that the GAMybRNAi construct used was effective at inhibiting GAMyb-induced α-amylase expression.

Figure 2. Barley HVA22 inhibits PCD/vacuolation in aleurone cells in the presence of GA. A, Schemes of gene constructs. Gray arrows represent the ORF (not drawn to scale), the expression of which is driven by the maize ubiquitin promoter, Ubi1. B, Confocal micrographs of aleurone cells cobombarded with DesRed and a construct expressing HVA22 or vector. Samples were preincubated in control buffer for 24 h, then transferred to buffer containing no hormone (Control) or

1 μM GA (GA). After another 48 h, aleurone cells were observed with a confocal microscope. C, Magnified images of representative cells from B. D, Percentage of vacuolated cells was calculated as described for Figure 1C. The results represent averages ± se (n = 6).
Taken together, the results from overexpression of GAMyb and GAMyb plus HVA22 show that GAMyb is required for the GA-mediated PCD, which is inhibited by HVA22 acting downstream from GAMyb.

HVA22 Is Not the Only Regulatory Protein for ABA Inhibition of GA-Induced PCD/Vacuolation

Since ABA inhibits GA-induced PCD and the expression of HVA22 is highly induced by ABA, we hypothesize that ABA induces HVA22 accumulation to inhibit PCD during seed development. To investigate whether HVA22 is absolutely necessary for ABA inhibition of PCD, the RNAi effector construct HVA22RNAi was generated to inactivate endogenous HVA22 mRNA (Fig. 5A) by inserting inverted repeats of a conserved TB2/DP1 domain of HVA22 (306 bp) into the GFP ORF (750 bp). When cells were cotransformed with HVA22 and HVA22RNAi effector constructs, the inhibitory effect of HVA22 on GA-induced vacuolation was completely abolished (Fig. 5B). This result confirmed that HVA22RNAi was effective at blocking HVA22 inhibition on PCD. However, when compared with the vector-only control, HVA22RNAi did not result in a significantly higher vacuolation level in aleurone cells treated with GA plus ABA (Fig. 5C), indicating that HVA22RNAi could not block the effect of ABA on GA-induced PCD.
These results suggest that HVA22 is not absolutely required for ABA-mediated inhibition on PSV vacuolation and PCD.

Localizations of HVA22 in ER and the Golgi Apparatus

What is the mechanism of HVA22 in regulating PCD? To address this question, the subcellular localizations of HVA22 in aleurone cells were examined. The coding region of barley HVA22 cDNA was fused in frame with GFP ORF at the N terminus (HVA22::GFP), and the resulting construct was cotransformed into aleurone cells with DesRed (Fig. 6A). To confirm that the HVA22::GFP fusion protein is functional, the effect of expressing HVA22::GFP on GA-induced PCD was analyzed. When compared with cells transformed with GFP, overexpression of HVA22::GFP significantly reduced the percentage of vacuolated cells by 20% and 40% under control and GA-treated conditions (Fig. 6B), respectively. This result demonstrates that the fusion protein HVA22::GFP is able to perform similar functions as native HVA22 proteins in vivo.

In the aleurone cells cotransformed with HVA22::GFP and DesRed, the HVA22::GFP fusion proteins produced punctate patterns and some network-like strings in both nonvacuolated (Fig. 6C, a and c) and vacuolated (Fig. 6C, g and i) cells, whereas DesRed alone produced the expected diffuse pattern (Fig. 6C, b and h). When GFP and DesRed were cotransformed in cells, both proteins generated exactly the same diffuse pattern in both nonvacuolated (Fig. 6C, d–f) and vacuolated (Fig. 6C, j–l) cells.

The punctate and network patterns were reminiscent of the localization of the ER and Golgi apparatus (Kim et al., 2001), as observed previously with HVA22 homologs in yeast and animal cells (Calero et al., 2001; Voeltz et al., 2006). In plant cells, the BiP::RFP fusion protein between red fluorescent protein (RFP) and portions of the chaperone binding protein (BiP) has been widely used as an ER marker (Kim et al., 2001; Song et al., 2006), and ST::mRFP, a chimeric protein between rat sialyltransferase and monomeric red fluorescent protein (mRFP), has been shown to be localized in the Golgi stacks (Kim et al., 2001). In order to further define the subcellular localizations of HVA22 protein, HVA22::GFP was cotransformed with BiP::RFP or ST::mRFP for comparison.

When transformed with both HVA22::GFP and BiP::RFP, the aleurone cells displayed the BiP::RFP fluorescence in filamentoid and patchy ER patterns (Fig. 7B), which is consistent with previous observations in Arabidopsis cells (Kim et al., 2001). Some portions of the HVA22::GFP-positive dim filaments and bright punctate stains were colocalized with fluorescence from BiP::RFP (Fig. 7, A–C, arrowheads). The intensity profile of the green and red fluorescence signals along the transect in Figure 7D further showed that the arrowhead-highlighted peaks of HVA22::GFP signals corresponded to the BiP::RFP signals located at the ER.

In aleurone cells transformed with HVA22::GFP and ST::mRFP, the red fluorescence of ST::mRFP was observed in the punctuate Golgi complexes and the space between the plasma membrane and cell walls (Fig. 7F). Most of the dot-like green fluorescent signals of HVA22::GFP overlapped with the red fluorescent signals of ST::mRFP to yield yellow signals (Fig. 7, E–G, arrowheads). The intensity profile of GFP and RFP signals along the transect in Figure 7H further demonstrated that those peaks of HVA22::GFP signals corresponded to the positions of ST::mRFP signals. These results suggest that barley HVA22 proteins are likely localized in the ER and Golgi apparatus.

Roles of Transmembrane Domains in HVA22 Function

According to Pfam prediction (http://pfam.sanger.ac.uk/family?id=TB2_DP1_HVA22&tab=speciesBlock), the barley HVA22 protein contains three potential transmembrane domains located in the TB2/DP1 region (Fig. 8A). To examine the function of these transmembrane domains in vivo, we generated effector
constructs expressing truncated forms of HVA22 proteins, ΔTM1, ΔTM2, and ΔTM3 (Fig. 8A). The individual transmembrane domains (TM1, TM2, and TM3) were deleted such that the remaining amino acids formed in-frame protein with the other two domains. When these truncated HVA22 proteins (ΔTM1, ΔTM2, and ΔTM3) were overexpressed with DesRed, none of these truncated proteins could inhibit GA-induced PCD and PSV vacuolation; in the same experiment, however, intact HVA22 protein reduced the vacuolation by around 60% compared with the vector-alone control (Fig. 8B).

It is possible that the truncated forms of HVA22 proteins (ΔTM1, ΔTM2, and ΔTM3) are not stable in vivo or are misexpressed in other subcellular compartments. To clarify these possibilities, the localizations of these truncated proteins were further investigated. The truncated forms of HVA22 were fused in frame with GFP at the N terminus (ΔTM1:GFP, ΔTM2:GFP, and ΔTM3:GFP) in the same manner as HVA22:GFP used in Figure 6A. As expected, the expression of HVA22:GFP resulted in distinct punctate and filamentoid patterns (Fig. 8C, a and c), in contrast to the diffused pattern of DesRed (Fig. 8Cb). The localizations of ΔTM1:GFP (Fig. 8Ce) and ΔTM3:GFP (Fig. 8Cm) were similar to those of the full-length protein, HVA22:GFP; however, 95% of cells (from a total of 40 cells observed) transformed with ΔTM2:GFP displayed weak green fluorescence and diffuse patterns as DesRed (Fig. 8C, i–k). The distributions of the signal intensities of green and red fluorescence of these representative cells further showed low intensity and diffuse patterns of ΔTM2:GFP signals (Fig. 8Cl) when compared with HVA22:GFP (Fig. 8Cd), ΔTM1:GFP (Fig. 8Ch), and ΔTM3:GFP (Fig. 8Cp). These observations highlight the importance of TM2 for its protein stability and correct subcellular localization in vivo. However, we cannot exclude the possibility that other sequences are also required for its function. Therefore, further studies will be required to examine the effects of other hydrophilic domains on PCD.

**Arabidopsis HVA22 Also Inhibits PCD/Vacuolation**

Electron microscopy showed that the ultrastructure of Arabidopsis aleurone cells is similar to that of cereal aleurone cells (Bethke et al., 2007). The same GA-induced PCD and PSV vacuolation were also observed in the Arabidopsis aleurone cells. It is possible that Arabidopsis HVA22 homologs perform similar functions to barley HVA22. In Arabidopsis, 16 HVA22
homologs were identified through blasting the TB2/DP1 domain of barley HVA22 (Supplemental Fig. S1). Among these HVA22 homologs, only AtHVA22A, AtHVA22B, AtHVA22C, and AtHVA22E are predicted to contain similar transmembrane domains as barley HVA22 (Supplemental Fig. S2). To investigate if these Arabidopsis homologs function similarly to barley HVA22, the reporter DesRed was cobombarded with effector constructs expressing AtHVA22A, AtHVA22B, AtHVA22C, AtHVA22D, and AtHVA22E driven by the maize Ubi1 promoter (Fig. 9A). In the presence of GA, overexpressing AtHVA22D proteins was most effective at reducing GA-induced PSV vacuolation, with its effect similar to that caused by barley HVA22 (Figs. 2D and 9B). AtHVA22B, AtHVA22C, and AtHVA22E proteins only provided some degree of inhibition on PCD, and AtHVA22A had no effect on vacuolation in aleurone cells.

DISCUSSION

The cereal aleurone layer is a secretory tissue in which hydrolytic enzymes are de novo synthesized and secreted for breaking down the reserves stored in starchy endosperm for the early stages of seedling
growth (Filner and Varner, 1967; Eastmond and Jones, 2005). The hydrolases are synthesized from amino acids previously incorporated in storage proteins, which originate from the ER and Golgi pathway and accumulate primarily in specific differentiated vacuoles, PSVs (Herman and Larkins, 1999; Vitale and Hinz, 2005). When the aleurone layer has completed its digestive function, PCD of aleurone cells occurs concurrently with extensive fusion of PSVs to generate large central vacuoles (vacuolation; Jones and Price, 1970; Bethke et al., 1999). GA is an enhancer, while ABA is a negative regulator, of this PCD process in aleurone cells (Bethke et al., 1999, 2007).

In this study, we provide evidence to show that expression of barley HVA22 specifically inhibits GA-induced PCD/vacuolation of aleurone cells (Figs. 1 and 2). The action of HVA22 is downstream of the transcription factor GAMyb (Fig. 3). Based on these results and previous signal transduction studies, we propose a model of HVA22 function as shown in Figure 10. HVA22 is the downstream ABA-induced membrane protein negatively regulating GA-induced PCD in barley aleurone cells. The synthesis of HVA22 is downstream from transcription factors ABI5/VP1, and the action of HVA22 is downstream from the GA-mediated transcription factor GAMyb (Casaretto and Ho, 2003).

GAMyb has been shown to be the principal transcription activator for hydrolase expression in aleurone cells (Gubler et al., 1995; Zentella et al., 2002; Kaneko et al., 2004). It is surprising that GAMyb is sufficient but may not be absolutely necessary for GA-

Figure 8. Transmembrane domains are necessary for HVA22 function. A, Schemes of gene constructs. White boxes indicate predicted transmembrane domains of HVA22 protein. Black boxes and arrows indicate the same N-terminal and C-terminal regions used in these constructs. B, Percentage of vacuolated cells in transformed aleurone tissues. The reporter DesRed was co-bombarded with empty vector, a construct expressing HVA22, or constructs expressing various HVA22 truncated forms (ΔTM1, ΔTM2, and ΔTM3) into aleurone cells. Samples were preincubated in control buffer for 24 h, then transferred to buffer containing no hormone (Control) or 1 μM GA. After another 48 h, aleurone cells were observed by confocal microscopy. Percentage of vacuolated cells was calculated as described for Figure 1C. The results represent averages ± se (n = 6). C, Confocal micrographs of transformed cells expressing HVA22::GFP (a-c), ΔTM1::GFP (e-g), ΔTM2::GFP (i-k), and ΔTM3::GFP (m-o). The intensity profiles of green and red fluorescence from representative cells expressing HVA22::GFP (d), ΔTM1::GFP (h), ΔTM2::GFP (l), and ΔTM3::GFP (p) are also shown.
induced PCD, since depletion of GAMyb expression via GAMybRNAi only decreased GA-induced vacuolation by 40% (Fig. 3). A similar result was also observed for its upstream repressor, SLN1, which is an important intermediate of GA signaling (Fig. 10; Gubler et al., 2002; Zentella et al., 2002). However, we cannot exclude the possibility that there is another unknown factor to activate the GA signaling pathway. Also, while GAMybRNAi is able to effectively block GA activation on $\alpha$-amylase expression (Fig. 4C), a relatively small amount of $\alpha$-amylase activity (7-fold compared with vector only) could still be observed when GAMyb and GAMybRNAi were cotransformed (Fig. 4B). These data suggest that some residual amount of GAMyb remained in vivo under these conditions, which could have triggered the downstream PCD.

Loss of function of HVA22 via transformation with the HVA22RNAi construct did not alleviate ABA inhibition of the GA effect (Fig. 5), suggesting that HVA22 is sufficient but not necessary for ABA inhibition of PCD. This could be explained by functional compensation by other HVA22-like genes or other synergistic components. This notion is supported by the fact that HVA22 genes constituted families of 51 and 16 genes in rice (Oryza sativa) and Arabidopsis, respectively (Supplemental Fig. S1). It is also worth noting that HVA22/Yop1p/DPI-deficient yeast or nematode worms grow normally (Brands and Ho, 2002; Voeltz et al., 2006; Audhya et al., 2007). The defective phenotypes were only observed in combination with mutation/deletion of another coordinated protein, Rtn4 or Sey1p, which does not share sequence similarity.

Alternatively, ABA could directly inhibit the expression of GAMyb that is important for downstream GA-mediated processes (Fig. 10). It has been shown that ABA could inhibit GAMyb transcription to prevent $\alpha$-amylase expression (Zentella et al., 2002). Why do cells need two ABA regulatory pathways in controlling PCD? When barley aleurone protoplasts were preincubated with GA for 24 h, adding ABA was no longer able to block $\alpha$-amylase expression (Bethke et al., 1999; Fath et al., 2001), demonstrating that ABA inhibition of the GA signaling pathway is dependent on timing and the fine balance between GA and ABA action (Finch-Savage and Leubner-Metzger, 2006). This could be particularly important for seedlings under stress, in which the GA-mediated processes are already under way yet need to be suppressed in response to stressful conditions. It is conceivable that the stress-induced ABA in turn suppresses PCD downstream from GAMyb, which is already induced by GA. Therefore, the stressed seedlings could still slow the nutrient mobilization processes that are not needed during stress. During late stages of seed development, the accumulation of HVA22 transcripts in barley embryo is correlated with endogenous ABA biosynthesis (Shen et al., 2001). Highly accumulated HVA22 mRNA is maintained in dry seeds until imbibition, whereas ABA is degraded to lower levels in fully mature seeds (Karssen et al., 1983). Thus, accumulation of HVA22 proteins could provide another control mechanism in aleurone cells to prevent premature GA-induced PCD to maintain dormancy when ABA is low in mature seeds. This hypothesis is further supported by the fact that the amount of HVA22 transcripts is correlated with the status of seed dormancy (Shen et al., 2001), which has been suggested to

Figure 9. Effects of overexpressing Arabidopsis ATHVA22 homologs on GA-induced PCD/vacuolation in barley aleurone cells. A, Schemes of gene constructs. Gray arrows represent the ORF (not drawn to scale), whose expression is driven by the maize ubiquitin promoter, Ubi1. B, Percentage of vacuolated cells in transformed aleurone tissues. The reporter DesRed was co-bombarded with empty vector or constructs expressing Arabidopsis ATHVA11A, ATHVA11B, ATHVA11C, ATHVA11D, or ATHVA11E. Samples were preincubated in control buffer for 24 h, then transferred to buffer containing no hormone (Control) or 1 $\mu$M GA. After another 48 h, aleurone cells were observed with a confocal microscope. Percentage of vacuolated cells was calculated as described for Figure 1C. The results represent averages $\pm$ se ($n = 6$).

Figure 10. Scheme of factors and pathways involved in GA/ABA-regulated PCD/vacuolation in aleurone cells. A repressor, SLN1, and an activator, GAMyb, are important regulators in the GA-mediated PCD and the associated PSV vacuolation. ABA negatively regulates PCD both upstream and downstream of GAMyb. The ABA-induced HVA22 is involved in the inhibition downstream of GAMyb.
be determined by the existence of aleurone tissues (Bethke et al., 2007).

Exclusive localizations of HVA22:GFP in the ER and Golgi stacks (Figs. 6 and 7) and the requirement of transmembrane domains for its function (Fig. 8) imply that barley HVA22 exerts its function in ER and Golgi membranes. These results are consistent with previous observations with yeast and animal HVA22 homologs, Yop1p and DP1. The Yop1p/DP1 proteins are predominantly localized in the ER and have been shown to interact with reticulons to shape peripheral ER tubules (Voeltz et al., 2006; Audhya et al., 2007; Hu et al., 2008). A subpopulation of Yop1p in S. cerevisiae is also localized in the Golgi and interacts with Yop1p (Calero et al., 2001), a component of the Yip1p/Yif1p/Yos1p complex that appears to be involved in vesicle formation (Heidtman et al., 2005). It has been proposed that Yop1p, in conjunction with Yip1p, facilitates Rab GTPase-mediated protein transport between the ER and the Golgi network (Calero et al., 2001). With its localization similar to that of yeast Yop1p, it is likely that HVA22 participates in the ER-to-Golgi secretory pathway in plants.

The vacuolation of PSVs is similar to homotypic vacuole fusion in yeast. Studies in yeast have shown that homotypic vacuole fusion requires cytosolic enolase, ATP, and several endomembrane secretary-related proteins (Wickner and Haas, 2000; Decker and Wickner, 2006). In particular, a GTPase, Ypt7p, is required to concentrate SNAREs and other proteins to activate the vacuole fusion machinery (Haas et al., 1995; Starai et al., 2007). Disrupting protein transport during the secretory pathway will result in nonfused fragmented vacuoles (Haas et al., 1995; Decker and Wickner, 2006). Interestingly, the yeast HVA22 homolog Yop1p also physically interacts with Ypt7p in vivo (Calero et al., 2001), and overexpressing Yop1p in yeast cells results in negative dominant defects in vacuole protein transport (Haas et al., 1995). Therefore, plant HVA22 protein may regulate homotypic vacuole fusion of PSVs by mediating vesicle trafficking between the ER, Golgi, and PSVs. This notion is further supported by the observation that in GA-treated aleurone cells, the ER and Golgi stacks lying adjacent to the PSVs proliferate vesicles that appear to fuse with the PSVs via autophagic internalization (Jones, 1969a, 1969b, 1987; Jones and Price, 1970; Herman and Larkins, 1999).

Combining these studies, we propose that ABA induces the biosynthesis of HVA22 to negatively regulate vesicle transport between the ER and Golgi or the Golgi to PSVs, to prevent premature degradation of PSVs and nutrient mobilization. When GA triggers germination, HVA22 proteins are rapidly degraded to facilitate the necessary protein trafficking for PSV metabolism and fusion.

Phylogenetic analyses show that AtHVA22D and AtHVA22E are closer to barley HVA22 than to AtHVA22A, AtHVA22B, and AtHVA22C (Chen et al., 2002). Consistent with this finding, AtHVA22D is more effective than other Arabidopsis HVA22 proteins at inhibiting the GA-induced PCD (Fig. 9). Although the level of expression and stability of Arabidopsis HVA22 proteins could influence these results, the significantly higher ability of AtHVA22D than other AtHVA22s at inhibiting PCD suggests that AtHVA22D may also function in ER/Golgi secretory transport, as proposed for barley HVA22. Interestingly, among all AtHVA22s, AtHVA22D is also the most induced by ABA and abiotic stresses in vegetative tissues (Chen et al., 2002). How the role of HVA22 in regulating vesicle trafficking contributes to plant responses to abiotic stresses warrants further investigation.

MATERIALS AND METHODS

Plant Materials

Barley (Hordeum vulgare ‘Himalaya’) seeds from 1998 and 2002 harvested at Washington State University in Pullman, Washington, were used in all experiments. Embryoless half-seeds were prepared as described previously (Shen et al., 1993).

Plasmid Construction

The reporter and effector constructs were generated as follows. (1) GFP, DesRed, GAMyb, Amy-GUS, and LUC plasmids were constructed as described previously (Cercos et al., 1999; Zentella et al., 2002). The same backbone plasmid (CYH8) was used, in which the maize (Zea mays) ubiquitin promoter and its first intron (Ub1) were cloned into the pSil site of pBluescript II SK + with the 3 ’ Nos terminator. (2) For HVA22 plasmid, the complete barley HVA22 ORF was amplified from the barley embryo cDNA using a pair of primers (5 ’-AGAAACCCGGGTCATGCGAAATCATG-3 ’ and 5 ’-ATACTAGTCAATGCACACCCATG-3 ’). The resulting 939-bp fragment was subcloned into Smal and Sph1 sites of CYH8. (3) For HVA1 plasmid, complete barley HVA1 ORF was released from a cDNA clone using NotI digestion, then was subcloned into the NotI site of CYH8. (4) For the HVA22RNAi construct, Gateway technology was used. A PCR product of 300 bp of HVA22 ORF (19–324 bp), including the conserved TB2/DP1 domain, was amplified using primers 5 ’-ATATACTAGTTCAATGACACGACC-3 ’ and 5 ’-ATACTAGTCAATGCACACCCATG-3 ’. The amplified fragment was cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting HVA22-pENTR construct was recombined with the Gateway destination vector pANDA-mini (Miki and Shimamoto, 2004) by LR (recombination between attL and attR sites) reaction using LR Clonase II (Invitrogen) following the manufacturer’s instructions. (5) For GAMybRNAi, a PCR product of 351 bp of the partial GAMyb ORF from barley was amplified using primers 5 ’-CACTCTCACCAGACACAAAACTACG-3 ’ and 5 ’-TACGCCGAGACCTGCTGATTCTC-3 ’. The amplified fragment was cloned into the pENTR/D-TOPO vector (Invitrogen). The resulting HVA22-pENTR construct was recombined with the Gateway destination vector pANDA-mini (Miki and Shimamoto, 2004) by LR (recombination between attL and attR sites) reaction using LR Clonase II (Invitrogen) following the manufacturer’s instructions. (6) For the ΔTM1 plasmid, a PCR product of 300 bp containing predicted transmembrane domains TM2 and TM3 of HVA22 was amplified using primers 5 ’-AACCCGGGATGCGCAGCGTACAACTGTCAG-3 ’ and 5 ’-ATATAGCTATTTAAGCGAATAGCTG-3 ’. Then, the ΔTM1 fragment was cloned into XmuI and Sph1 sites of CYH8 plasmid (7). For the ΔTM3 plasmid, a PCR product of 356 bp including predicted TM1 and TM2 of HVA22 was amplified using primers 5 ’-AGAAACCCGGGTCATGCGAAATCATG-3 ’ and 5 ’-ATACTAGTCAATGCACACCCATG-3 ’. Then, the ΔTM3 fragment was cloned into XmuI and Sph1 sites of CYH8 plasmid (7). For the ΔTM2 construct, TM3 of HVA22 was first amplified using primers 5 ’-ATACTAGTTCAATGGTGACACCCATG-3 ’ and 5 ’-ATACACTGTTCAATGGTGACACCCATG-3 ’. The 190-bp PCR product was then cloned into BamHI and Sph1 sites of the ΔTM1 plasmid to replace TM2, so that TM1 and TM3 were fused in frame. (9) For HVA22:GFP, ΔTM1:GFP, ΔTM2:GFP, and ΔTM3:GFP constructs, the complete HVA22 ORF, or corresponding gene fragments for ΔTM1, ΔTM2, and ΔTM3 were amplified using HVA22, ΔTM1, ΔTM2, and ΔTM3 plasmids as templates.
GACAAAACTATGGACTTC-3
GTACCTATAGTCATCATCA-3
TCATGGGCAAATCATG-3
or 20

For GUS assays, 50 mM
software.
The analysis of colocalization was performed using LSM 5 Image Examiner excitation at 543 nm with a HeNe laser beam. The GFP images were captured embryoless half-seeds and observed with a Zeiss CLSM510 confocal microscope.

in control shooting buffer for 0, 24, or 48 h depending on the experiment. After bombardment, the half-seeds were preincubated in a molar ratio of 1:10. After bombardment, the half-seeds were preincubated in control shooting buffer for 0, 24, or 48 h depending on the experiment. The half-seeds were homogenized in 1 mL of grinding buffer after 24 h of

2.5 constant of 100,000. All experiments consisted of three replicates, and the constant was repeated at least two times with similar results.

2:5 GMCCTCATGTAATCTGACA-3 and 5'-TATGCATTAGTCTGCACTAC-3). Except for Ub-AhIVA22B, no GFP signals were observed in the Xnol and Sphl sites of CyH8, whereas the AhIVA22B PCR product was cloned into Xnol and Sphl sites of CyH8. All of the plasmids were further confirmed by sequencing.

Confocal Microscopy

A role for Rab5 in

Confocal Microscopy

Aleurome layers were isolated by removing the starchy endosperm of the embryoless half-seeds and observed with a Zeiss CLSM510 confocal microscope. DeRed images were captured in the 560- to 615-nm range after excitation at 543 nm with a HeNe laser beam. The GFP images were captured in the 505- to 530-nm range after excitation at 488 nm with an argon laser beam. The analysis of colocalization was performed using LSM 5 Image Examiner software.

LUC and GUS Enzyme Assays

The half-seeds were homogenized in 1 ml of grinding buffer after 24 h of transformation (Shen et al., 1993). After centrifugation at 12,000 rpm for 10 min, 100 μl of supernatant was used for LUC assays using a luminometer (Lumat LB9507; Berthold Technologies). For GUS assays, 50 μl of supernatant was mixed with 150 μl of GUS assay buffer (Shen et al., 1993) and incubated for 20 h at 37°C in the dark; then, 50 μl of reaction mixture was diluted in 2 ml of 200 mM sodium carbonate stop buffer. GUS activity was measured with a fluorometer (TBS-380; Turner BioSystems) calibrated to 1.000 units with a standard made by diluting 50 μl of 1 μM 4-methylumbelliferone in 2 ml of 200 mM sodium carbonate. Relative GUS activity was calculated by dividing GUS activity by LUC activity of the respective samples and multiplying by a constant of 100,000. All experiments consisted of three replicates, and the entire experiment was repeated at least two times with similar results.

HVA22 Inhibits Vacuolation and Programmed Cell Death

Transient Expression via Particle Bombardment

Barley embryoless half-seeds were prepared and transformed transiently by particle bombardment as described previously (Shen et al., 1993). In general, 2.5 μg of reporter construct was co-bombarded with an effector construct as indicated in the figures in a molar ratio of 1:1 except for GAMyb. The GAMyb effector construct was cotransformed with reporter or other effector constructs in a molar ratio of 1:10. After bombardment, the half-seeds were preincubated in control shooting buffer for 0, 24, or 48 h depending on the experiment. Then samples were transferred to buffer in the presence or absence of 1 μM GA or 20 μM ABA for another 4 h before observation with a confocal microscope. More than 20 cells from each aleurome tissue and six to eight barley half-seeds were observed by confocal microscopy for each construct combination. Cells containing only one to three large vacuoles were classified as vacuolated cells, and the percentage of vacuolated cells was calculated in each aleurome tissue (vacuolated cells/total observed cells × 100%). The values shown in the figures represent average percentages ± se of vacuolated cells of six to eight barley aleurome tissues from independent half-seeds.

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HVA22 Inhibits Vacuolation and Programmed Cell Death


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This article was published with the name of corresponding author Tuan-Hua David Ho misspelled. The name has been corrected in the online version of the article.