Running Head: Adi3 Phosphorylation of Gal83

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The β-subunit of the SnRK1 complex is phosphorylated by the plant cell death suppressor Adi3

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ABSTRACT

The protein kinase Adi3 is a known suppressor of cell death and loss of its function has been correlated with cell death induction during the tomato (Solanum lycopersicum) resistance response to its pathogen Pseudomonas syringae pv. tomato. However, Adi3 downstream interactors that may play a role in cell death regulation have not been identified. We used a yeast two-hybrid screen to identify the plant SnRK1 (Sucrose non-Fermenting-1-Related Protein Kinase 1) protein as an Adi3 interacting protein. SnRK1 functions as a regulator of carbon metabolism and responses to biotic and abiotic stresses. SnRK1 exists in a heterotrimeric complex with a catalytic α-subunit (SnRK1), a substrate interacting β-subunit, and a regulatory γ-subunit. Here we show that Adi3 interacts with, but does not phosphorylate the SnRK1 α-subunit. The ability of Aid3 to phosphorylate the four identified tomato β-subunits was also examined and it was found that only the Gal83 β-subunit was phosphorylated by Adi3. This phosphorylation site on Gal83 was identified as Ser26 using a mutational approach and mass spectrometry. In vivo expression of Gal83 indicates it contains multiple phosphorylation sites, one of which is Ser26. An active SnRK1 complex containing Gal83 as the β-subunit and Snf4 as the γ-subunit was constructed to examine functional aspects of the Adi3 interaction with SnRK1 and Gal83. These assays revealed that Adi3 is capable of suppressing the kinase activity of the SnRK1 complex through Gal83 phosphorylation plus the interaction with SnRK1, and suggests this function may be related to the cell death suppression activity of Adi3.
Programmed cell death (PCD) is a genetically encoded, highly regulated process in multi- and single cell eukaryotic organisms (Lam, 2004; Brownlee, 2008; Deponte, 2008; Lane, 2008) and bacteria (Engelberg-Kulka et al., 2006; Lane, 2008). In multicellular organisms, PCD often occurs during developmental processes, imparting a positive effect by killing specific cells in the organ connected with the process (Lam, 2004). Without PCD, proper development is not achieved. In plants, flower and embryo development, seed coat formation, senescence, leaf shape formation, xylem formation, and resistance to pathogens all involve PCD (Lam, 2004). Thus, PCD plays a central role in many aspects of maturation and survival of plants.

Despite the many processes in plants that require PCD, identification of genes and signaling pathways involved in plant PCD has been difficult compared to mammalian systems (Lam et al., 2001; Hoeberichts and Woltering, 2003; Lam, 2004, 2008). However, in the past decade the number of genes identified to be involved in plant PCD control has increased substantially. Just to name a few, these genes range from the plant homologues for the mammalian PCD regulators Bax inhibitor-1 and the BAG proteins (Doukhanina et al., 2006; Watanabe and Lam, 2009) to mitogen-activated protein kinases and transcription factors (Zhang and Klessig, 2001; Ren et al., 2002; Pedley and Martin, 2005; Kaneda et al., 2009; Phan et al., 2011). However, the signaling pathways associated with the proteins encoded by these genes remain for the most part unresolved.

One of the plant genes identified to control PCD encodes the AGC Ser/Thr protein kinase Adi3 from tomato (Solanum lycopersicum), which functions as a cell death suppressor (Devarenne et al., 2006; Ek-Ramos et al., 2010). Adi3 was first characterized for its role in the hypersensitive response (HR) cell death induced during the resistance of tomato to its bacterial pathogen Pseudomonas syringae pv. tomato (Pst) (Bogdanove and Martin, 2000; Devarenne et al., 2006). We have used a variety of methods to analyze Adi3 cell death suppression (CDS) activity and parts of the Adi3 signaling pathway. A loss of Adi3 function by virus induced gene silencing (VIGS) causes spontaneous cell death lesions to form on leaves and stems, ultimately leading to death of the plant (Devarenne et al., 2006). Adi3 can prevent cell death when overexpressed in the presence of PCD-inducing conditions. 3-phosphoinositide-dependent protein kinase-1 (Pdk1) is the upstream kinase that phosphorylates Adi3 at Ser539 for activation of its CDS activity (Devarenne et al., 2006; Ek-Ramos et al., 2010). The phosphorylation of Adi3 by Pdk1 also directs Adi3 to the nucleus where its CDS activity is manifested (Ek-Ramos...
et al., 2010). Prevention of nuclear entry eliminates Adi3 CDS and thus, induces cell death (Ek-Ramos et al., 2010). Thus, it is hypothesized that prevention of Adi3 signaling and CDS, possibly by inhibiting nuclear entry, may be a mechanism by which cell death is initiated for PCD-requiring situations such as the HR response to Pst (Ek-Ramos et al., 2010).

Our studies on Adi3 CDS show remarkable similarity to the mammalian cell death (apoptosis) suppressor protein kinase B (PKB; aka Akt). PKB is also an AGC Ser/Thr kinase that is phosphorylated by Pdk1 for activation and nuclear localization, where PKB CDS activity occurs (Vivanco and Sawyers, 2002; Scheid et al., 2005; Miyamoto et al., 2009). Loss of PKB function by knockout or elimination of kinase activity causes spontaneous cell death and the PKB knockout is lethal (Dudek et al., 1997; Chen et al., 2001; Luo et al., 2003). PKB can prevent cell death when overexpressed in the presence of PCD-inducing conditions (Arico et al., 2002; Kulp et al., 2004; Zhu et al., 2004). These similarities in function, cell localization, and signaling between Adi3 and PKB suggest that Adi3 may be the functional homologue of PKB in plants (Devarenne et al., 2006; Ek-Ramos et al., 2010).

While many PKB substrates for CDS are known (Carnero, 2010), substrates for Adi3 have yet to be identified. Thus, we initiated a yeast two-hybrid (Y2H) screen to identify potential substrates of Adi3. This screen identified the α-subunit of the Sucrose non-Fermenting-1-Related Protein Kinase-1 (SnRK1) complex as an Adi3 interactor. SnRK1 is the plant homologue of the conserved protein complex known as Sucrose non-fermenting1 (Snf1) in yeast, and AMP-Activated Protein Kinase (AMPK) in mammals. These protein complexes regulate carbon metabolism and metabolic stress responses (i.e. low glucose or starvation) (Halford et al., 2003; Polge and Thomas, 2007; Halford and Hey, 2009; Hey et al., 2010). Snf1 controls the shift from glucose fermentation to the aerobic use of alternate carbon sources (Polge and Thomas, 2007). AMPK is activated under low ATP/glucose conditions and represses ATP consuming pathways while activating ATP producing pathways (Polge and Thomas, 2007; Halford and Hey, 2009). SnRK1 activates starch mobilization and metabolism under low glucose conditions such as during darkness (Halford et al., 2003; Polge and Thomas, 2007; Halford and Hey, 2009; Hey et al., 2010). Additionally, SnRK1 regulates metabolism in response to environmental stresses such as pathogen attack and herbivory, flooding, and cell death during development (Sreenivasulu et al., 2006; Coello et al., 2010; Hey et al., 2010; Cho et
al., 2012). Thus, SnRK1 appears to be a key regulator connecting metabolism and stress responses in plants (Halford and Hey, 2009; Hey et al., 2010).

The SnRK1 (and Snf1/AMPK) complex exists as a heterotrimer of an α-subunit Ser/Thr kinase called SnRK1, one of several possible β-subunits (Sip1, Sip2, or Gal83 in yeast), and a γ-subunit called Snf4 (Halford and Hey, 2009; Coello et al., 2010). The γ-subunit has been shown to regulate kinase activity of the complex (Jiang and Carlson, 1996), while the β-subunits regulate complex substrate specificity and cellular localization (Mitchelhill et al., 1997; Vincent and Carlson, 1999; Vincent et al., 2001, 2001; Warden et al., 2001). The signaling mechanisms exerted on the β-subunits for controlling function are not fully understood. But, at least for yeast Sip1 and the human β-subunit AMPKβ1, phosphorylation appears to be involved in controlling β-subunit function (Warden et al., 2001; Hedbacker et al., 2004). Recently, two kinases have been shown to phosphorylate yeast Gal83, but a connection to function has not been shown (Mangat et al., 2010) and it has yet to be shown that a plant β-subunit is phosphorylated. Here we present data showing that Adi3 interacts with the tomato SnRK1 α-subunit and the Gal83 β-subunit, that Adi3 can only phosphorylate Gal83, and we show that Adi3 can inhibit the kinase activity of the SnRK1 complex.

RESULTS

Identification of SnRK1 as an Adi3 Interacting Protein.

In an effort to identify Adi3-interacting proteins we carried out a yeast two-hybrid (Y2H) screen using a cDNA prey library that has been previously used to identify proteins that interact with the tomato resistance protein kinase Pto (Zhou et al., 1995). Approximately 15 million yeast transformants were screened for Adi3-interacting proteins using selection on Leu- plates and 1,366 transformants were followed-up in a β-galactosidase (LacZ) screen. The prey inserts from 85 random positive clones were sequenced and screened against GenBank by BLAST for identification. Of these clones, SnRK, encoding the α-subunit of the SnRK1 protein complex, was identified four times. The SnRK insert in the prey library was a partial ORF and a full-length ORF was identified by searching the tomato EST data base (http://solgenomics.net/) by BLAST with the SnRK Y2H fragment. Unigene SGN-U564382 was identified as containing a full-length SnRK
ORF and this sequence was amplified from tomato leaf tissue RNA by RT-PCR. A BLAST search against GenBank with the full-length SnRK sequence showed that it was identical to a previously identified tomato SnRK cDNA (Bradford et al., 2003). In Arabidopsis, SnRK proteins are separated into three distinct families, SnRK1, SnRK2, and SnRK3 (Halford and Hey, 2009). BLAST and alignment comparison of the protein encoded by the SnRK sequence cloned here with members of the Arabidopsis SnRK (AtSnRK) family indicated that it belongs to the SnRK1 family (Supplemental Fig. S1). The tomato gene identified here will be referred to as SlSnRK1 throughout this study.

The full-length SlSnRK1 ORF was used to confirm the Y2H interaction with Adi3 and test the interaction with kinase activity mutants of Adi3. SlSnRK1 does not autoactivate in the Y2H assay when expressed from either the prey or bait vectors (Fig. 1A). Our previous studies have shown that mutation of the Pdk1 phosphorylation site on Adi3 (S539) to Asp (Adi3S539D) confers constitutive kinase activity on Adi3, and mutation of Lys337 to Gln (Adi3K337Q) in the ATP-binding pocket eliminates Adi3 kinase activity (Devarenne et al., 2006). The interaction of SlSnRK1 with Adi3 was not abolished by either of these Adi3 kinase activity mutants (Fig. 1A). This was the case whether the proteins were in the bait or prey vectors (Fig. 1A) suggesting that kinase activity is not required for this interaction. The SlSnRK1 and Adi3 interaction was also tested by immunoprecipitation. GST-Adi3 immunoprecipitated with an α-GST antibody was not capable of pulling down MBP, but was capable of pulling down MBP-SlSnRK1 (Fig. 1B, compare lanes 5 and 6).

**Adi3 Also Interacts With Two SlSnRK1 β-Subunits.**

We also tested if Adi3 could interact with two of the previously identified SlSnRK1 β-subunits. First, cDNAs for these two tomato β-subunits, SlGal83 and SlSip1 (Bradford et al., 2003), were cloned. The reported SlGal83 sequence is not a full-length ORF and is missing a portion of the 5’ end (Bradford et al., 2003). Thus, we used the tomato EST and genomic databases to identify the remaining 5’ end of the SlGal83 sequence and to make sure the published SlSip1 sequence contained the full-length ORF. A BLAST search of the tomato ESTs with the published SlGal83 sequence identified a full-length ORF within unigene SGN-U564868, which indicated the published SlGal83 sequence was missing 51 bp from the 5’ end,
or 17 N-terminal amino acids (Supplemental Fig. S2). The original SlGal83 sequence also had a mis-identification of nucleotide 58 as guanine when EST and genomic sequence indicates nucleotide 58 is a cytosine (not shown). The full-length SlGal83 ORF was amplified by PCR from SGN clone cTOF-18-D18.

A BLAST search with the published SlSip1 sequence (Bradford et al., 2003) against the tomato genomic database identified the SlSip1 gene within genomic locus AC186291.2. The deduced ORF from this genomic sequence was longer than the published sequence and indicated the published SlSip1 ORF was missing 177 bp of 5’ sequence, or 59 N-terminal amino acids (Supplemental Fig. S2; Supplemental Fig. S3A). The full-length ORF sequence of SlSip1 was cloned by RT-PCR based on the deduced ORF sequence confirming the presence of this transcript in tomato (Supplemental Fig. S3A). Both of these cloned full length tomato sequences were used for all subsequent studies reported here.

The interaction of SlGal83 and SlSip1 with Adi3 was tested by α-GST IP as with SlSnRK1. The results indicated that both β-subunits were capable of interacting with Adi3 (Fig. 1B, lanes 7, 8). For reasons that will become apparent below, we made SlGal83 the main subject of our research and have shown that Adi3 also interacts with SlGal83 in the Y2H assay (Supplemental Fig. S3B). These results indicated that Adi3 is capable of interacting with several members of the SlSnRK1 complex.

**Adi3 Phosphorylates SlGal83**

Since Adi3 interacts with the SlSnRK1 α-subunit it is possible that Adi3 acts as an upstream activator of SlSnRK1. Thus, we analyzed Adi3 kinase activity toward SlSnRK1. First, a kinase-inactive SlSnRK1 was generated by mutating Lys48 to Gln, SlSnRK1K48Q (Fig. 2, lane 12). This Lys corresponds to the invariant Lys45 in AMPK required for ATP binding (Dyck et al., 1996) (Supplemental Fig. S1). Phosphorylation of SlSnRK1K48Q by the constitutively-active Adi3S539D was not seen (Fig. 2A, lane 14), suggesting Adi3 is not an upstream activator of SlSnRK1.

Because Adi3 can interact with SlGal83 and SlSip1 it is possible that Adi3 can phosphorylate these β-subunits. The β-subunits from yeast and mammals are known to be phosphorylated (Mitchelhill et al., 1997; Warden et al., 2001; Mangat et al., 2010), while phosphorylation of the plant β-subunits has not been reported to date. Thus, the ability of Adi3 to phosphorylate the
SIGal83 and/or SI*Sip1 β-subunits was examined. Kinase assays showed that both wild-type Adi3 and constitutively-active Adi3 SS39D were able to phosphorylate SIGal83 with Adi3 SS39D phosphorylating SIGal83 ~ 6 times more than wild-type (Fig. 2A, compare lanes 6 and 8). Interestingly, neither form of Adi3 was capable of phosphorylating SI*Sip1 (Fig. 2A, lanes 9-11) even though Adi3 can interact with SI*Sip1 (Fig. 1B, lane 8). This would suggest there is some catalytic specificity of Adi3 towards SIGal83 over that of SI*Sip1.

The phosphorylation of SIGal83, but not SI*Sip1 by Adi3 lead us to search the tomato genome for additional SI*SnRK1 β-subunits that may be phosphorylated by Adi3. The SIGal83 sequence was used to search the tomato genome by BLAST against the SGN Tomato Combined Database (whole genome, BAC, and unigene sequences) and two additional sequences with high similarity to SI*SnRK1 β-subunits were discovered and termed Tau1 and Tau2 (Supplemental Fig. S2). Additionally, BLAST of the Tau1 and Tau2 proteins against GenBank returned the Arabidopsis β-subunit AKINβ2 (E values 1E-90 and 3E-136, respectively) as a top hit suggesting these proteins are SnRK1 β-subunits. The Tau1 and Tau2 cDNAs were amplified from leaf RNA by RT-PCR (Supplemental Fig. S4) and the proteins derived from these ORFs appear to be more related to SI*Sip1 and the Arabidopsis β-subunit AKINβ2 than to SIGal83 (Fig. 2B). Next, the phosphorylation of Tau1 and Tau2 by Adi3 SS39D was tested using in vitro kinase assays, which showed that Adi3 did not phosphorylate Tau1 or Tau2 to a significant level and only phosphorylated SIGal83 (Fig. 2C).

Since Adi3 only phosphorylates SIGal83 and not the other β-subunits we focused on SIGal83, and confirmed that it is a functional SnRK1 β-subunit using yeast complementation that was not done in the initial SIGal83 study (Bradford et al., 2003). In yeast the Snf1 complex functions to allow growth on alternative carbon sources such as sucrose (Carlson et al., 1981; Polge and Thomas, 2007) and loss of the three yeast β-subunits (ScSip1, ScSip2, and ScGal83; sip1Δsip2Δgal83Δ yeast) does not allow for growth on sucrose (Schmidt and McCartney, 2000). Complementation of sip1Δsip2Δgal83Δ cells and restoration of growth on sucrose can be accomplished by introducing any one of the β-subunits (Schmidt and McCartney, 2000). Individually, each of the Arabidopsis β-subunits (AKINβ1, AKINβ2, AKINβ3) are also capable of complementing the sip1Δsip2Δgal83Δ cells (Gissot et al., 2004; Polge et al., 2008). We carried out this assay and showed that SIGal83-GFP was capable of restoring sip1Δsip2Δgal83Δ growth on sucrose, confirming complementation (Supplemental Fig. S5A).
confirmation of SIGal83 complementation of sip1Δsip2Δgal83Δ yeast, we tested for restoration of invertase activity, which is regulated by the Snf1 complex under low glucose conditions (Carlson et al., 1984). Our results show that SIGal83-GFP was able to restore basal and low glucose-induced invertase activity to sip1Δsip2Δgal83Δ yeast (Supplemental Fig. S5B). These studies confirm SIGal83 as a true SnRK1 β-subunit and that SIGal83-GFP is functional in vivo.

Identification of Serine 26 as the Adi3 phosphorylation site on SIGal83.

In an effort to identify the SIGal83 residue phosphorylated by Adi3 we carried out a kinase assay screen of several SIGal83 Ser mutants. Within the SIGal83 protein there are 28 Ser amino acids (Supplemental Fig. S2), 17 of which were mutated to Ala and tested for loss of phosphorylation by Adi3 using in vitro kinase assays. Once the assays were completed, the SIGal83 phosphorylation levels were normalized to the SIGal83 and Adi3 protein levels in each assay, and the amount of SIGal83 phosphorylation was expressed as a percentage of wild-type SIGal83 phosphorylation. The results indicate that while many of the mutations slightly increased or decreased the ability of Adi3 to phosphorylate SIGal83, only the Ser26A mutation completely eliminated phosphorylation by Adi3 (Fig. 3A, lane 3). There are 8 Thr residues in SIGal83 (Supplemental Fig. S2). Alanine mutation of one Thr residue did not eliminate Adi3 phosphorylation (data not shown) and the remaining 7 Thr were not tested since Ser26A was a complete knockout of Adi3 phosphorylation of SIGal83 (Fig. 3A). These results indicate that while Adi3 can interact with several members of the SI-SnRK1 complex, it can only phosphorylate SIGal83. The β-subunit protein alignment indicates that SI-Sip1, Tau1, and Tau2 do not contain a Ser corresponding to SIGal83 Ser26 (possibly marginally conserved in Tau2; Supplemental Fig. S2) supporting the inability of Adi3 to phosphorylate these proteins.

Phosphorylation of SIGal83 Ser26 was confirmed by mass spectrometry (MS) analysis. Trypsin digestion of SIGal83 will produce two possible peptides containing Ser26, S\text{NVESGIVEDHALNSR} and R\text{SNVESGIVEDHALNSR} (Ser26 bold and underlined), and MS/MS analysis of in vitro Adi3 phosphorylated, trypsin digested SIGal83 identified Ser26 phosphorylation in both peptides (Fig. 3B; Supplemental Fig. S6A). The in vivo phosphorylation of Ser26 was also analyzed by first expressing SIGal83-GFP in tomato protoplasts and immunoprecipitating the protein with an α-GFP antibody (Supplemental Fig. S6B, C). The
trypsin digested protein was analyzed by MS/MS and Ser26 phosphorylation was identified in the SNVESGIVEDHHALNSR peptide (Fig. 3C), but not the RSNVESGIVEDHHALNSR peptide. This data indicates that Adi3 phosphorylates SIGa83 Ser26 \textit{in vitro} and supports the possibility that Adi3 also performs this phosphorylation event \textit{in vivo}.

An additional SIGa83 phosphorylation site was identified \textit{in vitro} and \textit{in vivo} in peptide RSNVEpSGIVEDHHALNSR corresponding to Ser30 (Supplemental Fig. S7A, B) suggesting that Adi3 may also phosphorylate Ser30. The Ser30 to Ala mutation was not initially tested as shown in Fig. 3A. So, the SIGal83\textsuperscript{S30A} protein was produced and tested for loss of Adi3 phosphorylation as in Fig. 3A. The results indicate that the S30A mutation does not significantly reduce the SIGa83 phosphorylation by Adi3 \textit{in vitro} (Supplemental Fig. S7C). While Adi3 could be responsible for this phosphorylation event \textit{in vivo}, it remains to be positively determined. It should be noted that for both the \textit{in vitro} and \textit{in vivo} MS/MS analysis, peptides with the Ser26 phosphorylation were approximately twice as prevalent as those with Ser30 phosphorylation and no peptides were found with both Ser26 and Ser30 phosphorylation.

**Tomato Gal83 is Phosphorylated \textit{in vivo}**

In order to analyze the \textit{in vivo} phosphorylation status of SIGal83 we used an alteration to the standard SDS-PAGE by adjusting the ratio of \textit{bis}-acrylamide to acrylamide. This method has been used to distinguish different phosphorylation states of yeast phosphatidylinositol 4-kinase (Demmel et al., 2008). SIGal83-GFP transgenic \textit{Arabidopsis} plants were created and the SIGal83-GFP protein analyzed by \textalpha-GFP western blot using increasing ratios of \textit{bis}-acrylamide to acrylamide. The 1:200 \textit{bis}-acrylamide:acrylamide ratio was capable of separating five different forms of SIGal83-GFP and two of these forms are lost when expressing the SIGal83\textsuperscript{S26A}-GFP protein (Supplemental Fig. S8). This would suggest that the 1:200 SDS-PAGE/\textalpha-GFP western blot can be used to effectively separate and identify different modified forms of SIGal83.

Next, the \textit{in vivo} phosphorylation status of SIGal83 as expressed in tomato was analyzed. SIGal83-GFP was expressed in protoplasts, an extract made, the extract treated with \textlambda phosphatase, and SIGal83-GFP analyzed using 1:200 gels/\textalpha-GFP western blot. In the presence of \textlambda phosphatase SIGal83-GFP appeared as a single band (Fig. 4A, lane 1). However, in the
absence of λ phosphatase SlGal83-GFP appeared as at least four distinct protein bands, and by comparison to the λ phosphatase treatment this can be interpreted as one unphosphorylated form and three phosphorylated forms of SlGal83-GFP (Fig. 4A, lane 2).

The contribution of Ser26 phosphorylation to the SlGal83 phosphorylated protein bands was analyzed by mutating SlGal83 Ser26 to the non-phosphorylatable Ala (SlGal83S26A) and the phosphomimetic Asp (SlGal83S26D). Expression of the GFP fusions of both of these proteins in tomato protoplasts appeared to reduce the number of SlGal83-GFP phosphorylated forms; SlGal83S26A only had one phosphoprotein band (Fig. 4A, lane 3), while SlGal83S26D showed a reduction of one phosphoprotein band (Fig. 4A, lane 5). The phosphoprotein bands for both SlGal83S26A and SlGal83S26D can be removed by λ phosphatase treatment (Fig. 4A, lanes 4 and 6, respectively). This would suggest that Ser26 phosphorylation contributes to the in vivo phosphorylation status of SlGal83.

**Adi3 Phosphorylates SlGal83 in vivo.**

We looked for evidence that SlGal83 Ser26 is phosphorylated in vivo by Adi3 using a coexpression approach. SlGal83-GFP, SlGal83S26A-GFP, and HA-Adi3 were coexpressed in tomato protoplasts and the banding pattern of phosphorylated SlGal83-GFP analyzed by 1:200 gels/α-GFP western blot. In the absence of HA-Adi3, SlGal83-GFP and SlGal83-GFPS26A appeared as was seen in Fig. 4A (Fig. 4B, lane 2 and 3). However, in the presence of HA-Adi3, wild-type SlGal83-GFP protein appeared to shift upward (Fig. 4B, lane 4). Treatment of this sample with λ phosphatase reduced SlGal83-GFP to a single non-phosphorylated protein band (Fig. 4B, lane 6, compare to lane 4). In the presence of HA-Adi3, the SlGal83-GFPS26A protein appeared similar to that without HA-Adi3 (Fig. 4B, lane 5). Taken together, this data would suggest SlGa83 is phosphorylated by Adi3 in vivo. Additionally, it was seen that HA-Adi3 exists as several phosphoprotein bands that can be reduced to a single band with λ phosphatase treatment (Fig. 4B, middle panel).

**Functional Analysis of SlGal83 Ser26 Phosphorylation.**
In order to begin to analyze possible roles for Adi3 phosphorylation of SlGal83 we first utilized the \( \text{sip1}\Delta \text{sip2}\Delta \text{gal83}\Delta \) yeast complementation assay. The ability of the \( \text{SlGal83}^{S26A}\)-GFP and \( \text{SlGal83}^{S26D}\)-GFP proteins to complement the \( \text{sip1}\Delta \text{sip2}\Delta \text{gal83}\Delta \) cells was tested and the results indicate these proteins complement to an extent similar to that of wild-type SlGal83-GFP (Supplemental Fig. S5A). This suggests Adi3 phosphorylation of SlGal83 may not affect the function, at least in a heterologous system, of controlling growth on alternate carbon sources.

Given the role of Adi3 in suppression of cell death (Devarenne et al., 2006; Ek-Ramos et al., 2010) and that Adi3 can phosphorylate SlGal83, the ability of SlGal83 and its Ser26 phosphorylation mutants to suppress cell death was analyzed in tomato cells. It is known that high levels of NaCl are capable of inducing cell death in plants (Katsuhara and Kawasaki, 1996; Lin et al., 2006; Tuteja, 2007; Jiang et al., 2008; Affenzeller et al., 2009; Banu et al., 2009; Chen et al., 2009; Wang et al., 2010) and a functional Snf1 complex has been shown to be required for yeast cell survival in the presence of high NaCl (Hong and Carlson, 2007). We expressed SlGal83-GFP, SlGal83\(^{S26A}\)-GFP, SlGal83\(^{S26D}\)-GFP, and GFP-Adi3 in tomato protoplasts, treated them with 200 mM NaCl, and measured cell viability over a 5.5 hr time course. Both Adi3 and SlGal83 were capable of CDS activity and provided increased cell viability in response to NaCl compared to the vector transformed sample (Fig. 5A). The SlGal83 Ser26 phosphorylation mutants did not confer increased or decreased cell viability over wild-type SlGal83 (Fig. 5D). These results indicated that SlGal83 does have a role in cell death suppression, but phosphorylation of Ser26 may not play a role in controlling SlGal83 CDS activity.

Next, the affect of SlGal83 phosphorylation on SlSnRK1 complex kinase activity was tested. In order to carry out these assays, an \textit{in vitro} active SnRK complex must be assembled. Thus, the SlSnRK complex members studied here were analyzed for the formation of an active complex by testing kinase activity against the AMPK/SnRK1 SAMS peptide substrate (HMRSAMSGLHLVKRR; phosphorylation site bold and underlined) (Halford et al., 2003). We also cloned the tomato cDNA for \( \text{Snf4} \), which encodes the \( \gamma \)-subunit of the SlSnRK complex (Bradford et al., 2003) for inclusion in the kinase assays. The \( \alpha \)-subunit SlSnRK1 by itself showed limited SAMS phosphorylation (Fig. 5B, column 1). The phosphomimetic mutation of SlSnRK1 Thr175 (SlSnRK1\(^{T175D}\)), which corresponds to the identified phosphorylation activation site in AMPK (Thr172) and spinach and \textit{Arabidopsis} SnRK1 (Thr175) (Hawley et al., 1996; Sugden et al., 1999; Crozet et al., 2010) (Supplemental Fig. S1), conferred an increase in
SAMS phosphorylation (Fig. 5B, column 3). Addition of SlSnf4 marginally, but significantly increased SlSnRK1<sup>T175D</sup> SAMS phosphorylation (Fig. 5B, column 4). Inclusion of all complex subunits (SlSnRK1, SlSnf4, SlGal83) imparted a greater increase in SlSnRK1<sup>T175D</sup> SAMS phosphorylation (Fig. 5B, column 5). These assays show that the SlSnRK1 subunits comprise a functional complex. To the best of our knowledge, this is the first report of reconstituting an active plant SnRK complex <i>in vitro</i>.

The contribution of SlGal83 Ser26 phosphorylation towards SlSnRK1 kinase activity on the SAMS peptide was analyzed by including the SlGal83<sup>S26D</sup> protein in the complex or adding Adi3 to the complex. The results show that SlGal83<sup>S26D</sup> conferred a slight yet statistically significant decrease in SlSnRK1 SAMS phosphorylation (Fig. 5B, column 6), while the addition of Adi3<sup>S539D</sup> to the assay drastically lowered the phosphorylation of SAMS to a level close to that of SlSnRK1 alone (Fig. 5B, column 7). This drop in SAMS phosphorylation appears to partially depend on Adi3 kinase activity as inclusion of the kinase-inactive Adi3<sup>K337Q</sup> restored activity of the complex similar to SlSnRK1<sup>T175D</sup> alone, but not to the level of the full complex (Fig. 5B, column 8). This would suggest that even though Adi3 does not phosphorylate SlSnRK1 (Fig. 2A, lane 14), it may inhibit SnKR1 kinase activity through their interaction. This appears to be the case since kinase-active Adi3<sup>S539D</sup> or kinase-inactive Adi3<sup>K337Q</sup> reduced SAMS phosphorylation by SlSnRK<sup>T175D</sup> and SlSnRK<sup>T175D</sup> + Snf4 close to the level of SlSnRK1 alone (Fig. 5B, columns 9, 10, 11, 12). In order to analyze if the drop in complex kinase activity in the presence of Adi3 is due to an additional protein in the assay, the analysis was repeated with the addition of GST protein. This assay had strong kinase activity, but not to the level of the full complex (Fig. 5B, column 13). This would suggest that some loss of kinase activity in the presence of Adi3 could be due to the addition of an additional protein. To take this into account, the values in Fig. 5B were normalized to that of the assay in the presence of GST; i.e. the GST sample was set as 100% and the other samples were expressed as a percentage of this value. Fig. 5C shows that when expressed in this manner, the trends do not change.

We extended the SnRK1 SAMS phosphorylation assays to a more <i>in vivo</i> approach by expressing SlGal83-GFP or SlGal83<sup>S26D</sup>-GFP in tomato protoplasts, making extracts of these cells, and using the extract to phosphorylate the SAMS peptide. We found that the extract from SlGal83<sup>S26D</sup>-GFP expressing cells had greatly reduced SAMS phosphorylation compared to expression of SlGal83-GFP (Fig. 5D). This reduction in SAMS phosphorylation is much lower
than what was seen for the in vitro assay (Fig. 5B, C) suggesting that a more in vivo context is needed to better realize the effects of Ser26 phosphorylation. Taken together, these kinase assay data suggest that the Adi3 interaction with the SISnRK complex has the ability to inhibit the kinase activity of the complex. This may be mediated through two mechanisms, phosphorylation of SLGal83 and interaction with SISnRK1.

DISCUSSION

In the present study we present evidence for the interaction of Adi3 with the SnRK complex in tomato. Our finding that Adi3 can only phosphorylate the Gal83 SnRK β-subunit out of the four β-subunits identified in tomato has far-reaching implications since Snf1/AMPK/SnRK1 β-subunits control cellular localization and substrate specificity of the complex (Mitchelhill et al., 1997; Vincent and Carlson, 1999; Vincent et al., 2001, 2001; Warden et al., 2001). Additionally, β-subunit phosphorylation has been associated with regulation of some of these β-subunit functions (Mitchelhill et al., 1997; Warden et al., 2001; Hedbacker et al., 2004; Mangat et al., 2010), and the SnRK complex appears to link signaling connected with metabolism and stresses (Halford and Hey, 2009; Hey et al., 2010). Given the role of Adi3 in cell death control our studies add additional evidence for the connection of SnRK with stress signaling. Alternatively, Adi3 may also be involved in the direct regulation of metabolism through its interactions with the SISnRK1 complex.

A Role for Adi3 Phosphorylation in Regulating SnRK Complex Kinase Activity?

We have shown that Adi3 phosphorylates SLGal83 at Ser26 (Fig. 2) and explored the functional relevance of this phosphorylation event. While, several β-subunits have been shown to be phosphorylated in yeast and mammals (Warden et al., 2001; Hedbacker et al., 2004; Mangat et al., 2010), our studies appear to be the first report of phosphorylation for a plant β-subunit. An in vitro functional analysis showed that kinase-active Adi3 has drastic affects on the kinase activity of the SnRK 1 complex. If Adi3 phosphorylation of SLGal83 at Ser26 is controlling this large decrease in SnRK1 complex activity, the SLGal83S26D protein should also confer a decrease in kinase activity. A large reduction in SAMS phosphorylation in the presence
of SlGal83$^{S26D}$ was seen \textit{in vivo} (Fig. 5D), but was much less drastic \textit{in vitro} (Fig. 5C) suggesting there is an \textit{in vivo} role for Ser26 phosphorylation in controlling SnRK1 kinase activity.

Interestingly, the restoration of SAMS phosphorylation when including kinase-inactive Adi3$^{K337Q}$ would suggest that Adi3 kinase activity is at least partially required for this large inhibition of SnRK1 activity \textit{in vitro} and may suggest additional Adi3 phosphorylation sites on SlGal83 for controlling activity. It is possible that Ser30 is one of these sites since we identified phosphorylation of this SlGal83 residue both \textit{in vitro} and \textit{in vivo} by MS analysis. However, the inability of Adi3 to phosphorylate this site \textit{in vitro} raises doubt about the role of Ser30 phosphorylation. Additionally, the complete loss of Adi3 phosphorylation of the SlGal83$^{S26A}$ protein suggests Ser26 is the only Adi3 phosphorylation site on Gal83. Thus, the requirement of Adi3 kinase activity in the suppression of SnRK1 substrate phosphorylation still remains to be fully resolved.

While many studies have shown that phosphorylation of $\alpha$-subunits controls complex kinase activity in yeast, mammals, and plants (Hong et al., 2003; Nath et al., 2003; Sutherland et al., 2003; Woods et al., 2003; Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005; Shen and Hanley-Bowdoin, 2006; Hey et al., 2007; Shen et al., 2009), only one previous study has shown that phosphorylation of a $\beta$-subunit affects complex kinase activity. A phosphorylation mutant of the human AMPK$\beta_1$ $\beta$-subunit reduced AMPK complex kinase activity by 60\% (Warden et al., 2001). Thus, control of complex kinase activity by $\beta$-subunit phosphorylation may be more common than previously thought. This could be supported by determining if phosphorylation of the conserved Ser26 residue in the \textit{Arabidopsis} Gal83 homologue, AKIN$\beta_1$, affects AtSnRK1 complex kinase activity.

Our results also suggest that the interaction of Adi3 with SlSnRK1 is capable of suppressing SlSnRK1 kinase activity (Fig. 5B). In the absence of SlGal83 the kinase-active or -inactive forms of Adi3 are capable of suppressing SlSnRK1 kinase activity (Fig. 5B). This apparently contradicts the finding that the kinase-inactive Adi3 can restore activity of the complex in the presence of SlGal83. However, these results may indicate that the Adi3/SlGal83 interaction affects the ability of Adi3 to fully interact with and inhibit SlSnRK1. Eliminating SlGal83 from the assay would then allow for full interaction between Adi3 and SlSnRK1 and stronger activity inhibition. The interaction of Adi3 with SlSnRK1 may be inhibiting the ability of SlSnRK1 to bind the SAMS substrate or even ATP.
These data also help to explain the detection of SlSnRK1 as an Adi3 Y2H interactor even though Adi3 does not phosphorylate SlSnRK1 as well as shed light on the biological significance for this interaction. Given the role of Adi3 in the host response to \( Pst \) and the function of SnRK1 in stress signaling, Adi3 may be directing reallocation of cellular energy reserves by modulating SlSnRK1 kinase activity during the resistance response of tomato to \( Pst \). Studies using \textit{Nicotiana attuneata} show that photosynthate is reallocated to the roots in response to herbivore attack through the down-regulation of SnRK \( \beta \)-subunit expression (Schwachtje et al., 2006). Our results indicate that SIGal83 phosphorylation at Ser26 functions as an inhibitor of SlSnRK kinase activity. Down regulation of this \( \beta \)-subunit may thus play a role in facilitating the activation of SlSnRK1 and the metabolic modifications required to respond to pathogens. Phosphorylation of SIGAL83 by SlAdi3 offers an additional layer of control over SlSnRK activity, a specificity required given the involvement of this complex in regulating metabolic responses to several environmental stresses (Hong and Carlson, 2007; Hey et al., 2010; Cho et al., 2012).

**Multiple Roles for \( \beta \)-Subunit Phosphorylation.**

Snf1/AMPK/SnRK1 \( \beta \)-subunits appear to be phosphorylated on several amino acids and our studies also support phosphorylation at several residues on SIGal83. Expression of SIGal83-GFP in plant cells showed the existence of multiple phosphorylated protein bands based on our \( \lambda \) phosphatase treatments, one of which contains Ser26 phosphorylation (Fig. 4A and B). One of these phosphorylated bands may also contain Ser30 phosphorylation. This and the identity of any additional SIGal83 phosphorylation sites remains to be determined. Multiple phosphorylation sites have been found for other \( \beta \)-subunits. Mass spectral analysis of human AMPK\( \beta \)1 isolated from COS cells identified phosphorylation at Ser24/25, Ser108, and Ser182, but the responsible kinase has not been identified (Mitchelhill et al., 1997). Phosphorylation of AMPK\( \beta \)1 Ser24/25 and Ser182, but not Ser108, appears to prevent nuclear localization (Warden et al., 2001). ScGal83 is phosphorylated by both the \( \alpha \)-subunit Snf1 and casein kinase 2 (CK2), and while the exact sites of phosphorylation have not been identified they are predicted to be Ser64 or Ser65 for Snf1 and Ser87, Thr90 or Ser93 for CK2 (Mangat et al., 2010). The role for Snf1/CK2 phosphorylation of ScGal83 is not clear since deletion of the region containing both the Snf1 and CK2 phosphorylation sites did not affect glucose-regulated Snf1 function (Mangat et al., 2010).
et al., 2010). The situation for ScSip1 is similar. Protein kinase A (PKA) has been shown to be required for retaining ScSip1 cytoplasmic localization under high glucose conditions (Hedbacker et al., 2004). However, mutation of 4 potential PKA phosphorylation sites did not affect ScSip1 cellular localization (Hedbacker et al., 2004).

Taken together it appears that the role of Snf1/AMPK/SnRK1 β-subunit phosphorylation is not fully understood and will be an important area of research for the future. From our studies the full role of SlGal83 Ser26 phosphorylation by Adi3 is not clear. It appears to have only a minor role in controlling SlSnRK1 complex kinase activity. So, additional functions attributable to this phosphorylation event will be important to identify in the future. Given the role of β-subunits in controlling Snf1/AMPK/SnRK1 complex localization and phosphorylation playing a role in this function, it will be important to examine the contribution of phosphorylation by Adi3 in controlling SlGal83 cellular localization. Consequently, the full extent of the SlGal83 Ser26 phosphorylation event by Adi3 remains to be determined.

**Is There a Link Between Cell Death Control and Metabolism?**

An important aspect of PCD is the reallocation of cellular resources such as proteins and sugars. This is particularly true of the cell death that occurs during leaf senescence (van Doorn and Woltering, 2004, 2008; Guiboileau et al., 2010). In fact, the reuse of cellular materials was suggested as early as 1891 from the examination of cell death associated with xylem development (Lange, 1891). Thus, it may not be surprising that a gene controlling PCD would also be able to regulate how cells utilize and/or mobilize energy sources. This appears to be the case for mammalian PKB. While it is well known that PKB suppresses cell death by phosphorylating and inactivating proapoptotic proteins or activating antiapoptotic proteins (Luo et al., 2003; Carnero, 2010), PKB also functions in the regulation of metabolism through the control of glycolytic enzymes and glucose uptake (Plas and Thompson, 2002; Carnero, 2010). Such a connection between a specific plant gene controlling cell death and metabolism has been indirect at best. Our previous studies have shown that there are many striking activity and cellular localization similarities between Adi3 and PKB (Devarenne et al., 2006; Ek-Ramos et al., 2010). The studies presented here showing Adi3 inhibition of SlSnRK1 complex kinase activity adds one additional similarity between Adi3 and PKB since PKB is known to modulate
AMPK activity. While PKB and AMPK do not directly interact with each other, there is substantial crosstalk between the pathways. For example, activation of PKB has been shown to down regulate AMPK activity and thus a decrease in AMP/ATP cellular ratios (Kovacic et al., 2003; Hahn-Windgassen et al., 2005). Conversely, activation of AMPK has been shown to inactivate PKB-regulated glycolysis (Grabacka and Reiss, 2008). Combining our current and previous Adi3 studies raises the possibility that Adi3 functions similarly to PKB in cell death and metabolism control. Further studies on the role of Adi3 association with the SlSnRK1 complex, especially phosphorylation of Gal83, will be required to fully understand if there is a connection between Adi3-mediated cell death control and SlSnRK1 metabolism control.

**MATERIALS AND METHODS**

**Cloning of Tomato *SnRK1, Gal83, Sip1, Tau1, Tau2, and Snf4.***

All primers and restriction sites used in this study for ORF amplification, cloning, and mutagenesis are listed in Supplemental Table S1 and the primers used to amplify all genes were designed using sequence data obtained from the Sol Genomics Network (SGN) databases (http://solgenomics.net/). The ORFs for *SlSnRK1, SlSip1, SlSnf4, SlTau1,* and *SlTau2* were obtained by RT-PCR using cDNA generated with Superscript III (Invitrogen) from tomato total RNA isolated from 4-week-old leaves. Primers used to amplify *SlSnRK1* (accession #AF143743) were based on the unigene SGN-U564382. The cDNA for *SlSip1* (accession #AF322108) in unigene SGN-U575258 and reported in Bradford et al. (2003) appeared to lack a portion of the 5’ end of the cDNA when compared to homologous β-subunits from yeast and *Arabidopsis*. Consequently, the tomato genome sequence was searched on SGN for the *SlSip1* gene using unigene SGN-U575258. An *SlSip1* gene was found in genomic sequence SL2.31ch05:63330625..63325020 and primers were designed based on this sequence to amplify the ORF by RT-PCR. The reported *SlGal83* cDNA (accession #AY245177) lacked the 5’ end and the full length cDNA was identified in unigene SGN-U564868. Primers based on this unigene were used to amplify the ORF by PCR using SGN EST clone cTOF-18-D18 as a template. The Tau1 (accession #JQ846034) and Tau2 (accession #JQ846035) ORFs were isolated using primer sequences based on the unigenes U571217 and U565213, respectively.
The *SlSnf4* ORF (accession #AF419320) was amplified by PCR using the published sequence (Bradford et al., 2003). Mutagenesis of *SlSnRK1* and *SlGal83* was performed using Pfu Turbo Polymerase (Stratagene) and the primer pairs listed in Supplemental Table S1. Cloning of *Adi3* and its kinase activity mutants were described previously (Devarenne et al., 2006).

**Recombinant Protein Expression and Purification.**

The ORFs for *SlSnRK1*, *SlGal83*, and *SlSip1* were cloned as N-terminal MBP fusions into pMAL-c2 vector (New England Biolabs). Recombinant proteins were expressed in *E. coli* BL21 Star (DE3) as described previously (Devarenne et al., 2006) and purified using maltose binding resin (New England Biolabs) manufacturer protocols. For GST-Adi3, the Adi3 ORF was cloned into the pGEX-4T N-terminal GST fusion vector (GE-Healthcare) and protein was expressed and purified as recommended by the manufacturer. After elution, all fusion proteins were concentrated using Amicon Ultra centrifugal filters (Millipore) and added to buffer for final concentrations of 50% glycerol, 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 100 mM NaCl. Protein concentrations were quantified using Bio-Rad Protein Assay Kit before storage at -20°C.

**Yeast Two-Hybrid Assay**

Y2H assays were conducted using pEG202 for the bait vector and pJG4-5 for the prey vector as described previously (Devarenne et al., 2006). Constructs were transformed into yeast strain EGY48 containing the pSH18-34 reporter vector and analyzed for *LacZ* gene expression on X-Gal containing plates. Protein expression was confirmed by western blot. All other procedures for the Y2H assays and Y2H library screen for identifying Adi3 interactors followed standard procedures as previously described (Golemis et al., 2008).

**Yeast Complementation and Invertase Assays**

The ORF for *SlGal83* and its Ser26 mutants were fused to a C-terminal eGFP tag under the control of the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter in the modified vector MBB263. The yeast β-subunit knockout strain MCY4040 (MATα sip1Δ::KanMX6
sip2Δ3::LEU2 gal83::TRP1 his3-Δ200 leu2-3,112 trp1Δ1 ura3-52 lys2-801) (Vincent et al., 2001) was transformed with the SlGal83 constructs using the standard lithium acetate/PEG method. Transformants were screened on plates of complete minimal (CM) media with 2% glucose and lacking leucine, tryptophan, and uracil. Recovered colonies were grown in liquid CM 2% glucose medium for 48 hrs and 5-fold serial dilutions were spotted on selective media supplemented with either 2% glucose or 2% sucrose and incubated at 30˚C for 2 days (2% glucose) or 6-7 days (2% sucrose). Invertase assays were performed as previously reported (Celenza and Carlson, 1989; Bradford et al., 2003). Invertase activity of derepressed (0.05% glucose) and glucose-repressed (2% glucose) cells was estimated as a measure of the amount of sucrose metabolized into glucose using the Glucose (GO) Assay kit (Sigma) as described by the manufacturer.

**Pull Down Assays**

Immobilized glutathione beads (Thermo Scientific) were equilibrated by washing three times with 200 µl of binding buffer (50 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 5 mM EDTA). For each pull down 1 µg of either GST or GST-Adi3 and equivalent protein amounts of MBP, MBP-Gal83, MBP-Sip1, and MBP-SnRK1 were mixed in a final volume of 30 µl. Samples were incubated for 15 min at room temperature followed by addition of buffer pre-equilibrated glutathione resin to each sample and incubation for 1 hr at 4˚C on an orbital shaker. The resin with bound proteins was pelleted by centrifugation at 100 x g and washed 5 times with 200 µl of wash buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% Triton X-100, 5 mM EDTA). Bound proteins were eluted using 1x SDS-PAGE sample buffer, resolved by 12% SDS-PAGE, and analyzed by western blotting using α-GST (Santa Cruz Biotechnology) at 1:15,000 and α-MBP (New England BioLabs) at 1:5,000 for pull downs or 1:10,000 for loading controls.

**Kinase Assays**

_in vitro_ kinase assays were done in 30 µl reactions in Adi3 kinase buffer (10 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1mM DTT, 20 µM ATP) or S/SnRK1 kinase buffer (10 mM Tris-HCl pH 8, 10 mM MnCl2 or MgCl2, 1mM DTT, 20 µM ATP). S/SnRK1 autophosphorylation appeared
to be slightly stronger using MnCl₂ and therefore, was used for all *Sl*SNRK1 autophosphorylation assays. However, *Sl*SNRK1 substrate phosphorylation was comparable using MnCl₂ or MgCl₂ as a cofactor. Therefore, MgCl₂ was used for all *Sl*SNRK1 substrate phosphorylation experiments. Adi3 substrate phosphorylation assays contained 5 μg of purified MBP-Adi3 or MBP-Adi₃⁵３⁹Ｄ and 2 μg of MBP-Gal83, MBP-Gal83 mutants, or MBP-Sip1. For *Sl*SNRK1 kinase assays, 3 μg of MBP-SnRK1, MBP-SnRK1^K₄₈Q, or MBP-SnRK1^T₁₇₅D were used. Reactions were initiated upon addition of 0.25 μCi of γ-[³²P]ATP (6000Ci/m mole, Perkin Elmer) per sample and were incubated for 15 min at room temperature for Adi3 or 30 min at 30°C for *Sl*SNRK1. Reactions were terminated by addition of 4x SDS-PAGE sample buffer and samples resolved by 7.5% SDS-PAGE. Sample radioactive incorporation imaging and quantification was done with a phosphorimager (Bio-Rad Molecular Imager).

SAMS peptide (HMRSAM₆GLHLVKRR) phosphorylation assays were performed as described previously (Davies et al., 1989). Assay conditions for *Sl*SNRK1 phosphorylation of the SAMS peptide were as for the *Sl*SNRK1 substrate phosphorylation assays above plus 100 μM SAMS peptide (AnaSpec). Reactions were spotted on phosphocellulose p81 paper (Whatman), washed three times in 1% H₃PO₄, once in acetone, the paper dried, and the incorporated radioactivity counted using a Beckman LS5000TA scintillation counter. For SAMS phosphorylation with protoplast lysates, 4 x 10⁵ tomato protoplasts expressing empty pTEX vector, *Sl*GAL83-GFP, and *Sl*GAL83⁵₂⁶D-GFP were lysed by vortexing in a buffer containing 50mM Tris pH8.0, 1mM EDTA, 50mM NaCl, 8% Glycerol, 5mM DTT, 2% plant protease inhibitor cocktail (Sigma) and 2% plant phosphatase inhibitor cocktail (Sigma). Extracts were cleared by centrifugation at 4°C, 13,000 g for 10 minutes. Protein concentration was estimated as described above and lysates were adjusted to equal protein concentrations with lysis buffer. Reactions were done as described above, but using a buffer containing 40 mM HEPES-KOH pH 7.6, 10 mM MgCl₂, 1mM DTT, 200 μM ATP, 2 μCi of γ-[³²P]ATP, and 100μM SAMS peptide (buffer adapted from (Fragoso et al., 2009)). Reactions were initiated with the addition of 8 μl of the protein extract. Phosphate incorporation was analyzed as described above and the remaining lysates were used for α-GFP western blotting to evaluate expression efficiency of the proteins.

Mass Spectrometry
For sample preparation, coomassie stained gel bands were in-gel digested with trypsin overnight and phosphopeptides were enriched using a NuTip metal oxide phosphoprotein enrichment kit according to manufacturer’s instructions (Glygen, Columbia, MD).

For LC-MS/MS analysis, phosphopeptides were injected onto a capillary trap (LC Packings PepMap, Amsterdam, Netherlands) and desalted for 5 min with 0.1% v/v acetic acid at a flow rate of 3 µl/min. The samples were loaded onto an LC Packings C\textsubscript{18} PepMap nanoflow HPLC column. The elution gradient of the HPLC column started at 97% solvent A, 3% solvent B and finished at 60% solvent A, 40% solvent B for 30 min. Solvent A consisted of 0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H\textsubscript{2}O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H\textsubscript{2}O. LC-MS/MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). The instrument under Xcalibur 2.07 with LTQ Orbitrap Tune Plus 2.55 software was operated in the data dependent mode to automatically switch between MS and MS/MS acquisition. Survey scan MS spectra (from m/z 300 – 2000) were acquired in the orbitrap with resolution R=60,000 at m/z 400. During collisionally induced dissociation (CID), if a phosphate neutral loss of 98, 49, 32.66 and 24.5 m/z below the precursor ion mass was detected, there was an additional activation of all four neutral loss m/z values. This multistage activation was repeated for the top five ions in a data-dependent manner. Dynamic exclusion was set to 60 seconds. Typical mass spectrometric conditions include a spray voltage of 2.2 kV, no sheath and auxiliary gas flow, a heated capillary temperature of 200°C, a capillary voltage of 44V, a tube lens voltage of 165V, an ion isolation width of 1.0 m/z, a normalized CID collision energy of 35% for MS/MS in LTQ. The ion selection threshold was 500 counts for MS/MS. The mass spectrometer calibration was performed according to the manufacturer’s guidelines using a mixture of sodium dodecyl sulphate, sodium taurocholate, MRFA and Ultramark.

For the protein search algorithm, all MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.2.2). Mascot was set up to search a current \textit{Arabidopsis} database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met and phosphorylation of serine, threonine and tyrosine are specified as variable modifications. The MS/MS spectra of the identified
phosphorylated peptides were manually inspected to ensure confidence in phosphorylation site assignment.

**Phosphatase Treatment**

Gal83-GFP proteins were exercised in tomato protoplasts from pTEX for 22 hrs and were lysed in ice-cold extraction buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1% Triton X-100, 2 mM DTT, 2.5% plant protease inhibitor cocktail (Sigma), and 6 μM epoxymicin (Enzo Life Sciences). Lysates were split into two fractions; one for phosphatase treatment and one for a no treatment control. Both fractions were adjusted to 3 mM MnCl$_2$ in λ phosphatase buffer (50mM HEPES pH7.5, 100mM NaCl, 2mM DTT, 0.01% Brij-35) in a final volume of 100 μl. The no treatment fraction was additionally adjusted to 2% phosphatase inhibitors (Sigma, phosphatase inhibitor cocktail 1). Reactions were started with the addition of 800 units of λ phosphatase (New England BioLabs), incubated at 30˚C for 30 min, and reactions terminated by addition of 1x SDS-PAGE sample buffer. Samples were then resolved by 7.5% SDS-PAGE with a 1:200 bis-acrylamide :acrylamide ratio and analyzed by α-GFP western blotting.

**Protoplast Protein Expression and Cell Death Assays**

The ORFs for *Gal83* and *Gal83$^{Ser26}$* were cloned into the *BamHI* and *SalI* restriction sites of pTEX-eGFP (Ek-Ramos et al., 2010) to yield an in frame C-terminal GAL83-GFP fusion under the control of the 35S promoter. Cloning of *Adi3* into pTEX-eGFP for an N-terminal tagged GFP-Adi3 was previously described (Ek-Ramos et al., 2010). The resulting constructs were purified using CsCl gradient centrifugation. Protoplasts were isolated from expanded leaves of 4-week-old PtoR tomato plants and transformed as previously reported (Devarenne et al., 2006; Ek-Ramos et al., 2010) using 8 x 10$^5$ protoplasts and 25 μg of plasmid DNA. For NaCl-induced cell death experiments transformed protoplasts expressing proteins for 18 hrs were suspended in 200 μl of WI buffer (0.5M mannitol, 4 mM MES pH5.7, 20mM KCl) with or without 200 mM NaCl, incubated in the dark at 25˚C, and aliquots taken over a 5.5 hr time-course. Cell viability was estimated by treating 30 μl protoplast aliquots with 0.05% Evans blue for 5 min and counting a minimum of 200 cells as previously described (Devarenne et al., 2006; Ek-Ramos et
al., 2010). Cell viability estimates are a measurement of at least three independent transformation experiments. Protein expression was confirmed by western blot with $4 \times 10^5$ transformed protoplast resuspended in 1x SDS-PAGE sample buffer and boiled at 95°C for 5 min. GFP-fusion proteins were detected with an HRP-conjugated \( \alpha \)-GFP antibody (Santa Cruz Biotechnology) at 1:1000.

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JF895513 (SlGal83), JF8955212 (SlSip1), JQ846034 (SlTau1), JQ846035 (SlTau2)

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Alignment of SnRK proteins from tomato and *Arabidopsis*.

**Supplemental Figure S2.** Alignment of SnRK complex \( \beta \)-subunits.

**Supplemental Figure S3.** RT-PCR amplification of SlSip1 and Adi3/SlGal83 yeast two-hybrid interaction.

**Supplemental Figure S4.** RT-PCR amplification of SlTau1 and SlTau2.

**Supplemental Figure S5.** SlGal83 complementation of sip1Δsip2Δgal83Δ yeast.

**Supplemental Figure S6.** MS identification of SlGal83 S26 phosphorylation and \( \alpha \)-GFP immunoprecipitation of SlGal83-GFP.

**Supplemental Figure S7.** MS identification of SlGal83 S30 phosphorylation.

**Supplemental Figure S8.** Separation of SlGal83-GFP phosphoproteins by SDS-PAGE with varying bis-acrylamide:acrylamide ratios.
Supplemental Table S1. List of primers used in this study.

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FIGURE LEGENDS

Figure 1. Adi3 interaction with SnRK1 complex members. A, Adi3 and SnRK1 interact in the Y2H assay. The indicated bait and prey constructs were expressed in yeast and tested for expression of the lacZ gene on X-Gal plates (blue = interaction). B, Adi3 interacts with SnRK1 complex members by immunoprecipitation. Top panels, GST or a GST-Adi3 fusion protein was incubated at 4°C for 1 hr with MBP fusion proteins of SnRK1, Gal83, or Sip1, immunoprecipitated with an α-GST antibody, and the proteins associated with GST-Adi3 analyzed by α-MBP western blot. Bottom panels, a 1/10 aliquot of each MBP fusion protein was analyzed by α-MBP western blot for loading control.

Figure 2. Adi3 phosphorylates Gal83. In A and C top panels, phosphorimage; bottom panels, Coomassie stained gel. Quantity One software was used to normalize the phosphorylation levels to the protein levels in each assay. A, Analysis of SnRK1 α- and β-subunit phosphorylation by Adi3. Kinase-active and inactive MBP-Adi3 proteins were tested for phosphorylation of MBP-Gal83, MBP-Sip1, and kinase-inactive MBP-SnRK1K48Q using γ-[32P]ATP in in vitro kinase assays. Gal83 phosphorylation values are reported as a percentage of wild-type Adi3 phosphorylation of Gal83 and are representative of two independent experiments. B, Phylogenetic relationship between tomato and Arabidopsis β-subunits. Proteins were aligned using Clustalw (Larkin et al., 2007) and produced tree was analyzed using Treeview (Page, 1996). Line indicates number of amino acid substitutions per site. C, Adi3 only phosphorylates the Gal83 β-subunit. Kinase-active MBP-Adi3S539D was tested for phosphorylation of MBP-Gal83, MBP-Sip1 MBP-Tau1, and MBP-Tau2 as in (A). Values are averages of three independent experiments. Error bars are standard error. One asterisk indicates significant decrease in β-subunit phosphorylation as compared to Gal83 phosphorylation (Student’s t test, p < 0.01).

Figure 3. Adi3 phosphorylates Gal83 at Ser26. A, Adi3 phosphorylates Ser26 of Gal83. Kinase-active MBP-Adi3S539D was used to phosphorylate the indicated MBP-Gal83 Ser to Ala mutants using γ-[32P]ATP in in vitro kinase assays. Quantity One software was used to normalize the phosphorylation levels to the protein levels in each assay. Gal83 phosphorylation
values are reported as a percentage of wild-type Gal83 phosphorylation and are the average of three independent experiments. Error bars are standard error. One asterisk and two asterisks indicate significant increase or decrease, respectively, in phosphorylation of Gal83 Ser to Ala mutants compared to wild-type Gal83 phosphorylation (Student’s t test, p < 0.05). Top panels, phosphorimage; bottom panels, Coomassie stained gel. B, MS identification of Gal83 Ser26 in vitro phosphorylation by Adi3. in vitro Adi3 phosphorylated Gal83-MBP as in (A) was digested with trypsin, passed over an IMAC column, and eluted peptides analyzed by MS/MS. C, MS identification of Gal83 Ser26 in vivo phosphorylation. Gal83-GFP was expressed in tomato protoplasts, α-GFP immunoprecipitated, the protein digested with trypsin, passed over an IMAC column, and eluted peptides analyzed by MS/MS. pS, Phospho-Ser; dN, deaminated Asn.

**Figure 4.** In vivo phosphorylation status of Gal83. In both A and B, proteins were separated by SDS-PAGE using a 1:500 bis-acrylamide:acrylamide ratio followed by α-GFP or α-HA western blot. A, Gal83 is phosphorylated in tomato protoplasts. Total protein extracts from Gal83-GFP expressing protoplasts were treated with and without λ-phosphatase and analyzed by α-GFP western blot. Black arrow heads indicate different Gal83-GFP phosphorylated forms. B, Adi3 phosphorylates Gal83 in vivo. Protoplasts expressing the indicated combinations of HA-Adi3 and Gal83-GFP were analyzed by α-GFP for analysis of the phosphorylation status of Gal83-GFP, and α-HA western blot.

**Figure 5.** Functional analysis of Gal83 Ser26 phosphorylation mutants. A, Gal83 confers cell viability in the presence of high NaCl. Tomato protoplasts expressing GFP-Adi3 or the indicated Gal83-GFP constructs for 18 hrs were treated with 200 mM NaCl and cell viability determined by Evans blue staining over a 5.5 hr time course. Values are averages of at least three independent experiments. Error bars are standard error. Data analysis was carried out using Duncan’s multiple-range test. Samples with the same letter above the bars are not significantly different (p < 0.05). Protein expression detected by α-GFP western blot is shown on the right. B, SnRK1 substrate phosphorylation with Gal83S26D mutant and Adi3. Kinase-active and inactive MBP-SnRK1 proteins were tested for phosphorylation of the SAMS peptide in combination with MBP-Snf4, MBP-Gal83, and MBP-Adi3 using γ-[32P]ATP in in vitro kinase assays. Values are shown as pmole of phosphate incorporated/mg of SnRK1 protein/min and are
averages of three independent experiments. Error bars are standard error. One and two asterisks indicate significant increase or decrease, respectively, in SAMS phosphorylation as compared to phosphorylation by SnRK1T175D alone (Student’s t test, $p < 0.01$). SDS-PAGE gel shows proteins put into the assay. C, Expression of the data in (B) as a percentage of the GST sample, column 13. All other information as in (B). D, SAMS phosphorylation by protoplast extracts expressing SlGal83. The indicated SlGal83-GFP proteins were expressed in protoplasts for 16 hrs, an extract made, and tested for phosphorylation of SAMS as in (B). Values are averages of three independent experiments and error bars are standard error. One asterisk indicates significant decrease in SAMS phosphorylation as compared to the SlGal83 sample (Student’s t test, $p < 0.01$).
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