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FCS-Like Zinc Finger 6 and 10 repress SnRK1 signalling in Arabidopsis

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SUMMARY

SNF1-Related Protein Kinase 1 (SnRK1) is a central regulator of plant growth during energy starvation. The FCS-Like Zinc finger (FLZ) proteins are recently identified adaptor proteins which facilitate the interaction of SnRK1 with other proteins. In this study, we identified that two starvation-induced *FLZ* genes, *FLZ6* and *FLZ10*, work as repressors of SnRK1 signalling. The reduced expression of these genes resulted in an increase in the level of SnRK1α1, which is the major catalytic subunit of SnRK1. This resulted in a concomitant increase in the phosphorylated protein and SnRK1 activity in the *flz6* and *flz10* mutants. FLZ6 and FLZ10 specifically interact with SnRK1α subunits in the cytoplasmic foci which colocalized with the endoplasmic reticulum. In physiological assays, similar to *SnRK1α1* overexpression line, *flz* mutants showed compromised growth. Further, the growth promotion in response to favourable growth conditions was found to be attenuated in the mutants. The enhanced SnRK1 activity in the mutants resulted in a reduction in the level of phosphorylated RIBOSOMAL S6 KINASE and the expression of *E2Fa* and its targets indicating that TARGET OF RAPAMYCIN-dependent promotion of protein synthesis and cell cycle progression is impaired. Taken together, this study uncovers a plant-specific modulation of SnRK1 signalling.

INTRODUCTION

Eukaryotes possess conserved energy sensing mechanisms which enable them to optimise growth according to energy availability (Rolland *et al.*, 2002; Dobrenel *et al.*, 2016; Broeckx *et al.*, 2016). SUCROSE NON-FERMENTING 1/ AMP-ACTIVATED PROTEIN KINASE/SNF1-RELATED PROTEIN KINASE 1 (SNF1/AMPK/SnRK1) are the conserved regulators of growth during energy starvation in yeast, mammals and plants respectively. They are heterotrimeric serine-threonine kinases composed of α kinase subunit and β and γ regulatory subunits (Broeckx *et al.*, 2016). Similar to AMPK, the plant homologue SnRK1 is a pivotal regulator of growth during energy deficit (Baena-González *et al.*, 2007).

In mammals, during energy starvation, AMPK is activated through the phosphorylation of the T-loop present in the kinase subunit (Shaw *et al.*, 2004; Hawley *et al.*, 2005). Phosphorylation at the T-loop was also found to be essential for the activation of SnRK1 (Baena-González *et al.*, 2007; Shen *et al.*, 2009; Crozet *et al.*, 2010; Rodrigues *et al.*, 2013). Functional analysis has identified that SnRK1 share many downstream phosphorylation targets with AMPK (Broeckx *et al.*, 2016). Both AMPK and SnRK1 can phosphorylate REGULATORY-ASSOCIATED PROTEIN OF mTOR (RAPTOR), which is a regulatory subunit of the kinase TARGET OF RAPAMYCIN (TOR) suggesting that the negative regulation of TOR activity by AMPK is possibly conserved across different eukaryotic lineages (Gwinn *et al.*, 2008; Nukarinen *et al.*, 2016).

Compared to AMPK, conserved motifs present in the α-hook region were found to be diverged in SnRK1 kinase subunits suggesting that despite the functional conservation, T-loop-dependent activation mechanism by adenylates might not be conserved in SnRK1 (Emanuelle *et al.*, 2016; Broeckx *et al.*, 2016). As a consequence, the T-loop phosphorylation of SnRK1 was found to be insensitive to AMP and ADP treatments or starvation, and resistant to general and AMPK-specific phosphatases (Baena-González *et al.*, 2007; Emanuelle *et al.*, 2015). The homology modelling of SnRK1 suggests that unlike AMPK, SnRK1 seems to be constitutively active and protein turnover might be a regulating factor of SnRK1 activity (Broeckx *et al.*, 2016).

Protein-protein interaction studies identified that the Arabidopsis FCS-Like Zinc finger (FLZ) proteins interact with the kinase subunits of SnRK1 and may act as adaptors to facilitate the interaction of SnRK1 with other proteins (Arabidopsis Interactome Mapping Consortium, 2011; Nietzsche *et al.*, 2014). They are found to be small proteins with solitary FCS-Like Zinc finger (FLZ) domain which functions as the canonical interaction module (Jamsheer K and Laxmi, 2014; Nietzsche *et al.*, 2014; Jamsheer K *et al.*, 2015). The expression of *FLZ* genes is highly regulated by energy status and abiotic stress (Jamsheer K and Laxmi, 2014). Functional analyses identified that FLZ proteins are involved in the regulation of abiotic stress, ABA-responses and growth and development (He and Gan, 2004; Chen *et al.*, 2013; Hou *et al.*, 2013). The transcriptional regulation of *FLZ* genes by sugars and the interaction of FLZ proteins with kinase subunits of SnRK1 suggest that their role in plant growth and stress adaptation is possibly mediated through the interaction with SnRK1 signalling.

Here, we provide molecular and physiological evidences for the role of two FLZ proteins, FCS-LIKE ZINC FINGER 6 (FLZ6/AT1G78020) and FCS-LIKE ZINC FINGER 10 (FLZ10/AT5G11460) in controlling SnRK1 signalling through the inhibition of the SnRK1α1 level. In a feedback loop, SnRK1 induces the transcription of these genes. FLZ6/10 interactions with SnRK1α colocalize with the endoplasmic reticulum (ER) and through the modulation of the SnRK1 pathway, these proteins are also involved in the SnRK1-TOR antagonistic interaction in response to energy availability.

RESULTS

Tissue- and developmental stage-specific expression of *FLZ6* and *FLZ10*

In an earlier study, using qRT-PCR we found that *FLZ6* is expressed more in flowers, silique, and seedling stages. *FLZ10* also showed almost similar expression pattern (Jamsheer K *et al.*, 2015). To get a more detailed picture of the tissue- and developmental stage-specific expression pattern, we constructed promoter: GUS reporter lines for both the genes (Figure 1). In 3 DAG, *FLZ6* showed expression in the hypocotyl and a small amount of GUS activity was also observed in cotyledons. No activity was observed in the roots. *FLZ10* showed most activity in the stelar region of shoot and root (Figure 1a). In 5 DAG, the expression of *FLZ6* was mostly concentrated in the hypocotyl region and cotyledons. The expression of *FLZ10* in the 5 DAG was concentrated mainly in the root-shoot junction, cotyledon and root (Figure 1b and c). During lateral root development, diffused expression of *FLZ10* was observed in the young primordia while, in the older primordia, no expression was found in the tip as observed in primary root. No expression of *FLZ6* was observed during lateral root development (Figure 1d). In rosette plants, the promoters of *FLZ6* and *FLZ10* showed the highest activity in the old, senescing leaves and showed gradual reduction in new leaves (Figure 1e). A similar expression pattern was also observed in the leaves excised from bolted rosette where older rosette leaves showed maximum expression and cauline leaves showed the least expression (Figure 1f). *FLZ10* showed expression in the pistil and anthers of the flower bud (Figure 1g). In the open flower, the expression of *FLZ6* was found in pistil, pollen and filament. *FLZ10* also showed expression in pistil and pollen while no expression was observed in the filament (Figure 1h). A small amount of GUS activity was observed in the silique tip and stalk (Figure 1i).

The *in planta* interaction of FLZ6 and FLZ10 with SnRK1α subunits occurs in the cytoplasmic foci which colocalizes with endoplasmic reticulum

To identify the *in planta* interaction site of FLZ6 and FLZ10 with SnRK1 kinase subunits, BiFC assay was conducted in the onion epidermal cells. Strong fluorescent signals were observed in cytoplasmic foci and the signal was completely abolished in the negative control experiments without FLZ proteins (Figure 2a, S1a and S2). DAPI staining identified that these interactions predominantly occur in the periphery of the nucleus (Figure 2a). Similar results were also observed in the BiFC assay in Arabidopsis mesophyll protoplasts confirming the results obtained from the onion cells (Figure S3). The sinuous patterns and its proximity with the nucleus suggest that the interaction site might be the ER associated with the nucleus. To test this, we employed a widely used ER-specific marker (ER-rk) which is constructed by the fusion of mCherry with ER- signal peptide of WALL-ASSOCIATED KINASE 2 in the N-terminus and an ER retention peptide at the C-terminus (Nelson *et al.*, 2007). In the subcellular localization assay, apart from the mesh-like pattern observed in the periphery of the cell, ER was found to be associated with the nucleus as well strengthening the hypothesis that SnRK1α-FLZ6/10 interaction might be happening in the same foci (Figure S4). Indeed we found colocalization of ER-rk signal with the BiFC signal between FLZ and SnRK1α interaction (Figure 2b, Figure S1b). To confirm the ER colocalization, we used an ER-specific stain, ER-Tracker Red. In the assays, BiFC signals were found to be colocalized with the ER-Tracker Red signal confirming the colocalization of FLZ-SnRK1α interaction with ER (Figure 2c). Although the interaction with SnRK1α subunits was found to occur in specific cytoplasmic bodies, in the subcellular localization assay, both the proteins were localized in nucleus and cytoplasm both in onion and Arabidopsis cells (Figure S5).

Mapping of FLZ6/10 interacting region in SnRK1α1

The SnRK1 kinase subunits are composed of the N-terminal catalytic domain (CD) which is responsible for the kinase activity and the C-terminal regulatory domain (RD) which recruits β and γ subunits and other regulatory proteins (Broeckx et al., 2016). In AMPK and SNF1, the RD possesses an autoinhibitory domain (AID) which inhibits the kinase activity. In SnRK1, this region is not conserved (Shen et al., 2009). In the AID region, SnRK1 kinase subunits possess short ubiquitin-associated (UBA) domain which mediates the interaction

with proteins related to ubiquitin pathway (Farrás et al., 2001). The C-terminal domain (αCTD) is responsible for the interaction with β and γ subunits and 2C protein phosphatases (PP2Cs) (Rodrigues et al., 2013; Broeckx et al., 2016). To map the FLZ6/10-binding regions in SnRK1α1, constructs corresponding to CD (1-271), RD (272-512), UBA (291-332), AID Like (AIDL; 291-380) and αCTD (463-512) regions were cloned in AD vector and FLZ6 and FLZ10 were cloned in BD vector (Figure 2d). FLZ6 showed strong interaction with full-length SnRK1α1 and weak interaction with CD region (Figure 2e). The FLZ10 showed strong interaction with both full-length SnRK1α1 and the RD region alone. Further, it also showed weak interaction with AIDL and UBA regions in the RD (Figure 2e).

ABA and SnRK1 promote the transcription of *FLZ6* and *FLZ10*

We have previously shown that *FLZ6* is induced during prolonged sucrose starvation. *FLZ10* was also found to be induced in response to mild starvation; however, prolonged starvation repressed its transcription. Further, *FLZ6* is induced in response to 2-Deoxy-d-glucose (2DG) treatment which causes energy depletion by blocking glycolysis (Jamsheer K and Laxmi, 2015). To analyse whether *FLZ10* is also regulated by the reduction in cellular energy, its expression was checked in seedlings treated with increasing concentrations of 2DG. The level of *FLZ10* was found to be increased in response to 2DG treatment in a dose-dependent manner confirming that like *FLZ6*, the *FLZ10* level is also induced by energy starvation (Figure 3a). The marker gene for energy starvation, *DARK INDUCED 6 (DIN6)*, also showed similar trend indicating that *FLZ10* is also a starvation-inducible gene (Figure 3a) (Baena-González *et al.*, 2007).

The interaction between ABA and SnRK1 signalling is well established at molecular, genetic, and physiological levels and ABA is a positive regulator of SnRK1 signalling in Arabidopsis (León and Sheen, 2003; Radchuk *et al.*, 2010; Jossier *et al.*, 2009; Rodrigues *et al.*, 2013). Similar to energy starvation, *FLZ6* and *FLZ10* were found to be positively regulated by ABA treatment (Figure 3b).

The expression analysis identified that the expression of *FLZ6* and *FLZ10* are induced during SnRK1-activated conditions. During energy starvation, SnRK1 through downstream transcription factors such as bZIPs, alters the expression of a large number of genes (Baena-González *et al.*, 2007; Mair *et al.*, 2015). The expression of *FLZ6* and *FLZ10* are induced

during energy starvation (Figure 3a; (Jamsheer K and Laxmi, 2015). To understand whether the SnRK1 signalling has any role in controlling the expression of these genes during energy starvation, we analyzed their transcript level in a previously reported overexpression line *SnRK1α1 OE2* before and after energy starvation (Baena-González *et al.*, 2007). *DIN6* was used as a marker for starvation. The level of both *FLZ6* and *FLZ10* was found to be slightly increased in the overexpression line even in the normal growth conditions. Upon sugar starvation for 24 hours, the level of *FLZ6* and *FLZ10* was upregulated in both the WT and *SnRK1α1 OE2*; however, the extent of upregulation was significantly higher in the *SnRK1α1 OE2* indicating that SnRK1 promotes the expression of *FLZ6* and *FLZ10* during energy starvation (Figure 3c).

The *flz* mutants show SnRK1α1 overexpression like phenotype

To understand the role of *FLZ6* and *FLZ10* in plant growth, we identified T-DNA mutants of both genes. The homozygous lines were screened and the T-DNA insertion position was identified by sequencing the T-DNA insertion junction. The *flz6.1* was found to have T-DNA insertion at 26 bp upstream of 5'UTR while *flz10.1* has T-DNA insertion at 18 bp upstream of 5'UTR which resulted in a significant reduction in the level of *FLZ6* and *FLZ10* in their respective mutants (Figure 4a and b). These lines were grown in 0.5X MS medium with 1% sucrose for 10 days to assess the impact of reduced expression of respective genes in the mutants. The mutants displayed reduced biomass and lateral roots and shorter primary root (Figure 4c and d). The ectopic expression of *SnRK1α1* also results in reduced growth in 1% sucrose medium (Baena-González *et al.*, 2007). This phenotype was repeated in our culture conditions also (Figure 4e and f). To verify whether the phenotype obtained is due to the reduced expression of *FLZ6* and *FLZ10*, we identified another set of T-DNA insertion mutants. The insertion in 89 bp upstream of 5'UTR in *flz6.2* and in the beginning of the second exon in *flz10*.2 culminated in reduced expression of corresponding genes (Figure S6a and b). Since the insertion in *flz10.2* is in second exon and the promoter and the first exon are intact, we analysed whether the first exon is expressing using a primer specific to the first exon. Interestingly, a 2.5-fold upregulation in the expression of the first exon was observed in *flz10.2* (Figure S6c). In the phenotypic analysis, like *flz6.1*, *flz6.2* also showed reduced primary root length, lateral root and biomass. The *flz10.2* showed reduced lateral roots and biomass; however, no difference was observed in the primary root length (Figure S6d and e).

Taken together, the phenotypic analysis indicates that the reduced expression of *FLZ6* and *FLZ10* negatively affect overall seedling growth.

The *flz* mutants accumulate SnRK1α1 and show enhanced SnRK1 activity

The phenotypic analysis suggests that the reduced growth in the *flz* mutants might be due to alteration in the level of their interacting protein, SnRK1α1. To test this, we estimated the level of SnRK1α1 in the *flz6.1* and *flz10.1* mutants grown under standard growth conditions using a specific antibody. The immunoblot analysis identified an elevated level of SnRK1α1 in both mutants (Figure 5a and b). Previous reports show that ectopic expression of *SnRK1α1* is enough to produce increased kinase activity in *A. thaliana* and rice (Baena-González *et al.*, 2007; Cho *et al.*, 2012). Although the T175 phosphorylation at the T-loop of SnRK1 is not responsive towards AMP or starvation, it is indispensable for SnRK1 activity (Baena-González *et al.*, 2007; Shen *et al.*, 2009; Crozet *et al.*, 2010; Rodrigues *et al.*, 2013; Emanuelle *et al.*, 2015). To check whether the increased level of SnRK1α1 in the *flz* mutants amounts to increase in the level of T-175 phosphorylated protein, we estimated the level of phosphorylated SnRK1α1 using anti-phospho-AMPKa (T172) antibody. We found that both *flz6.1* and *flz10.1* lines accumulated more T-175 phosphorylated SnRK1α1 (Figure 5c and d). However, in the comparison of total and phosphorylated SnRK1α1, we couldn't find any significant change in the level of phosphorylated SnRK1α1 indicating that the elevated level of the total SnRK1α1 in the mutants is responsible for the increase in the phosphorylated SnRK1α1 (Figure 5e). To test whether this increased level of SnRK1α1 in the mutants is due to enhanced transcription, we checked the level of *SnRK1α1* and its redundant paralogue *SnRK1α2* in mutants. No significant difference in the transcript level of *SnRK1α1* or *SnRK1α2* in the mutants was observed indicating that the increased level of SnRK1α1 in the *flz* mutants could be due to the increased stability of SnRK1α1 in the mutants (Figure 5f).

To identify whether this increased SnRK1α1 in the mutants resulted in increased SnRK1 activity, we estimated the endogenous SnRK1 activity of WT and *flz* mutants using AMARA peptide assay. A significant increase in SnRK1 activity was observed in the mutants suggesting that the increased accumulation of SnRK1α1 culminated in enhanced endogenous SnRK1 activity (Figure 5g). SnRK1 through downstream transcription factors, regulates the transcription of a broad array of genes in response to energy starvation (Baena-González *et*

al., 2007). We checked the level of two previously known SnRK1-induced genes, *DIN6* and *TREHALOSE-6-PHOSPHATASE SYNTHASE S8 (TPS8)* in the mutants. The level of both *DIN6* and *TPS8* were found to be significantly higher in the mutants in comparison to WT (Figure 5h). The overexpression of *SnRK1α1* confers ABA hypersensitive phenotype (Jossier *et al.*, 2009). Consistent with this, the *SnRK1α1 OE2* showed hypersensitivity towards the ABA-mediated inhibition of primary root growth (Figure S7). Similarly, the *flz* mutants were also found to be hypersensitive to this response (Figure 5i and j). Taken together, our molecular and physiological assays demonstrate that reduced expression of *FLZ6* and *FLZ10* results in the accumulation of SnRK1α1 and increased SnRK1 activity.

The *flz* mutants show impaired growth acceleration in response to energy abundance

Although overexpression of *SnRK1α1* shows decreased growth when grown in sucrose-rich medium, they show better growth in the sucrose-deficient medium (Baena-González *et al.*, 2007). To test whether the increased SnRK1 activity in the *flz* mutants results in similar physiological response, the 5DAG WT, *SnRK1α1 OE2*, *flz6.1* and *flz10.1* seedlings grown in 1% sucrose were transferred to 0.5X MS medium containing 0.25, 0.5, 1, and 2 percentage of sucrose and growth was monitored on the 5th day after the transfer. In our assay, there was no significant difference in the fresh weight, primary root length and lateral root number of *SnRK1α1 OE2* in 0.25 and 0.5 percentages of sucrose in comparison to the WT. However, at higher concentration of sucrose, *SnRK1α1 OE2* failed to show growth acceleration as observed in the WT seedlings (Figure S8).

Interestingly, the *flz* mutants also showed a trend similar to *SnRK1α1 OE2* (Figure 6a). The growth of WT seedlings enhanced linearly in response to increased availability of sucrose. However, mutants showed a very different trend. The difference in growth of WT and mutants were found to be minimal when grown in 0.25% sucrose. No difference in primary root length and lateral root numbers was observed while there was a significant reduction in the overall biomass (Figure 6b). However, this reduction was found to be smaller compared to the biomass difference when grown in higher percentages of sucrose. As the amount of sucrose increased in the media, the mutant plants showed a more prominent difference in the phenotypic parameters studied (Figure 6b).

We verified these observations by modulating the level of endogenous sugars by growing plants in light abundant growth regime. The photosynthetic rate and sugar accumulation are directly dependent on light quality (Wingler *et al.*, 2005; Jänkänpää *et al.*, 2012). The 5 days old seedlings grown under normal light (60 µmol m^{-2} s^{-1}) condition were grown for another 5 days under high light (HL, 105 µmol m^{-2} s^{-1}) regime, and three growth parameters were studied (Figure 7a-d). In the HL regime, *SnRK1α1 OE2* showed a high degree of reduction in fresh weight, primary root length and lateral root number in comparison to WT (Figure 7a and b). Similarly, in the HL regime, *flz* mutants displayed a significant decrease in all three growth parameters studied (Figure 7c and d).

The *flz* mutants are impaired in TOR signalling

In the physiological assays, similar to *SnRK1α1 OE2*, due to the increased SnRK1 activity, *flz* mutants also showed better growth under energy limited growth conditions. However, in the energy abundant growth conditions, they failed to show growth acceleration to the extent observed in WT. Light and photosynthetically derived sugar promotes the seedling growth through the action of TOR signalling (Ren *et al.*, 2012; Xiong *et al.*, 2013) TOR kinase is activated by energy abundance and work antagonistic of SnRK1, while the mammalian counterpart of SnRK1 i.e., AMPK inhibits the TOR activity through various mechanisms (Horman *et al.*, 2002; Inoki *et al.*, 2003; Gwinn *et al.*, 2008; Ma and Blenis, 2009). The available evidence indicates that SnRK1 represses TOR signalling in plants (Baena-González *et al.*, 2007; Nukarinen *et al.*, 2016; Cho *et al.*, 2012; Li and Sheen, 2016). The phosphorylation status of the RIBOSOMAL S6 KINASE (S6K), a direct target of TOR in controlling translation is a widely used read-out of TOR activity in plants (Xiong *et al.*, 2013; Ahn *et al.*, 2015; Nukarinen *et al.*, 2016; Pfeiffer *et al.*, 2016). To find out whether the increased SnRK1 activity affects the TOR activity in favourable growth conditions, we estimated the level of phosphorylated and total S6K in WT, *SnRK1α1 OE2*, *flz6.1* and *flz10.1* seedlings grown in HL regime. Consistent with previous reports, the level of phosphorylated S6K was found to be reduced in *SnRK1α1 OE2* (Figure 7e). The *flz* mutants also showed reduction in the level of phosphorylated S6K indicating that elevated SnRK1 activity prevents the promotion of TOR activity in the mutants. No major difference in the level of total S6K was observed indicating that SnRK1 has inhibitory role on the phosphorylation status of the S6K (Figure 7e).

In response to energy, TOR phosphorylates the E2Fa transcription factor which results in the enhanced transcription of S-phase cell cycle genes (Xiong *et al.*, 2013). Ectopic expression of *SnRK1α1* resulted in a reduction in the expression of *E2Fa* and downstream cell cycles genes indicating that SnRK1 also negatively regulate TOR-E2Fa mediated cell cycle progression (Figure 7f). To test whether the TOR-E2Fa signalling is perturbed in *flz* mutants, we estimated the transcript level of *E2FA* and downstream genes. Similar to *SnRK1α1 OE2*, *flz* mutants showed a significant decrease in the level of *E2Fa* and its targets suggesting that TOR-E2Fa signalling is also downregulated in these mutants (Figure 7g).

DISCUSSION

SnRK1 is a central regulator of growth during energy deficit. Previously we demonstrated that energy status and SnRK1 regulate the expression of *FLZ* genes (Jamsheer K and Laxmi, 2015). Here, we show that two FLZ proteins, FLZ6 and FLZ10 negatively regulate the SnRK1α1 level. The knock-down mutants were found to be accumulating more SnRK1α1 which resulted in an increase in SnRK1 activity. Due to this, *flz6* and *flz10* mutants displayed reduced growth in the normal conditions and this phenotype was observed in two independent mutant lines confirming the role of FLZ6/10 in plant growth through regulating SnRK1 signalling. Interestingly, *flz10.2* showed increased expression of the first exon which could be due to the 35S promoter in the T-DNA which is observed in many other mutant lines (Deprost *et al.*, 2007; Waterworth *et al.*, 2007; Ülker *et al.*, 2008). The *flz10.2* showed reduced biomass and lateral root number, but there was no significant change in primary root length suggesting that the truncated transcript in this line might be functional to some extent. The phylogenetic analysis suggests that *FLZ* genes are originated in the terrestrial plants (Jamsheer K *et al.*, 2015). The regulation of SnRK1 signalling by *FLZ* genes might be originated in land plants which provide an additional layer of control over the SnRK1 signalling in plants (Figure 8).

Unlike AMPK, SnRK1 is proposed to be constitutively active suggesting that protein turnover might be an important mechanism which regulates SnRK1 activity in plants (Broeckx *et al.*, 2016). SnRK1 was found to be interacting with many ubiquitination-related proteins and has been demonstrated to be degraded by proteasome pathway (Bhalerao *et al.*, 1999; Farrás *et al.*, 2001; Lee *et al.*, 2008; Ananieva *et al.*, 2008; Carvalho *et al.*, 2016).

FLZ6 and *FLZ10* levels were found to be induced by ABA and during energy starvation through SnRK1 signalling. This suggests an interesting postulate that FLZ6 and FLZ10 might be working as repressors of SnRK1α1 during energy deficit and this might be important in preventing hyperactivation of SnRK1. Interestingly, an analogous mechanism is known to exist in yeast to control the SNF1 activity. During prolonged energy starvation, SNF1 positively regulates REG2, a regulatory subunit of PROTEIN PHOSPHATASE 1 which leads to the inactivation of SNF1 through dephosphorylation. This helps yeast to check the hyperactivation of SNF1 and thereby helps rapid recovery from the stress once cells enter favourable growth conditions (Maziarz *et al.*, 2016). This control over SNF1 activity is essential for the survival of yeast because the unregulated SNF1 activity is lethal for the organism (Ruiz *et al.*, 2011; Ruiz *et al.*, 2013). Even in mammals, the extent of AMPK activation is tightly controlled by the extent of energy deficit (Xiao *et al.*, 2011; Li *et al.*, 2015). Intriguingly, the expression analysis of *FLZ6* and *FLZ10* identified overlap with the expression pattern of SnRK1 subunits (Schmid *et al.*, 2005; Bitrián *et al.*, 2011; Gao *et al.*, 2016; Polge *et al.*, 2008). This overlap in the expression domain suggests that *FLZ6* and *FLZ10* are expressed along with SnRK1 subunits in many tissues which may be important in preventing the hyperactivation of SnRK1 during different developmental stages in plants.

The colocalization of the FLZ6/10 interaction with SnRK1α subunits in ER gives clues about the biological significance of this interaction. SnRK1α subunits are reported to be localized in nucleus, cytoplasm and chloroplast (Broeckx *et al.*, 2016). The ER-colocalization and preferential association of this interaction with nucleus suggest that the FLZ6/10-SnRK1 complex might be spatially localized to the site of protein synthesis. TOR is detected in ER and in the fractions of the ribosomes in mammals and plants (Liu and Zheng, 2007; Dı´az-Troya et al., 2008; Oh et al., 2010; Schepetilnikov et al., 2013). The active TOR phosphorylates S6K1, which results in its dissociation from eukaryotic initiation factor 3 which ultimately leads to the promotion of protein synthesis (Holz et al., 2005; Schepetilnikov et al., 2013). On the basis of our results, we suggest that FLZ6 and FLZ10 form a complex with SnRK1 in the vicinity of ER and through modulating SnRK1α1 level, regulate TOR-SnRK1 antagonistic interaction (Figure 8). The negative regulation of S6K phosphorylation and transcription of E2Fa and its targets observed in the *flz6* and *flz10* mutants substantiate this hypothesis. Intriguingly, the direct physical interaction of other FLZ proteins with RAPTOR1B is previously reported (Arabidopsis Interactome Mapping

Consortium, 2011). It would be interesting to analyze whether FLZ6 and FLZ10 directly interact with RAPTOR1B and regulate TOR activity independent of SnRK1.

Mapping of FLZ6 and FLZ10 interaction sites in SnRK1α1 identified that FLZ10 interacts strongly with the RD region which is responsible for the interaction of other subunits and regulatory proteins (Broeckx *et al.*, 2016). Although FLZ6 did not show any interaction with RD region alone, it showed only weak interaction with CD region indicating that RD region might also have a role in mediating this interaction. Although both *FLZ6* and *FLZ10* mutants showed elevated SnRK1α1, they interacted with different regions of SnRK1α1. The functional significance of this divergence in the interaction region is yet to be identified.

FLZ6 and *FLZ10* are phylogenetically distant FLZ proteins (Jamsheer K *et al.*, 2015). Interestingly, reduced expression of these genes in their respective mutants resulted in the accumulation of SnRK1α1 suggesting that neither of these genes can completely replace the other in function. This could be attributed to the divergence in the expression pattern (Jamsheer K and Laxmi, 2015; Nietzsche *et al.*, 2014). In summary, this work highlights the presence of a plant-specific regulatory component of the eukaryotic energy gauge, SnRK1.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Mutants of *FLZ6* (*flz6.1*/ SALK_039103C; *flz6.2*/ SALK_090349) and *FLZ10* (*flz10.1*/ SALK_106003C; *flz10.2*/ SALK_073232) were obtained from ABRC (https://abrc.osu.edu/). *SnRK1α1 OE2* was obtained from Prof. Filip Rolland (Metabolic Signalling group, KULeuven) (Baena-González *et al.*, 2007). From the seeds obtained from ABRC, *flz6.1* and *flz10.1* plants with insertion exclusively in the gene of interest were identified by PCR-based screening employing the method described in the SIGnAL (http://signal.salk.edu/tdnaprimers.2.html). The homozygous lines for the insertion were obtained through segregation and used for all the analysis. Using the PCR-based screening method, additional alleles (*flz6.2* and *flz10.2*) were also confirmed for homozygous insertion and used for the phenotypic analysis. The insertion position was identified through sequencing. The primers used for screening and sequencing are listed in Table S1. Seeds were surface sterilised and stratified at 4 °C for 48h in the dark. The stratified seeds were

grown vertically on square petri plates containing 0.5X MS medium with 0.8% agar or in pots prepared with 3:1 ratio of agro peat and vermiculite. All experiments were done in climate-regulated growth chambers under 16:8 hours photoperiod with 22°C ± 2°C temperature and 60 µmol m^{-2} s^{-1} light intensity.

Physiological Assays

The *flz* mutants and *SnRK1α1 OE2* were grown in the standard growth and medium conditions for 10 days. Images of seedlings grown in plates were taken using Nikon Coolpix digital camera (Nikon Corporation) after 10 days of growth. The lateral root number was measured under Nikon SMZ1500 stereomicroscope (Nikon Corporation). The primary root length of seedlings was measured using the Image J program (http://imagej.nih.gov/ij/). For fresh weight measurement, 10-days old seedlings were harvested and fresh weight was measured with Sartorius TE64 balance. The data obtained is the average of at least 15 seedlings unless specified and all the experiments were repeated 3 times yielding the same results.

For the sugar-availability assay, 5 days old 0.5X MS grown seedlings were transferred to 0.5X MS medium supplemented with 0.25, 0.5, 1, and 2 percentages of sucrose for another 5 days. Similarly, 5 days old 0.5X MS grown *flz* mutants and WT seedlings were transferred to 0.5X MS medium with 1% sucrose and grown under 21 and 105 µmol m^{-2} s^{-1} light intensity growth conditions for phenotypic analysis and immunodetection with the S6K antibodies. For the ABA-sensitivity assay, 5 days old 0.5X MS grown seedlings were transferred to 0.5X MS medium supplemented with or without 5 µM ABA. All the measurements and calculations were done as described above.

Chemical Treatments for qRT-PCR

The expression of genes in their respective mutants was studied in 5 days old seedlings grown in 0.5X MS medium with 0.8% agar. The expression of *DIN6*, *TPS8*, *E2FA* and *E2FA*-downstream genes were studied from the same sample. For 2DG treatment, 5 days old *Col-0* seedlings were subjected to 2DG (25 and 50 mM) treatments in 0.5X liquid MS medium for 3h at 140 rpm in dark. Similarly, the 5 days old *Col-0* seedlings were treated with 10 µM

ABA in 0.5X liquid MS medium for three different (30 min and 3 and 6 hrs) time points in the light. To study the response of *FLZ* genes towards sugar starvation in L*er* and *SnRK1α1 OE2*, the 5 days old seedlings grown on 0.5X MS with 1% sucrose were subjected to starvation treatment in 0.5X MS liquid medium without sucrose in dark for 24 hours in 22 °C at 140 rpm.

Gene Expression Analysis

RNA isolation was done using RNeasy Plant Mini Kit (Qiagen, USA) and cDNA was prepared from equal amount of RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The qRT-PCR reactions were performed with 1:50 diluted cDNA using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, USA) in Step One Plus or ViiA 7 Real-Time PCR System (Applied Biosystems, USA). The primers were prepared by PRIMER EXPRESS v3.0 (Applied Biosystems, USA). The relative quantification of mRNA level was calculated using ΔΔCT method (Livak and Schmittgen, 2001). The primers used for qRT-PCR are given in Table S1.

Promoter: GUS Line Construction and GUS Assay

Promoter: GUS transcriptional fusion construct of *FLZ10* was constructed using 2 kb 5' UTR upstream region. The *p:: FLZ6-GUSA* line was constructed in an earlier study (Jamsheer K and Laxmi, 2015). The promoter fragment was cloned into the pMDC164 vector (Curtis and Grossniklaus, 2003). The primers used for cloning are listed in Table S1. The clones were transformed into *Col-0* plants using floral dip method (Clough and Bent, 1998). Transformants were identified on 0.5X MS selection medium containing 15 µg/ml hygromycin A. Three independent homozygous lines showing similar expression pattern were identified at T3 generation.

Subcellular Localization Study

The *FLZ6*, *FLZ10* were cloned into pEG104 and pEG101 vectors using Gateway technology (Earley *et al.*, 2006). The ER-rk (CD3-959) construct was obtained from ABRC (Nelson et

al., 2007). The constructs were bombarded into onion peel using PDS-1000 Helios Gene Gun (Bio-Rad). DAPI staining was done as described previously (Jamsheer K and Laxmi, 2014). *Arabidopsis* mesophyll protoplasts were isolated by Tape-*Arabidopsis* Sandwich method and transformed as described previously (Wu *et al.*, 2009). The cells were visualised in TCS SP2 (AOBS) laser confocal scanning microscope 24 hours after the bombardment.

Bimolecular Fluorescent Complementation

BiFC experiments were performed using onion epidermis system and Arabidopsis mesophyll protoplasts using pSAT4-DEST-N-(1-174)-EYFP-C1 and pSAT5-DEST-C-(175-END) EYFP-C1 vectors (Tzfira et al., 2005). The *FLZ* genes were cloned in pSAT4-DEST-N-(1-174)-EYFP-C1, and *SnRK1α1* and *SnRK1α2* were cloned in pSAT5-DEST-C-(175-END)-EYFP-C1 using Gateway cloning (Invitrogen, CA). The primers used for cloning are listed in Table S1. The PDS-1000 Helios Gene Gun (Biorad) was used for cotransformation in onion epidermis. The interactions were visualised in TCS SP2 (AOBS) laser confocal scanning microscope (Leica Microsystems) or AxioImager M2 Imaging System (ZEISS), 24 hours after the bombardment. The Arabidopsis mesophyll protoplasts were cotransformed as described previously (Wu *et al.*, 2009). The samples were washed thrice in Hank's balanced salt solution (HBSS) without phenol red before proceeding for ER-Tracker Red dye (Invitrogen) staining. The staining was performed in 1μM ER-Tracker Red dye solution prepared in HBSS for 30 minutes at 30^{o}C in dark. After staining, the samples were washed in HBSS and visualized. Simultaneously, a negative control experiment was performed with pSAT4-DEST-N (1–174) EYFP-C1 and SnRK1α1 and SnRK1α2 cloned in pSAT5-DEST-C (175-END) EYFP-C1.

Protein Extraction, Western Blot Detection and SnRK1 Activity Assay

The total protein was extracted in extraction buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.47 mM KH_2PO4, and 10% glycerol) supplemented with 1/500 (v/v) plant-specific protease inhibitor cocktail and phosphatase inhibitor cocktail 3 (Sigma). The concentration was estimated by Quick Start Bradford reagent (Biorad). An equal volume of protein was loaded for detection. The SnRK1α1 antibody (anti-AKIN10, Agrisera), Phospho-

AMPK-alpha pThr172 antibody, p70 S6 Kinase, Phospho-p70 S6 Kinase pThr389 antibody (Thermo Pierce) and monoclonal anti-Tubulin-Tyrosine antibody (Sigma) were used for immunodetection. After detection, membranes were subjected to Ponceau S (0.1%) staining. All Western blot experiments were repeated at least three times. The quantifications were performed by Image Studio Lite v5.2 (Li-Cor Bioscience). The endogenous SnRK1 activity was estimated from the immunoprecipitated SnRK1α1 from WT and mutants by AMARA peptide assay as described previously (Rodrigues *et al.*, 2013) and radioactivity was measured using the Tri-Carb 2900TR Liquid Scintillation Analyser (Perkin-Elmer Inc.).

Yeast Two-Hybrid Assay

The *FLZ6* and *FLZ10* were cloned in pGBKT7g and full-length and partial *SnRK1α1* were cloned in pGADT7g through Gateway technology (Stellberger *et al.*, 2010). The respective BD and AD constructs were transformed in Y2H Gold strain and the transformed colonies were selected on -Trp/ -Leu medium and screened on -Trp/ -Leu/ -His/ -Ade/ +AbA/ +X-alpha-Gal medium.

Accession numbers

Sequence data from this article can be found in TAIR database under the following accession numbers: *FLZ6* (AT1G78020), *FLZ10* (AT5G11460), *SnRK1α1* (AT3G01090), *SnRK1α2* (AT3G29160), *DIN6* (AT3G47340), *TPS8* (AT1G70290), *S6K1* (AT3G08730), *S6K2* (AT3G08720), *E2FA* (AT2G36010), *MCM3* (AT5G46280), *MCM7* (AT4G02060), *ORC6* (AT1G26840), *ETG1* (AT2G40550), *PCNA1* (AT1G07370) and *UBQ10* (AT4G05320).

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Author contributions

M.J.K. and A.L. conceived and designed the experiments; M.J.K. and A.L. supervised the experiments; M.J.K. performed most of the experiments; M.J.K. M.S. and D.S. performed the physiological experiments; M.J.K. and C.T.M performed the localization and mutant screening experiments; M.J.K, B.N.S, and S. J. performed the Y2H and qRT-PCR experiments; M.J.K. and A.L. analysed the data; M.J.K. wrote the article; A.L. supervised and complemented the writing.

SHORT SUPPORTING INFORMATION LEGENDS

Figure S1. *In planta* interaction of FLZ6 and FLZ10 with SnRK1 kinase subunits in onion epidermis.

Figure S2. Negative control experiments for BiFC.

Figure S3. *In planta* interaction of FLZ6 and FLZ10 with SnRK1 kinase subunits in Arabidopsis mesophyll protoplasts.

Figure S4. Localization of ER-rk in onion epidermis.

Figure S5. Subcellular localization of FLZ6 and FLZ10.

Figure S6. Phenotypic analysis of *flz6.2* and *flz10.2* mutants.

Figure S7. ABA sensitivity of *SnRK1α1* overexpression line.

Figure S8. Phenotypic analysis of *SnRK1α1 OE2* grown under different concentrations of sucrose.

Table S1. Primers used in this study.

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FIGURE LEGENDS

Figure 1. Tissue- and developmental stage-specific expression pattern of *FLZ6* and *FLZ10*.
(a) and (b) Expression of *FLZ6* and *FLZ10* in 3 DAG (a) and 5 DAG (b) seedlings. (c) Expression in 5 DAG cotyledon. (d) Expression during different stages of lateral root. (e) Expression in the leaves of 32 DAG old rosette; leaves are arranged according to their age and position in the rosette (f) Expression in leaves of 40 DAG old plant bearing flowers and silique: from left to right, the first leaf is the oldest rosette leaf, the second leaf is a mature rosette leaf, and the third leaf is a cauline leaf. (g) Expression in flower bud. (h) Expression in open flower. (i) Expression during silique development.

Figure 2. Interaction of FLZ6 and FLZ10 with SnRK1 kinase subunits.
(a) Interaction of FLZ6 and FLZ10 with SnRK1α1 and SnRK1α2 in BiFC in onion epidermis. (b) Colocalization analysis of the interactions with ER marker protein. The whole cell representation of the interaction shown in Figure 3a and 3b is given in Figure S1. (c) Colocalization analysis of the interactions with ER-Tracker Red. FLZ proteins were fused to the amino (YFP (1-174)) terminus of YFP while SnRK1α subunits were fused with the carboxyl (YFP (175-end)) terminus of YFP. Wavelengths used for activation/detection (absorption/emission) of the fluorophores: YFP (514/530 nm), DAPI (351/450 nm), mCherry (575/610 nm), ER-Tracker Red (587/615 nm). (d) Domain structure of SnRK1α1. (e) Interaction of FLZ6 and FLZ10 with full-length and different parts of SnRK1α1 in Y2H system.

Figure 3. Transcriptional regulation of *FLZ* genes by starvation, ABA and SnRK1.
(a) qRT-PCR analysis of *DIN6* and *FLZ10* transcript levels in response to 2DG treatments. (b) qRT-PCR analysis of *FLZ6* and *FLZ10* transcript levels in response to ABA treatments (c) qRT-PCR analysis of *DIN6*, *FLZ6* and *FLZ10* transcript levels in response to 24 hours of sugar starvation (SS) in WT and *SnRK1α1 OE2*. *UBQ10* was used as the endogenous control. The bars represent the average of three biological replicates and error bars represent SE. Asterisk indicates a significant difference of expression ($P<0.05$, Student's t-test).

Figure 4. Phenotypic analysis of *flz6.1* and *flz10.1* mutants.
(a) The T-DNA insertion position in the *flz6.1* and *flz10.1*. (b) The relative transcript levels of *FLZ6* and *FLZ10* in their mutants. The qRT-PCR experiments were repeated thrice and bars represent the average and error bars represent SE. *UBQ10* was used as endogenous control. Asterisk indicates a significant difference in the expression ($P<0.05$, Student's t-test). (c) The phenotype of 10 DAG old *flz* and WT seedlings grown in 0.5X MS medium containing 1% sucrose. (d) Primary root length, lateral root number and fresh weight of *flz* mutants and WT seedlings at 10 days. (e) The phenotype of *SnRK1α1 OE2* and WT at 10 days in 0.5X MS medium containing 1% sucrose. (f) Primary root length, lateral root number and fresh weight of *SnRK1α1 OE2* and WT seedlings. Physiological experiments were repeated thrice with at least 15 seedlings in each experiment. The data shown is the average from a representative biological replicate and error bar represent SD. The 'Δ' symbol indicates a significant difference in the parameter compared to WT in two-tailed Student's t-test ($P<0.001$).

Figure 5. FLZ6 and FLZ10 regulation of SnRK1 activity.
(a) and (b) The level of SnRK1α1 in the mutants at the seedling (5 DAG) stage. The level of SnRK1α1 in the *flz* mutants was quantified with respect to WT using α-tubulin as the normalization control. The Asterisk indicates a significant difference in SnRK1α1 level in two-tailed Student's t-test (P<0.05). (c) and (d) The level of phosphorylated SnRK1α1 (Thr175) in the *flz* mutant in the seedling (5 DAG) stage. The level of phosphorylated SnRK1α1 (Thr175) in the *flz* mutants was quantified with respect to WT using α-tubulin as the normalization control. The Asterisk indicates a significant difference in the SnRK1α1-Thr175 in two-tailed Student's t-test (P<0.05). (e) The level of SnRK1α1-Thr175 in the *flz* mutants in comparison to the total SnRK1α1 level. (f) The transcript level of *SnRK1α1* and *SnRK1α2* in the *flz* mutants at the seedling (5 DAG) stage (g) The SnRK1 activity in WT and *flz* mutants at seedling (5 DAG) stage assayed by AMARA peptide assay. The experiment was repeated thrice and the asterisk indicates a significant difference in the SnRK1 activity in two-tailed Student's t-test (P<0.05). (h) The level of SnRK1-induced genes in the mutants and WT in the seedling (5 DAG) stage. The qRT-PCR experiments shown is an average of three biological replicates and error bar represent SE. *UBQ10* was used as the endogenous control. Asterisk indicates a significant difference in expression (P<0.05, Student's t-test). (i) The phenotype of WT and *flz* mutants in response to ABA treatment. (j) The primary root length of mutants and WT in response to ABA treatment. The numbers above the column indicate the percentage inhibition of primary root length. The experiments were repeated thrice with at least 10 seedlings in each treatment. The 'Δ' symbol indicates a significant difference in the primary root length after treatment in two-tailed Student's t-test (P<0.001).
Figure 6. Phenotypic analysis of *flz* mutants grown under different concentrations of sucrose.
5 DAG *flz* mutants and WT seedlings grown in 0.5X MS medium with 1% sucrose were transferred to medium containing different sucrose concentrations. (a) The phenotype of *Col-0* and *flz* mutants grown in 0.5X MS medium containing different concentrations of sucrose for 5 days. (b) The fresh weight, primary root length, and lateral root number of *flz* mutants and *Col-0* grown in 0.5X MS medium containing different concentrations of sucrose. All experiments were repeated thrice and the data shown is the average from a representative biological replicate and error bar represents SD. At least 15 seedlings were used for each treatment. The 'Δ' symbol indicates a significant difference in the parameter in two-tailed Student's t-test (P<0.001).
Figure 7. Regulation of TOR activity in *flz* mutants**.**
(a) The phenotype of L*er* and *SnRK1α1 OE2* grown in 0.5X MS medium with 1% sucrose in high light (HL; 105 µmol m–2 s–1) regime. (b) The fresh weight, primary root length, and lateral root number of L*er* and *SnRK1α1 OE2* grown in HL regime. (c) The phenotype of *Col-0* and *flz* mutants grown in 0.5X MS medium with 1% sucrose in HL regime. (d) The fresh weight, primary root length, and lateral root number of *flz* mutants and *Col-0* grown in HL regime. All experiments were repeated thrice and the data shown is the average from a representative biological replicate and error bar represent SD. At least 15 seedlings were used in each treatment. The 'Δ' symbol indicates a significant difference in the studied parameter in two-tailed Student's t-test (P<0.001). (e) The level of phosphorylated (S6K-P) and total S6K in *SnRK1α1 OE2, flz6.1* and *flz10.1* in HL regime in comparison to WT. The numbers indicate the relative level of S6K-P in *SnRK1α1 OE2, flz6.1* and *flz10.1* lines in comparison to total S6K. (f) and (g) The transcript level of *E2FA* and its targets in *SnRK1α1 OE2* and *flz* mutants in comparison to WT in seedling (5 DAG) stage. *UBQ10* was used as the endogenous control. The bars represent the average of three biological replicates and error bars represent SE. Asterisk indicates a significant difference in expression in two-tailed Student's t-test (P<0.05, Student's t-test).
Figure 8. A model depicting the position of FLZ6 and FLZ10 in SnRK1 and TOR signalling.

ABA and energy starvation promote the transcription of *FLZ6* and *FLZ10*. SnRK1 also induces their transcript level during energy starvation. FLZ6 and FLZ10 interact and repress SnRK1α1. Through this repression of SnRK1, FLZ6 and FLZ10 promote TOR signalling which induces growth in favourable conditions.