SIZ1-Dependent Post-Translational Modification by SUMO Modulates Sugar Signaling and Metabolism in Arabidopsis thaliana

Pedro Humberto Castro1,4, Nuno Verde1,2,4, Tiago Lourenço1,2, Alexandre Papadopoulos Magalhães1,2, Rui Manuel Tavares3, Eduardo Rodrı´guez Bejarano3 and Herlânder Azevedo3,*

1BioSystems & Integrative Sciences Institute (BioISI), Plant Functional Biology Center, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
2CIBIO, InBIO—Research Network in Biodiversity and Evolutionary Biology, Universidade do Porto, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
3Instituto de Hortofruticultura Subtropical y Mediterránea ‘La Mayora’, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Departamento de Biología Celular, Genética y Fisiología, Universidad de Málaga, Campus Teatinos, 29071 Málaga, Spain
4These authors contributed equally to this work.

*Corresponding author: E-mail, hazevedo@cibio.up.pt; Fax, +351-252661780.

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Post-translational modification mechanisms function as switches that mediate the balance between optimum growth and the response to environmental stimuli, by regulating the activity of key proteins. SUMO (small ubiquitin-like modifier) attachment, or sumoylation, is a post-translational modification that is essential for the plant stress response, also modulating hormonal circuits to coordinate developmental processes. The Arabidopsis SUMO E3 ligase SAP and Miz 1 (SIZ1) is the major SUMO conjugation enhancer in response to stress, and is implicated in several aspects of plant development. Here we report that known SUMO targets are over-represented in multiple carbohydrate-related proteins, suggesting a functional link between sumoylation and sugar metabolism and signaling in plants. We subsequently observed that SUMO-conjugated proteins accumulate in response to high doses of sugar in a SIZ1-dependent manner, and that the null siz1 mutant displays increased expression of sucrose and starch catabolic genes and shows reduced starch levels. We demonstrated that SIZ1 controls germination time and post-germination growth and the response to environmental stimuli, by regulating the activity of key proteins. SUMO (small ubiquitin-like modifier) attachment, or sumoylation, is a post-translational modification that is essential for the plant stress response, also modulating hormonal circuits to coordinate developmental processes.

Keywords: Arabidopsis • 2-Deoxyglucose • Glucose • Post-germination growth arrest • SIZ1 • SUMO.

Abbreviations: ABIS, ABA-insensitive 5; AMPK, AMP-activated protein kinase; 2-DG, 2-deoxyglucose; EIN3, ethylene insensitive 3; GO, Gene Ontology; HMWC, high molecular weight SUMO conjugate; HXK, hexokinase; MS, Murashige and Skoog; PGGA, post-germination growth arrest; PTM, post-translational modification; qPCR, quantitative reverse transcription–PCR; SnRK, SNF1-related kinase; SSR, SUMO stress response; SUMO, small ubiquitin-like modifier; TF, transcription factor.

Introduction

Sugars are essential components of the cell, playing a structural role in many organic molecules, acting as pivotal elements of cell primary metabolism, and serving as protective elements in response to both abiotic and biotic stimuli. It is therefore not surprising that disruption of sugar homeostasis has severe consequences on development and survival. For instance, in humans these disturbances can result in metabolic-associated diseases such as diabetes and obesity (Lustig et al. 2012). In plants, sugars control many aspects of growth and development (Eveland and Jackson 2012, Lastdrager et al. 2014), and their homeostatic levels are tightly controlled and integrated in a plethora of hormonal circuits, especially those involving ABA and ethylene (Gazzarrini and McCourt 2001, Rolland et al. 2006). Since sugars need to be perceived in such a way that allows rapid adjustments to cellular metabolism, an increasingly relevant issue in glycobiology involves sugar sensing and the role of sugars as signaling molecules. Several approaches to identify the components of sugar perception were based on forward and reverse genetic strategies. So far, only a few signaling components have been described in plants, involving hexokinase 1 (HXK1), sucrose non-fermenting 1 (SNF1)-related kinase 1 (SnRK1), target of rapamycin 1 (TOR1) and regulator of G-protein signaling 1 (RGS1). These control carbohydrate homeostasis and are triggered by different conditions (Hanson and Smeekens 2009, Sheen 2014). HXK1 has dual, yet uncoupled roles: (i) it phosphorylates glucose into glucose 6-phosphate which is the entry point for glycolysis; and (ii) it acts as a glucose sensor (Moore et al. 2003). In the presence of excess glucose, HXK sugar sensing results in the down-regulation of the plant’s photosynthetic capacity, via formation of an inhibitory complex in the nucleus that binds to the promoter of photosynthesis-related genes (Cho et al. 2006). TOR and SnRK1 are likely to function in a regulatory nodule with antagonistic...
roles (Robaglia et al. 2012). AMP-activated protein kinase (AMPK)/SNF1/SnRK serine/threonine kinases act as a convergence point for various metabolic, hormonal and stress signals during growth and development, responding to the cellular energy charge sensed by relative AMP and ATP concentrations (Crozet et al. 2014). The Arabidopsis thaliana genome encodes 38 SnRks, of which SnRK1.1 (AKIN10/KIN10), SnRK1.2 (AKIN11/KIN11) and SnRK1.3 (AKIN12/KIN12) represent the orthologs of the budding yeast (Saccharomyces cerevisiae) SNF1 and mammalian AMPK metabolic sensors (Baena-Gonzalez et al. 2007, Crozet et al. 2014). In response to sugar deprivation, AKIN10/11 modulates transcription to promote catabolism, namely starch mobilization during darkness (Baena-Gonzalez et al. 2007). RGS1, a regulator of GTPase activity, is also a player in glucose signaling events and its inactivation occurs by phosphorylation-induced endocytosis in response to glucose (Urano et al. 2012).

Due to their sessile nature, plants have to withstand any potentially limiting environmental stress. When responding to an external stimulus, de novo protein production can be a costly and time-consuming process. Alternatively, post-translational modifications (PTMs) of existing proteins can lead to the re-modeling of protein activity in a timely fashion. PTMs by small peptides are common, rapid and reversible strategies to adjust growth and reprogram the cell to adverse conditions (Beltrao et al. 2013). One such peptide, that structurally resembles ubiquitin, is the small ubiquitin-like modifier (SUMO). SUMO is covalently attached to a target protein by an enzymatic cascade (E1–E2–E3), and can be removed by the action of SUMO proteases (Gareau and Lima 2010). SUMO has been identified across eukaryotes as an important regulator of several cellular mechanisms, including the maintenance of genome integrity, subcellular trafficking, transcription modulation and regulation of the cell cycle (Hay 2005). One key objective of current SUMO research is the identification of the pool of SUMO targets and the establishment of the molecular consequences of SUMO attachment. Several proteomic analyses have already been carried out to determine the sumoylome (Castro et al. 2012, Filosa et al. 2013, Impens et al. 2014), and functional analysis of SUMO conjugates highlighted that SUMO controls nuclear-associated processes and particularly proteins involved in stress responses (Castro et al. 2012, Cubenas-Potts and Matunis 2013). Environmental stresses trigger the rapid accumulation of SUMO conjugates, a mechanism designated the SUMO stress response (SSR), to co-ordinate transcriptional programs (Lewicki et al. 2014). In plants, functional characterization of SUMO pathway components revealed that this PTM is essential in response to extreme temperature, low water availability, high salinity and nutrient imbalance (reviewed by Castro et al. 2012). On key player in the SSR response is the SUMO E3 ligase SAP and Miz 1 (SIZ1), that acts as the major sumoylation enhancer in response to stress, not only in plants but also in yeast (Ishida et al. 2009, Lewicki et al. 2014).

The present work addresses SUMO control of sugar signaling events during plant development. We characterized the SIZ1 knockout mutant in response to exogenous sugar treatments and placed SIZ1 as a novel and important regulator of correct developmental adjustment to sugar, involving both osmotic and sugar signaling pathways. The accumulation of SUMO conjugates in response to sugars is SIZ1 dependent, and SIZ1 seems to be involved in the transcriptional regulation of several sugar metabolic genes. We specifically implicated SIZ1 with glucose-controlled developmental traits, including post-germination growth and root development. Possible mechanisms integrating SUMO and glucose-dependent signaling are further discussed.

Results

Sumoylation is induced by sugar supplementation

In a previous study, we observed that Gene Ontology (GO) analysis of known Arabidopsis SUMO conjugates was enriched in proteins involved in the carbohydrate stimulus response (Castro et al. 2012). To address this issue further, we updated the set of SUMO conjugates to include recent proteomics-based SUMO conjugates (Lopez-Torrejon et al. 2013, Miller et al. 2013, Park et al. 2013), to a now total of 503 Arabidopsis genes, and performed gene network analysis for GO enrichment associated with metabolic pathway analysis (Fig. 1). As a result, we observed that response to carbohydrate categories were once again enriched, clustering with seed developmental processes (e.g. seed maturation, dormancy, germination and photomorphogenesis) and post-embryonic development relating to shoot development. A different GO cluster was associated with carbohydrate metabolic processes, including glucose and glycolytic metabolism. We observed that many of the proteins underlying this GO enrichment were associated with transcription regulation (Supplementary Table S1), including the transcription factors (TFs) ABA-insensitive 5 (ABI5) and ethylene insensitive 3 (EIN3), previously reported to be important modulators of sugar signaling pathways (Arenas-Huertero et al. 2000, Yanagisawa et al. 2003). This suggests that sumoylation may be enhanced in response to carbohydrate stimulus to regulate TF activity. With this in mind, we treated Arabidopsis seedlings with 4% and 8% concentrations of both glucose and sucrose, and monitored SUMO conjugation levels by Western blot analysis against SUM1/2, the main Arabidopsis SUMO peptides (Fig. 2). We observed that sugar supplementation generated an increase in high molecular weight SUMO conjugates (HMWCs). This increase seemed to operate on global SUMO conjugate levels, as no specific protein bands were observed (Fig. 2). Also, 4% concentrations were already sufficient to trigger a massive increase in HMWCs. Given that low water potential is a known consequence of high amounts of sugars, we also treated Arabidopsis seedlings with equivalent concentrations of mannitol, to serve as an osmotic control (Fig. 2). Mannitol also induced sumoylation, suggesting that sugar-dependent SUMO conjugate accumulation can be attributed, at least partially, to an osmotic stress effect. In yeast, SIZ1 was shown to be the main SUMO E3 ligase responsible for SSR (the steady-state increase in SUMO conjugate levels) (Lewicki et al. 2014). Since the Arabidopsis ortholog SIZ1 was also shown to be the major sumoylation enhancer in
response to stress (Ishida et al. 2009), we also characterized SUMO conjugation profiles in the siz1-2 null mutant background. The siz1 mutant displayed a significant reduction of HMWC levels in the presence of carbohydrate stimulus (Fig. 2), indicating that Arabidopsis SIZ1 is essential for sugar-induced target sumoylation.

**SUMO regulates expression of sugar-associated genes and starch accumulation**

Since TFs are one of the main targets of SUMO modification (Filtz et al. 2014), we investigated how expression of sugar-associated genes was regulated by sumoylation. For that purpose, we analyzed the microarray-based transcriptome of the mutant siz1-3 (Catala et al. 2007), and observed that several sucrose and starch degradation genes were up-regulated (Fig. 3A). Specifically, siz1 had increased expression of genes encoding sucrose-phosphate synthase, sucrose phosphatase, cell wall invertase, fructokinase and α- and β-amylase. Corroborating gene expression data, the siz1 mutant was previously shown to have reduced levels of sucrose and other significant sugars such as glucose, fructose, xylose and maltose (Park et al. 2012, Tomanov et al. 2014). Based on this evidence, we monitored starch levels at the beginning and end of the light photoperiod, via tissue staining with Lugol solution. At the beginning of the light photoperiod, both wild-type and siz1 plants showed little starch accumulation, and no difference between these genotypes seemed to be present (Fig. 3B). Conversely, by the end of the light photoperiod, when starch levels are expected to be increased, wild-type plants showed the anticipated starch staining while siz1 was defective in starch accumulation (Fig. 3C). Soluble starch levels were also enzymatically quantified in leaves of 4-week-old plants (Fig. 3D). The siz1 mutant showed less starch content in comparison with wild-type plants, especially at the end of the light photoperiod, corroborating the in situ starch staining. The overall results suggest that SIZ1 is a positive regulator of starch levels.

**The siz1 mutant has altered sugar-dependent germination and post-germination development**

To correlate sumoylation with sugar perception, we screened for sugar-associated phenotypes, sowing siz1 seeds onto media.
supplemented with different types and concentrations of sugars. By scoring germination parameters, one can then establish whether genotypes are sensitive or insensitive to sugars (Rolland et al. 2006). Seed germination was measured 10 d after stratification by quantifying cotyledon greening (Fig. 4A, B). With relation to sucrose, we observed that increasing concentrations seemed to have no effect on the wild-type’s germination rate, while \textit{siz1} displayed reduced germination for sucrose concentrations beyond 3%, placing \textit{siz1} as a sucrose-sensitive mutant. Meanwhile, glucose supplementation negatively affected germination of both genotypes, but, once again, \textit{siz1} germination was more sensitive, making \textit{siz1} a glucose over-sensitive (\textit{glo}) mutant. When compared with sucrose, less glucose was required to inhibit germination of \textit{siz1} by half (\textasciitilde4% glucose) (Fig. 4B). Mannitol produced a germination inhibition profile that resembled that of glucose, suggesting that osmotic stress is determining the germination rate reduction in \textit{siz1}. However, when we measured the relative Chl content of 10-day-old seedlings (Fig. 4C), we could observe that sucrose and glucose, but not mannitol, led to a significant decrease of Chl in \textit{siz1}. One consequence of sugar signaling is the shutdown of photosynthesis-related genes and Chl levels (Mortain-Bertrand et al. 2008), aimed at decreasing sugar production. Therefore, the present results suggest that, at early post-germination developmental stages, \textit{siz1} displays enhanced responses to sugar-induced inhibition of photosynthetic pigment content that is independent of osmotic stress.

Next, we wanted to verify how sugars impacted on green cotyledon formation irrespectively of genotype-dependent seed fitness; therefore, the time-course kinetics were plotted in relation to the number of seedlings that formed green cotyledons, ruling out non-germinated seeds. Measurements were made (i) in the absence of sugars, (ii) in the presence of a standard sucrose concentration used in vitro (1.5%) and (iii) in the presence of sugar concentrations that inhibited close to 50% germination in \textit{siz1} (5% sucrose, 4% glucose and 4% mannitol) (Fig. 5A). Sugar supplementation delayed the development of green cotyledons of both the wild-type and \textit{siz1} in comparison with the control, sugar-free condition, and results from sucrose 1.5% and 5% supplementation indicate a dose dependency of the phenotype. Similar results were observed when we scored radicle emergence from the seed coat as a germination parameter (Supplementary Fig. S1). It is noteworthy that in germination-inhibiting concentrations, the \textit{siz1} mutant displayed a constitutive germination delay in relation to the wild-type, independently of sugar supplementation (Fig. 5A). A similar phenotype was observed with a second, independent mutant allele (\textit{siz1-3}; Supplementary Fig. S2A). Additionally, we observed that \textit{siz1} but not wild-type seedlings showed growth arrest soon after germination (Fig. 5B), a mechanism designated as post-germination growth arrest (PGGA) (Lopez-Molina et al. 2001), and, most importantly, PGGA was significant in the presence of sucrose and glucose, but not mannitol (Fig. 5C; Supplementary Fig. S2B), implicating...
sumoylation in early post-germination developmental stages via sugar signaling events.

**Glucose compromises siz1 root growth and morphology**

In addition to germination, sugars are also known to control other aspects of plant growth and development (Rolland et al. 2006). To determine the impact of SUMO–sugar interplay at the root level, siz1 seedlings were grown vertically and transferred to half-strength Murashige and Skoog (MS) medium supplemented with previously established growth-inhibitory sugar concentrations. Root growth was measured once every 2 d for an 8 d period. When grown in glucose, siz1 not only displayed a significant root growth reduction compared with the wild-type, but also produced a higher number of lateral roots (Fig. 6A–C; Supplementary Fig. S2C, D). Conversely, siz1 root growth in the control condition or in media supplemented with sucrose or mannitol was similar to that of the wild-type (Fig. 6A), indicating that glucose-dependent root growth inhibition is not caused by an osmotic effect. Interestingly, siz1 lateral root formation was negatively correlated with primary root growth. Most significantly, we detected a morphological defect in glucose-grown siz1 roots, characterized by the formation of abnormal root hairs with basal bulges along the root surface (Fig. 6D; Supplementary Fig. S2E). This trait was previously reported by Karve et al. (2012) in Arabidopsis seedlings transferred to high concentrations of glucose. Thus, our results suggest that the lack of a functional SIZ1 results in higher root sensitivity to glucose, which places SIZ1 as a positive regulator of root growth and development in the presence of glucose.
SIZ1–sugar interplay acts mostly downstream of HXK

Considering that the present results establish siz1 as a glo mutant and that the literature has supported a central role for glucose in signaling events (Sheen 2014), we next experimented on medium supplemented with the glucose analog 2-deoxyglucose (2-DG), which allows us to map glucose-sensing events on siz1 (Gibson 2000, Azevedo et al. 2014). Because 2-DG acts as a potent signaling molecule, the range of concentrations used was significantly reduced, allowing us also to uncouple observed phenotypes from osmotic effects. Supplementation with 0.5 mM 2-DG did not interfere with germination and green cotyledon formation, since both the wild-type and siz1 responded similarly in the presence or absence of 2-DG (Fig. 7A). However, PGGA was significantly affected by 2-DG (Fig. 7B, C), supporting the notion that this mechanisms is highly dependent on sugar signaling. The results also evidenced that the increased PGGA phenotype of siz1 was only slightly increased in the presence of 2-DG, which suggests that it is sugar dependent, but for the most part it is independent of 2-DG-related signaling events. In support of this, Chl contents were also not significantly affected by 2-DG supplementation (Fig. 7D). Interestingly, 2-DG induced significant changes in root morphology (increased primary root width, increased number and length of root hairs), but no differential

Fig. 4 The siz1 mutant is sugar sensitive. (A) Morphology of representative seedlings, 7 d after sowing onto half-strength MS medium supplemented with 5% sucrose, 4% glucose or 4% mannitol (control represents no supplementation). The scale bar represents 1 cm. (B) Germination levels (green cotyledon percentage), 7 d after sowing onto MS medium supplemented with different types and concentrations of sugars. Percentages were determined in relation to the total number of sown seeds. Error bars represent the SEM (n = 5 plates with ~25 seedlings each). Dashed lines indicate the sugar percentage that matches 50% of cotyledon greening. (C) Relative Chl content of seedlings, 7 d after sowing onto half-strength MS medium supplemented with different types and concentrations of sugars. Relative values are normalized to the control (0% sugar) within each genotype. Error bars represent the SEM (n = 5 plates with ~25 seedlings each).
phenotype was detected between the wild-type and siz1, namely the presence of basal bulges from defective root hairs that are visible in 4% glucose (Fig. 7E). The glucose analog 2-DG is known to undergo cellular uptake and phosphorylation, but it is not metabolized in the cell (Gibson 2000). Therefore, our results suggest that sensing mechanisms involving sugar import and the HXK glycolytic pathway entry point are not the major components of SUMO–sugar interplay, potentially placing these events as independent of HXK.

**SIZ1 regulates the expression of sugar signaling genes**

Sugars levels are capable of reprogramming gene expression by triggering the transcription of specific subsets of marker genes...
To differentiate between sugar- and osmoticum-dependent signaling mechanisms in the *siz1* background, RNA was extracted from 10-day-old seedlings grown in the absence or presence of high concentrations of both glucose and mannitol, and subjected to quantitative reverse transcription–PCR (qPCR) for a specific set of marker genes. Supplementary Fig. S3 depicts absolute gene expression variation under these experimental conditions, whereas Fig. 8 highlights the comparison between glucose- and mannitol-grown plants, allowing us to resolve the significance of sugar- vs. osmoticum-dependent signaling. First, we analyzed expression of the marker genes *SIS7* and *TBL26*, respectively involved in ABA biosynthesis and the RGS1-mediated G-protein signaling pathway, but which were also reported to be involved in sugar signaling (Huang et al. 2008, Urano et al. 2012). Both were up-regulated in *siz1* (Fig. 8), suggesting that ABA may be a signaling component involved in *siz1* sugar sensitivity. However, since our previous phenotypic data clearly demonstrated the uncoupling of sugar and osmotic-dependent signaling events in specific seedling developmental features, we explicitly monitored expression of the sugar sensing hubs *HXK1* and *AKIN10*, observing that *HXK1* was significantly up-regulated in *siz1*, whereas *AKIN10* was up-regulated though not statistically significantly in comparison with the wild-type. Since *HXK1* is a major glucose sensor and was up-regulated in *siz1*, we selected four marker genes: *RBCS* and *PLD1/C11*, involved in the *HXK1*-dependent pathway, and *cwINV* and *CHS*, involved in a *HXK1*-independent pathway (Jossier et al. 2009). Gene expression variation was not significantly different compared with the wild-type, with the exception of *CHS* (Fig. 8). The results suggest that an *HXK1*-dependent signaling pathway is not differentially expressed in *siz1* when comparing glucose and mannitol

![Fig. 6 Root growth rate and morphology in different sugar-supplemented media.](image-url)

(A) In vitro root growth rate of 7-day-old seedlings transferred to different types and concentrations of sugar-supplemented media. (B) Morphology of 2-week-old seedlings. (C) Number of lateral roots. (D) Representative root tip and root hair morphology of 2-week-old seedlings grown on media with 4% glucose; arrows highlight defective root hairs in *siz1*. Error bars represent the SEM (n ≥ 12). Asterisks represent statistically significant differences of mutants in relation to the wild-type (unpaired t-test; *P < 0.05; ***P < 0.001). The scale bar indicates 1 cm (B) and 0.5 mm (D).
supplementation. We subsequently studied expression of PR2 and ASN1, which are marker genes for a metabolic-dependent pathway (Baena-Gonzalez et al. 2007, Jossier et al. 2009). In contrast to previous marker genes, both genes were differentially expressed in siz1 (Fig. 8). Interestingly, PR2 and ASN1 are regulated by AKIN10, suggesting the existence of interplay between SIZ1 and AKIN10-dependent signaling. In light of this result, we monitored AKIN10 protein levels under these experimental conditions (Fig. 9). We observed that glucose-, but not mannitol-grown plants, displayed increased AKIN10 levels. Most significantly, AKIN10 levels were higher in the siz1 mutant background, suggesting a role for SIZ1 in the maintenance of AKIN10 homeostatic levels.

**Discussion**

**Sumoylation is involved in early development via osmotic and sugar-dependent mechanisms**

Plant sugar signaling has been extensively studied over the last decades due to its critical importance in plant metabolism, development and agricultural production, yet many molecular mechanisms controlling sugar perception remain obscure (Lastdrager et al. 2014, Ruan 2014). In the present work, we report that lack of the SUMO E3 ligase SIZ1 resulted in both sucrose and glucose sensitivity phenotypes in the early stages of development. The use of the osmolyte mannitol allowed us to resolve whether observed phenotypes were dependent on an osmotic stress component of exogenous application of sugars. Consequently, we were able to implicate SIZ1 in two separate events: (i) breaking of dormancy and radicle emergence; and (ii) post-germination development towards formation of green cotyledons (Fig. 10). Regarding the former, the siz1 mutant displayed a delay in germination time in comparison with the wild-type that was enhanced by glucose and sucrose, yet also by mannitol, suggesting it is dependent on osmotic stress (Fig. 10). This implicates SIZ1 in water availability responses, which is consistent with previous reports demonstrating SIZ1 importance in drought stress processes (Catala et al. 2007, Miura et al. 2012). A possible involvement of ABA, a key hormone in the control of germination (Nakashima and Yamaguchi-Shinozaki 2013), is supported by the fact that both ABA-dependent and -independent
mechanisms are involved in the SUMO–drought association (Castro et al. 2012). We also detected that siz1 has a constitutive germination defect in which 10–20% of seeds do not germinate, independently of the medium’s osmotic conditions. We have observed that siz1 seed viability also decreases significantly with seed age (data not shown). These observations support a key role for sumoylation in embryo and seed development, which is manifested by the embryo lethality of SUMO pathway
components upstream of SIZ1 and the embryo lethality of the SUMO E3 ligase double mutant siz1 mms21 (Saracco et al. 2007, Ishida et al. 2012). Secondly, we observed that siz1 exhibits extensive PGGA on glucose- and sucrose-supplemented media, in which cotyledons fail to develop and green (Fig. 10). This phenotype does not appear on mannitol-containing media, discarding the idea that the PGGA may be a consequence of low water availability in the medium. We also observed that siz1 sensitivity to sucrose and glucose (but not mannitol) manifests on seedling Chl content. We propose that SIZ1 is important for both seed germination and green cotyledon/photomorphogenic development, but only the latter is dependent on sugar-mediated signaling (Fig. 10). This reinforces previous observations that time of germination and PGGA are two uncoupled developmental processes (Dekkers et al. 2004).

**Glucose is central in SUMO–sugar interplay**

The present data suggest that glucose is the sugar responsible for siz1 sensitivity, and siz1 can therefore be classified as a glo mutant. Sucrose is likely to produce the post-germination growth arrest of siz1 by being rapidly converted into glucose and fructose, and in fact we showed that sucrose catabolic enzyme-coding genes (including a cell wall invertase) are up-regulated in siz1. Moreover, we demonstrated that starch levels in siz1 are lower than in the wild-type, which suggest deregulation of starch metabolism. In support of this, previous evidence suggests an important role for post-translation modification in the control of starch metabolic enzymes (Smith et al. 2004). Other reports have quantified sugar levels in siz1, confirming that glucose and sucrose are constitutively lower (Park et al. 2012, Tomanov et al. 2014). Additionally, we observed that (i) HXK1 was up-regulated in siz1 in high glucose conditions, therefore contributing to glucose phosphorylation and the feeding of glycolysis; and (ii) the lack of phenotypes in the presence of the non-metabolized glucose analog 2-DG suggests the involvement of sugar signaling events independently of HXK. Globally, the results suggest that SIZ1 acts as a negative regulator of sugar catabolic activity, and that signaling events are probably due to a metabolism-dependent rather than HXK signaling-dependent pathway.

Glucose levels can also intervene in root development (Mishra et al. 2009). In this work, root hair formation

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**Fig. 10** The SUMO E3 ligase SIZ1 controls early seedling developmental events in response to sugar content. High exogenous sugar levels increase SUMO conjugate accumulation in a SIZ1-dependent pathway; meanwhile SIZ1 negatively regulates the transcript levels of starch and sugar metabolic genes, which may prevent sugar catabolism. SIZ1-dependent sumoylation differentially affects two separate events during germinative/seedling development. SIZ1 acts as a positive regulator of seed breaking of dormancy leading to radicle emergence, via osmotic-dependent signaling. Via sugar-dependent signaling, SIZ1 acts as a positive regulator of post-germination and photomorphogenic development, inhibiting post-germination growth arrest (PGGA). Known SUMO conjugates include several transcription regulators associated with these developmental stages, such as ABI5 and EIN3/EIL1. We propose that SIZ1 modulation of development may involve sumoylation of these targets. Additionally, SIZ1 may also contribute to decrease AKIN10 protein levels, shutting down carbon starvation responses when sugar supplementation is sufficient.

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Possible mechanisms for sugar regulation by SUMO

We demonstrated that SUMO conjugates accumulate after exogenous application of sugars. No specific bandshift was observed, indicating that several targets are sumoylated in response to sugars. This is consistent with the increasing awareness that many SUMO targets are components of protein complexes and often involved in transcription regulation (Cubenas-Potts and Matunis 2013). Accordingly, we observed the presence of important TFs that are both sumoylated and involved in the carbohydrate response (Supplementary Table S1), of which we highlight ABI5 and EIN3 (Fig. 10). The ABI5 null mutant displays insensitivity to exogenous treatment with ABA and glucose, acting as a positive regulator of ABA- and glucose-induced developmental arrest in the early stages of development (Arenas-Huerto et al. 2000, Brocard et al. 2002). ABI5 protein levels are tightly controlled by ubiquitination via the ubiquitin E3 ligase Keep On Going (KEG). In response to ABA, KEG is autoubiquitinated, allowing ABI5 to be degraded via the ubiquitin E3 ligase Keep On Going (KEG). In response to sugars, ABI5 protein turnover is regulated by the E3 ubiquitin ligase complex made up of the F-box proteins EIN3-binding F-box protein 1 (EBF1) and EBF2 (Potuschak et al. 2003, Gagne et al. 2004), which are related to the yeast Glucose repression-resistant 1 protein (Grr1p) F-box involved in the regulation of glucose signaling (Flick and Johnston 1991, Gagne et al. 2004). One may hypothesize that SIZ1 involvement in sugar-mediated signaling may operate via SIZ1 control of EIN3/EIL1 stability, without affecting its TF activity.

Sugar sensors such as HXK1 and SnRK1s (e.g. AKIN10/11) are also potential SUMO targets. Arabidopsis HXK1 interacted with Arabidopsis SCE1 in a yeast-based screening and was shown to be sumoylated in vitro (Elrouby and Coupland 2010). However, our gene expression analysis of the marker genes RBCS and PLD1α (Jossier et al. 2009) revealed that the HXK1 signaling pathway is not altered in siz1 in response to glucose. Simpson-Lavy and Johnston (2013) recently showed that yeast Snf1p, the AKIN10/11 ortholog, was SUMO modified, resulting in its inactivation and degradation modulated by SUMO-targeted ubiquitin ligases (STUbLs). Arabidopsis AKIN10 was also shown to interact with the SUMO E2 SCE1, and was sumoylated in vitro (Elrouby and Coupland 2010), placing AKIN10/11 as a highly likely SUMO target. PGGA was shown to be positively regulated by AKIN10 (Jossier et al. 2009) and negatively regulated by SIZ1, suggesting that SIZ1 may be a negative regulator of AKIN10/11 (Fig. 10). Taking into account that AKIN10 expression did not change significantly in siz1 in response to glucose, it is possible that SIZ1 controls AKIN10 activity at the protein level. Here, we observed that AKIN10 protein levels were increased in the siz1 mutant background, which is consistent with PGGA phenotypic data, and further supports a role for SIZ1 in AKIN10/11 homeostasis. Simultaneously, we observed repression of ASN1 expression in siz1, even though ASN1 is a known marker for AKIN10 activity (Baena-Gonzales et al. 2007). This suggests increased complexity in the interplay between SIZ1 and AKIN10 signaling events, in which SIZ1 may affect AKIN10 activity in addition to its protein turnover, or may target additional proteins downstream of AKIN10. Further studies should help resolve this issue.

Concluding remarks

In the present work we establish for the first time an association between SUMO-dependent PTM and sugar metabolism and signaling. Since no significant alteration of the SUMO conjugate pattern was observed in the siz1 mutant background, this SUMO E3 ligase seems to be essential for SUMO conjugation in response to sugars, reinforcing its role as the major facilitator
of SUMO conjugation in plant (Cheong et al. 2009, Ishida et al. 2009) and non-plant systems (Lewicki et al. 2014). As a central molecular mechanism, sumoylation works as a fast strategy to reprogram protein activity, often impacting on transcriptional control (Cubenas-Potts and Matunis 2013), and in plants it is significantly involved in various responses to environmental stimuli (Castro et al. 2012). Energy deficiency is associated with most environmental perturbations, when mechanisms are required to monitor and trigger appropriate metabolic, growth and developmental responses (Baena-Gonzalez 2010), and in this regard sugar levels need to be tightly controlled, especially in the allocation between source and sink tissues. We demonstrated that many of the currently known plant SUMO targets are implicated in carbohydrate metabolism and that SUM1/2 conjugates accumulated in response to sugar shock treatment, implicating sumoylation in the response to sugar fluctuations. In support of SUMO–sugar interplay, the human SUMO protease SENP2 acts as a negative regulator of aerobic glycolysis metabolism by decreasing the expression levels of key glycolytic enzymes that sumoylation seems to be generally implicated in nutritional responses, adding sugar to the previously reported roles for sumoylation in phosphate (Miura et al. 2005), nitrate (Park et al. 2011), copper (Chen et al. 2011) and, more recently, sulfur homeostasis (Tomanov et al. 2014).

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana mutant lines are in the ecotype Columbia (Col-0) background. The T-DNA insertion mutants siz1-2 (SALK_065397) and siz1-3 (SALK_034008) (Miura et al. 2005) were ordered from the NASC European Arabidopsis Stock Centre (arabidopsis.info). Synchronized Arabidopsis seeds were stratified at 4 °C, in the dark, for 3 d. Seeds were surface sterilized by incubation in 70% (v/v) ethanol for 5 min following by 10 min in 20% (v/v) commercial bleach and then washed four times with sterilized ultra-pure water. Seeds were sown onto MS medium (full-strength MS macro- and micronutrients, 4.302 g l⁻¹, MES (0.5 g l⁻¹) and sucrose (15 g l⁻¹), pH 5.7) with 1.2% (w/v) agar for vertical growth or 0.8% (w/v) agar for horizontal growth. Plants were grown in growth chambers with a 16 h light/8 h dark cycle under cool white light (80 μE m⁻² s⁻¹ light intensity) at 22–24 °C. For standard growth, in vitro grown 7-day-old seedlings were transferred to a soil: vermiculite (4:1) mixture, and maintained under identical growth conditions, with regular watering. Sugar-induced stress was generated by supplementing half-strength MS nutrient medium with different types and concentrations of sugars or the glucose analog 2-DG (Sigma-Aldrich), as indicated in the Results. Germination was quantified by scoring two parameters, root emergence and double green cotyledon appearance, every day for 10 d, using a stereomicroscope, as previously described (Silva-Correira et al. 2014). For root growth assays, 7-day-old seedlings were grown vertically in full-strength MS and then transferred to half-strength MS medium supplemented with different types and concentrations of sugars. Three plants per genotype were transferred to each plate. Root elongation was measured every 2 d for 8 d, and data were fitted to establish a root growth rate (mm d⁻¹).

Starch staining and quantification

Plants were incubated in 70% (v/v) ethanol followed by 96% (v/v) ethanol at 95 °C, until all pigments were extracted. Plants were washed with water and then incubated in Lugol solution [1% (w/v) iodine (I₂); 2% (w/v) potassium iodide (KI)], in the dark, for 10 min. Lugol solution was removed and the tissues were washed three times with deionized water to eliminate background staining. Soluble starch content was determined using the Starch Assay Kit (ABCAM), following the manufacturer’s instructions.

Pigment extraction and quantification

To determine Chl and carotenoid contents, plant shoots were incubated in 80% (v/v) acetone at 1 h in the dark. The plant material was vortexed, spun down, and the supernatant was used to measure absorbances at 470, 645 and 663 nm in a microplate spectrophotometer (SpectraMax 340PC; Molecular Devices). Pigment contents were estimated as previously described (Arnon 1949, Lichtenthaler and Buschmann 2001), using the following formulas: total Chl, C_Total = 20.2 A470 + 8.02 A645 total carotenoids, C_carotenoid = (1,000 A663 – 1.82 (12.7 A663 – 2.69 A645)) / 85.02 (22.90 A663 – 4.68 A645)]/198.

RNA extraction, cDNA synthesis and quantitative RT–PCR

RNA was extracted from 10-day-old seedlings using an RNasy Plant Mini kit (QIAGEN). RNA quantity and quality were assessed using both a Nanodrop ND-1000 spectrophotometer and standard agarose gel electrophoretic analysis. Afterwards, 1 µg of each RNA sample was treated with DNase I (Sigma-Aldrich) and the reaction was used for cDNA synthesis using oligo(dt)18 and SuperScript III Reverse Transcriptase (Invitrogen). For qPCR, SsoFast EvaGreen Supermix (BioRad) was used in the reaction mixture as per the manufacturer’s instructions, and run in a CFX96 Real-Time System (Bio-Rad). Primers were designed using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure specific amplification within the Arabidopsis genome, and obeyed the following guidelines: 100–250 bp PCR amplification product size; 50–60% GC content; approximately 60°C Tm. Primers used are described in Supplementary Table S2, and ACT2 (At4g18780) was used as a reference gene (Lozano-Duran et al. 2011). Relative expression values were calculated according to the Pfaffl method (Pfaffl 2001).

Protein extraction and immunoblotting

For detection of HMWCs by immunoblotting, seedlings were grown in vitro for 2 weeks, in MS medium containing 1.5% sucrose. Sugar shock was induced by submerging approximately 10 plantlets in different sugar solutions. A vacuum was then applied for 3 × 5 min periods, at 50 kPa, to promote sugar infiltration into plant tissues. Seedlings were incubated for 1 h in the growth chamber under standard conditions, and subsequently frozen in liquid nitrogen. Tissues were ground in a microtube in liquid nitrogen with the help of polypropylene pestles. Protein extracts were obtained by adding 1 ml of extraction buffer [50 mM Tris-Base; 150 mM NaCl; 0.2% (v/v) Triton X-100; 1 tablet of Complete Mini Protease Inhibitor Cocktail (Roche) per 10 ml of buffer] per gram of tissue fresh weight, and incubating for 1 h at 4 °C with agitation. Microtubes were centrifuged twice for 30 min at 16,000 g and 4 °C. After each centrifugation, the supernatant was carefully transferred to a new microtube. Protein samples were stored at –80 °C. Protein samples were loaded onto a 4%–12% acrylamide gel and electrophoresed at 150 V. After electrophoresis, proteins were transferred to a PVDF membrane (Bio-Rad) using a semi-dry transfer apparatus at 0.25 mA per membrane at 4°C for 3 h. After transfer, membranes were blocked in 5% (w/v) nonfat milk in TBS for 1 h, followed by incubation in primary antibodies at the appropriate dilutions. Membranes were blocked with 5% (w/v) nonfat milk in TBS for 1 h, and then incubated in primary antibodies at the appropriate dilutions. Membranes were washed three times with Tris-buffered saline and 0.1% (v/v) Tween-20 (TBST) buffer. The primary antibodies used were anti-SUMO1 (ABCAM) anti-AIN1 (Agrisera) (for details see Supplementary Methods S1).

Bioinformatic analysis

GO network and Enrichment Pathway analysis for known Arabidopsis SUMO targets was performed using the ClueGo plugin on Cytoscape (Bindea et al. 2009). ClueGO analysis incorporated Gene Ontology for Biological Process, BioCyc and KEGG Ontologies/Pathways. The pathway’s restriction was set to P < 0.05, and a GO tree interval of 4–6 was used to specify GO terms. The Connectivity score (Kappa Score) was set to 0.5. Analysis of siz1-3 differentially expressed genes (Catala et al. 2007) involved in carbohydrate metabolism pathways was performed by running differentially expressed genes in MapMan (Thimm et al. 2004), complemented with metabolic pathway data from the Plant Metabolic Network database (www.plantece.org).
Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


Ishida, T., Yoshimura, M., Miura, K. and Sugimoto, K. (2012) MMS21/HPY2 and SIZ1, two Arabidopsis SUMO E3 ligases, have distinct functions in acid-, and stress-mediated SIZ1 phenotypes.


