Final Report of the OTKA research grant NN-107838

Project title: Does SNRK1 provide a signalling link from cellular sucrose and energy status to the regulation of cell proliferation by direct regulation of the RBR E2F transcriptional complex?

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Introduction

Coupling growth and cell proliferation with the available nutrient and energy supply is fundamental for cellular homeostasis, but in plants the mechanisms are little understood. The aim of this co-operative OTKA NN project between the Koncz group (Cologne) and the Magyar group (Szeged) was to find connections between two evolutionary conserved regulatory pathways both depending on the available sugar; the sucrose non-fermenting 1 (SNF1)/AMP-activated kinase 1 (AMPK1)/Snf1-related kinase 1 (SnRK1), which operates as an integrative metabolic sensor that maintains energy balance at both cellular and systemic level, and the E2F-RB transcriptional regulatory mechanism, which keeps the balance between cell proliferation and differentiation thus regulating plant growth. Previously the Koncz group demonstrated that SnRK1 is an integral part of the SCF ubiquitin ligase complex, and also regulates protein stability by direct phosphorylation of target proteins (Farrás et al., 2001). Earlier our group discovered that RBR is targeted by CDK-based kinases, mainly CDKA;1 (Magyar et al., 2012), but S6K1, one of the effectors of TOR kinase pathway was also able to interact with and phosphorylate RBR proteins (Henriques et al., 2010; Henriques et al., 2013). Our starting hypothesis was that SnRK1 could participate in the regulation of E2F-RBR proteins thereby their functions.

Results

1. RBR phosphorylation is regulated by sucrose and light and SnRK1 might be involved in this regulation

So far RBR phosphorylation in plants could only be followed using the S807 phosphosite-specific animal Rb antibody that detects a single RBR site (RBR^{Ser911} - Magyar et al., 2012). We showed that the phosphorylation of this site on the *Arabidopsis* RBR is initiated by sucrose and CYCD3;1 overexpression, and the phosphorylated RBR on Ser911 cannot bind to E2F transcription factors. Sugar level is naturally changing during the diurnal cycle, increasing during the day and dropping during the night. Previously we have seen that the level of P-RBR was low at night, and it was further decreased if the night was extended, while RBR became hyper-phosphorylated just few hours after dawn. We followed the RBR phosphorylation level during a 12h light/12h dark cycle both in wild type *Arabidopsis* Col, and in starchless *pgm* mutants (phosphoglucomutase or *pgm*). Starch is the major carbon source for night growth in *Arabidopsis*; therefore *pgm* mutants running into carbon starvation every night resulted in growth arrested mutant plants. It is known that *pgm* mutant contains very high levels of sugars at the end of the day and very low levels of sugars at the end of night. Phosphorylation of RBR follows a light and sucrose diurnal rhythm in both WT and mutant plants (Figure 1), however there were much bigger fluctuations in the P-RBR levels between day and night in the *pgm* mutants than in the WT reflecting different sucrose levels within these plants. In addition, RBR was almost completely un-phosphorylated in the *pgm* mutant from the middle of the night and remained very low till three hours after the morning. All these are signs of starvation in the mutant. We concluded that sucrose plays an important role in RBR phosphorylation as seen earlier. On the other hand, the phosphorylation level of RBR was higher during daylight than during the night indicating that another factor such as light could also be involved in the regulation of RBR activity.

Therefore we looked for changes in RBR phosphorylation minutes after starting the light period (Figure 1B). Eight days old seedlings were grown in the absence of externally added sugar in short day condition (8h light/16h dark). As the results show in Figure 1B, RBR was phosphorylated very quickly after light went on (5 minutes), and it increased for 1 hour when it was stabilized at a high level. That indicates that RBR is phosphorylated in a light dependent manner. In agreement, we have seen that photoreceptor Phytochrome A could bind to RBR in seedlings grown in light (our data not shown - unpublished). When this experiment

was carried out in the dark where seedlings were immersed in liquid medium supplemented with 2% sucrose we saw increased RBR phosphorylation but at later time points (after 1 hour and 4 hours) indicating that light and not sugar is responsible for the observed fast RBR phosphorylation (Figure 1C). Then we inhibited ATP-generating light reactions of photosynthesis by adding DCMU into the liquid medium that resulted in the complete inhibition of RBR phosphorylation (Figure 1D).

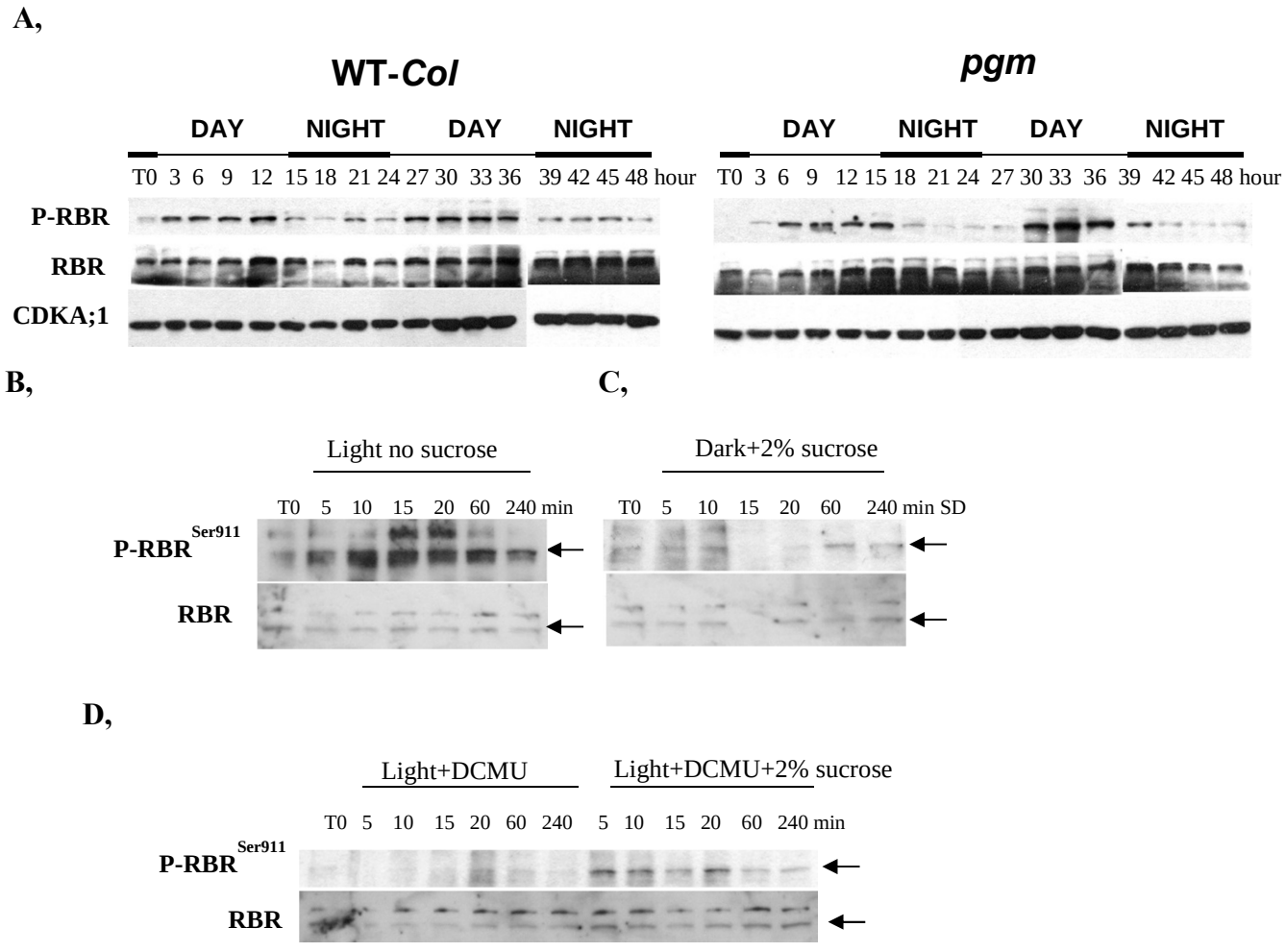


Figure 1. RBR protein is phosphorylated in sucrose and light dependent manner. (A) Ten days old seedlings were grown on ½ strength growth medium in a 12 h light/12 h dark cycle. Samples were taken at every 3 hours for two days. Western blot was done with the indicated specific antibodies. (B) Seedlings were grown in short day conditions for 8days in the absence of sucrose. Samples were taken at the indicated time point minutes (min) after the light went on, and P-RBR and RBR levels were detected by specific antibodies in a protein immune blot assay. (C) Seedlings were maintained in the dark and samples were taken at the indicated time points. (D) Seedlings were treated with DCMU (20µM) for one hour before T0 or transferred into sucrose (2%) containing medium combined with DCMU. Arrows indicate the specific protein bands.

When this experiment was repeated in the presence of 2% sucrose RBR was found to be rapidly phosphorylating again similarly to the light experiment (Figure 1B). Previously it was shown that DCMU activates SnRK1 kinase, which can be inhibited by stimulating glycolysis through the external addition of sucrose. All together these data indicates that RBR can function as a sugar and energy sensor to make a signal from sugar-energy levels to genes involved in the regulation of growth and cell proliferation; so SnRK1 might be involved in the regulatation of RBR function in energy low situations.

2. AKIN10 interacts with RBR but against expectations they form a complex in non-stressed and nutrient rich conditions.

How plant SnRK1 can regulate RBR functions? As AKIN10 is a kinase first we were interested in whether AKIN10 was able to phosphorylate RBR since RBR is exquisitely regulated by multiple phosphorylation events. For this purpose we collaborate with the Mészáros group at Semmelweis University in Budapest, as they optimized a wheat germ based cell free *in vitro* translation system to produce SnRK1, RBR and E2FA-C and ABI5 as a well-known substrate for AKIN10 in sufficient quantities. Although ABI5 was efficiently phosphorylated by the AKIN10 kinase, neither RBR nor E2F proteins were observed to be phosphorylated *in vitro* by AKIN10 (data not shown). In mouse the AMPK kinase phosphorylates the Rb protein, but specifically in the brain. It was suggested that AMPK plays a developmental role in the brain by regulating cell proliferation and differentiation most likely through Rb. Inhibiting the function of SnRK1 in plants significantly repressed growth with reduced meristem size indicating that plant SnRK1 could also play a regulatory role in meristem maintenance. In RBR there are around 20 phosphorylation sites that can be predicted to be phosphorylated, and with mass spectrometry (MS) we verified 14 of those phospho-sites by pull downs of RBR through GFP tag (our unpublished result). Two of these RBR phosphorylation sites (S712, S911) are sucrose regulated. Interestingly, AMPK1 phosphorylates a serine residue close to the 911 site and that site is present in the plant RBR. Currently we are purifying AKIN10 from seedlings through the GFP tag by using anti-GFP antibodies and *in vitro* kinase assays are going to be carried out by using in vitro purified RBR protein as substrate.

Parallel we have studied the interaction between RBR and AKIN10 by using the translational GFP-fused version of AKIN10 (pAKIN10:gAKIN10-YFP) generated earlier in the Koncz lab.

A week old *Arabidopsis* seedlings grown in long day conditions (16h light/8h dark) were transferred to liquid medium supplemented with either sucrose (1%) or without (0%) exactly 1 hour after the light period has started. They were incubated for another 12 hours. There was a weak but specific interaction between AKIN10 and RBR since the GFP alone was unable to precipitate any detectable amount of RBR protein from the control constitutive GFP-expressing line (p35S:GFP - Figure 2). Interestingly, the interaction was the strongest in the T0 sample, where the seedlings were grown on solid agar surface in the presence of 1% sucrose. These data indicated a weak interaction between AKIN10 and RBR proteins: this complex is very unstable and stress conditions, such as hypoxia might inhibit the complex formation.

In a separate experiment we further analysed the interaction between AKIN10 and RBR; again we used transgenic AKIN10-GFP expressing *Arabidopsis* seedlings grown under short day conditions (8h light/16hdark) for ten days in the presence of 1% sucrose. When the light period was started seedlings were transferred into liquid medium not supplemented with sucrose (nutrient limited condition) or treated with TOR-kinase inhibitor AZD for another three hours. Earlier we have shown that removing the externally added sucrose efficiently inhibits cell proliferation. Non-treated seedlings grown on agar surface in the presence of sucrose (1%) were also incubated for three more hours in light as a control. GFP expressing seedlings were used to determine specific interactions with AKIN10. Protein complexes were immunopurified using anti-GFP antibodies coupled to with very small magnetic beads (MACS® Technology, Miltenyi) digested in column with trypsin, and analyzed in a single run on the mass spectrometer. The results are summarized in Table 1. Accordingly, plant SnRK1 can be present in distinct complexes. As expected AKIN10 was found in complex with ß and γ subunits of the heterotrimeric kinase complex, however they were not equally represented. For example, in contrast to KINß1 and ß2, KINß3 was hardly detectable in complex with AKIN10 in this experimental system. As expected, nutrient limited conditions stimulate the SnRK1 complex formation, which was further stabilized when the TOR kinase was simultaneously inhibited (Table 1). Interestingly, members of the class II trehalose phosphate synthase (TPS) family, TPS5, TPS7 and TPS10 were also found to be associated with AKIN10, and the nutrient limited condition further accelerated the binding of TPS7 and TPS10 to AKIN10. Class II TPSs (AtTPS5-11) have a synthase and a phosphatase domain, but the active sites are less well-conserved compared with class I TPS (AtTPS1-4) and they lack both synthase (TPS) and phosphatase (TPP) activity. It was suggested that these TPSs

might rather have a regulatory function than an enzymatic one, and our data further support this hypothesis.

RBR was also found in complex with AKIN10, however it's binding was inhibited in nutrient limited conditions (or hypoxia), and it was further repressed when the TOR kinase was inactivated by AZD treatment. Interestingly, in our experiment the Eukryotic release factor (eRF1-2) showed a similar AKIN10 binding pattern with RBR. Recently it was shown that eRF1-2 participates in glucose signalling, which can link this regulator to the SnRK1 pathway.

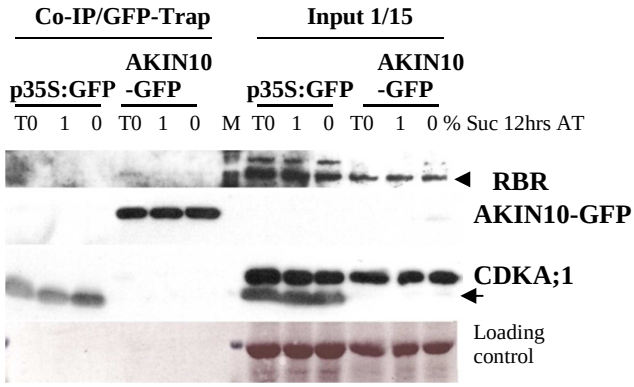


Figure 2. AKIN10-GFP interacts with RBR protein. GFP-containing proteins were immunoprecipitated from AKIN10-GFP or GFP expressing transgenic *Arabidopsis* lines after 7days grown in long day conditions in the presence of 1% externally added sucrose and samples were collected one hour after the light turned on (T0) or seedlings were transferred into liquid medium in the presence (1%) or absence (0%) of sucrose and were incubated for another 12 hours as indicated. The co-precipitated (Co-IP) proteins were analysed by using specific antibodies against RBR, CDKA;1 or GFP as indicated. Arrowhead marks RBR, arrow indicates GFP protein.

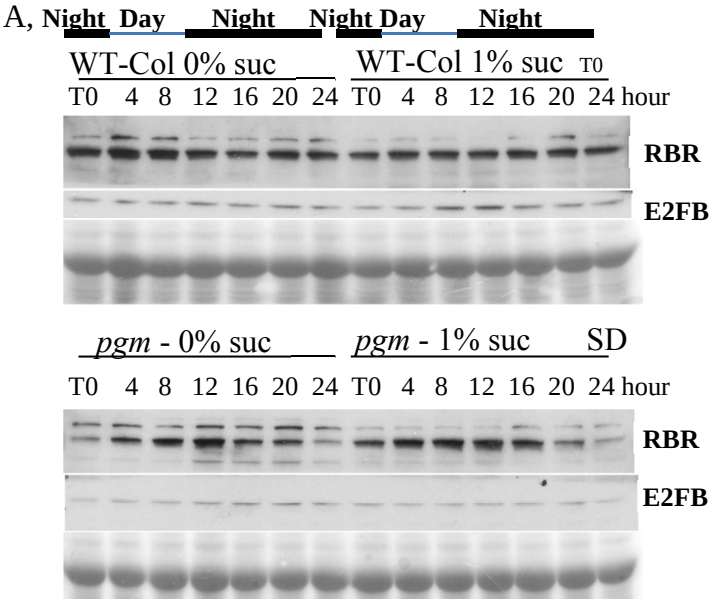
We suggest that the association of RBR with AKIN10 represents a new type of SnRK1 complex with proposed cell proliferation and/or developmental function. Our data indicates that this complex functions in normal, non-stressed conditions. Repressing the SnRK1 function in *Arabidopsis* by silencing SNF4 resulted in growth arrested seedlings even though they were grown in the presence of external sucrose and under continuous light conditions (data not shown). It indicates that plant SnRK1 might also have a non-metabolic function as it was previously demonstrated in animals.

Identified interactors of AKIN10		Peptide count of pull downs by GFP antibody		
		Control	Sucrose-free	Sucrose-free+ AZD
AKIN10	At3g01090	287	491	611
SNF4	At1g09020	151	178	195
KINß1	At5g21170	12	28	38
KINß2	At4g16360	47	50	55
KINß3	At2g28060	3	4	7
TPS7	At1g60410	28	77	92
TPS10	At1g60140	25	60	54
TPS5	At4g17770	6	6	7
RBR	At3g12280	35	12	8
ERF1-2	At1g12920	7	2	0
AGO1	At1g48410	1	10	4
RGGA	At4g16830	0	13	7

Table 1. Components of *Arabidopsis* SnRK complexes from seedling at early developmental stage. Immunopurified proteins were analysed by LC-MS/MS (see details in Kobayashi et al, 2015). Control represents 10 days old AKIN10-GFP expressing seedlings grown on 1% sucrose in SD light/dark conditions and harvested three hours after the morning light came on. At dawn seedlings were transferred into liquid medium in the absence of externally added sucrose or in the presence of TOR-kinase inhibitor AZD and they were incubated for three more hours. Numbers indicate the peptide count for the respective proteins. None of these proteins were identified during the analysis of GFP-expressing control plants.

3. The RBR protein level is regulated by AKIN10

According to our starting hypothesis AKIN10 might regulate the stability of RBR and E2F proteins. Previously it was shown that RBR protein stability was decreased in plants cells grown in nutrient limited conditions. Therefore we studied whether the RBR protein abundance was changed in starving *Arabidopsis* seedlings. Thus we utilized the starchless pgm mutant, where in the absence of starch seedlings starve during the night. We followed RBR and E2FB protein levels through a complete diurnal cycle under short day conditions in the presence (1%) or absence (0%) of external sucrose (8h light/16h dark). In the pgm mutant, the level of RBR protein peaked between 8-12 hours and fall to very low level by dawn (Figure 3A). In contrast, the RBR level did not show any fluctuation in the wild type control seedlings. Accordingly, the RBR protein abundance follows the sucrose levels.



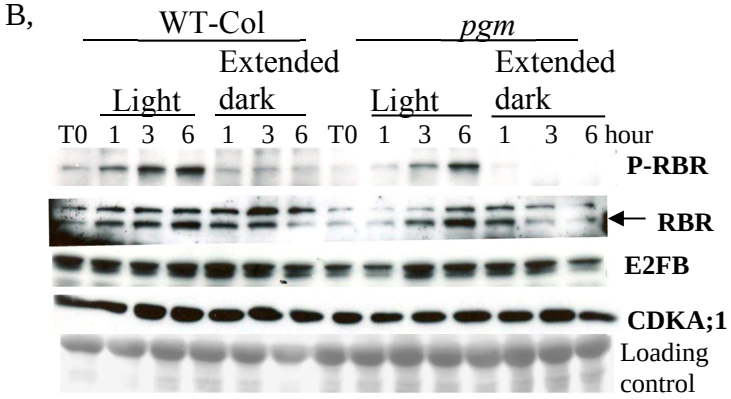


Figure 3. The RBR protein level is sensitive to carbon starvation. (A) RBR and E2FB protein levels were monitored during a complete day-night cycle in WT-Col and *pgm* mutant seedlings grown in short day conditions in the presence or absence of external sucrose (1% or 0% respectively) by using immunoblot assays. Samples were taken at the indicated time points (hour). (B) WT Col and *pgm* mutant seedlings were subjected to extended night and samples were taken at the indicated time points (hour). Control samples were also taken after the light period was started as indicated. Western blot was carried out by using specific antibodies as indicated. Arrow indicates specific RBR protein band.

When pgm seedlings were grown in the presence of 1% sucrose the RBR protein level was more stable and comparable with the wild type RBR level (Figure 3A). Thus we concluded that in young developing seedlings the RBR protein level is negatively influenced by sucrose starvation.

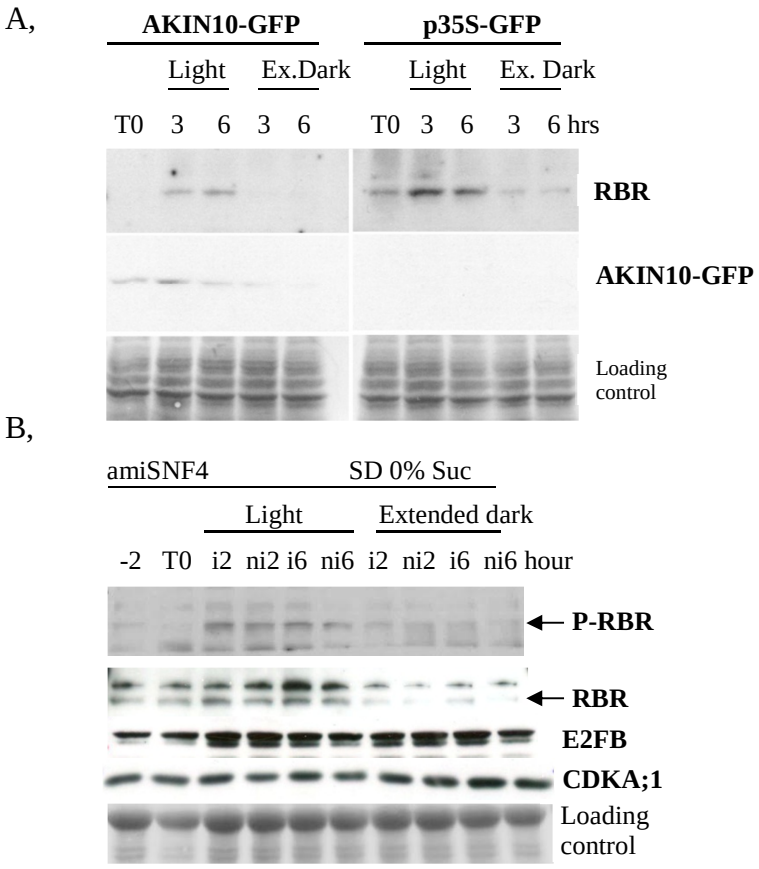


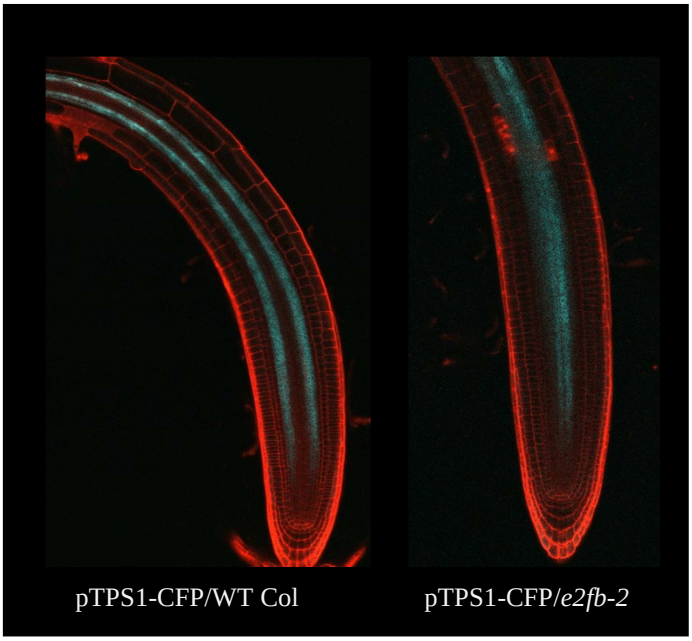
Figure 4. AKIN10 could negatively influence the RBR protein accumulation. (A) The RBR protein level was followed by western blot using a specific antibody against RBR. Samples were taken from AKIN10-GFP or p35S-GFP expressing seedlings (6days after germination) three and six hours (hrs) after morning (Light) or from extended dark (Ex. Dark). Plants were grown in short day (SD) conditions. (B) RBR protein abundance was followed in amiSNF4 seedlings in the presence or absence of an inducer (i or ni, respectively; 10µM ß estradiol). Seedlings were grown on SD and samples were taken 2 hours before the light period has started (-2) or when it just started (T0), 2, 6 hours into the light or 2, 6 hours in the extended dark period. Immunoblots were done with

specific antibodies as indicated. Ponceau staining membrane was used as loading control. Arrow indicates the specific protein bands.

During extended night the RBR level was also decreased to a very low level even in the wild type further supporting that the RBR protein is sensitive to carbon starvation (Figure 3B). In contrast to the RBR protein, the activator transcription factor E2FB was much less sensitive to the nutrient levels (Figure 3). We also followed the RBR protein level in the AKIN10-GFP expressing seedlings noticing a generally weaker RBR protein signal than in the similarly aged control line (Figure 4A). When the night was extended the RBR protein level was completely diminished in the AKIN10-GFP seedlings in comparison to the GFP expressing line indicating that AKIN10 could stimulate the degradation of RBR protein (Figure 3B). Than we utilized the conditional amiSNF4 line previously established in the afore-mentioned Koncz laboratory. Although we have seen a slight increase in the RBR amount after an extended night as an effect of SNF4 silencing, generally the RBR protein level was not significantly influenced by the induction, which could indicate that the observed decrease in the RBR protein accumulation level (especially during extended night) was not caused by the SnRK1 action. However, it turned out that the amiSNF4 line was partially silenced (personal discussion with Koncz) so further studies are needed to make the final conclusion.

4. Trehalose phosphate synthase 1 (TPS1) promoter activity is influenced by E2FB and SnRK1

A,



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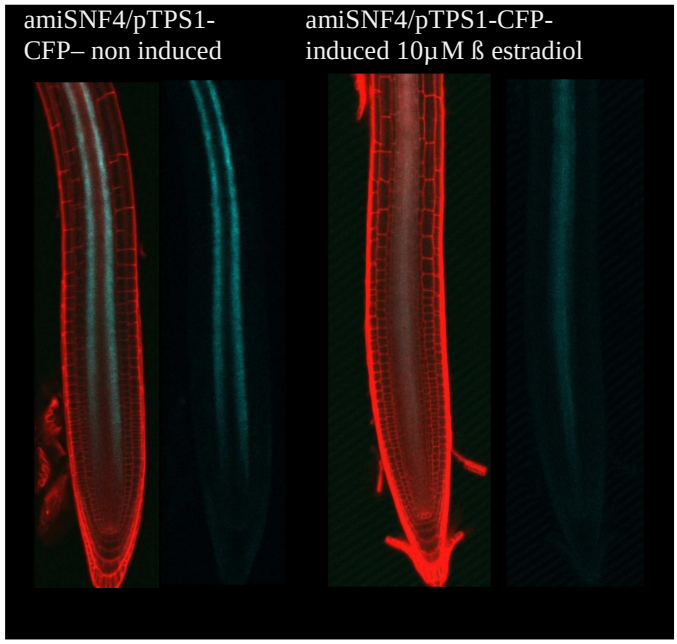

Figure 5. TPS1 promoter (pTPS1) activity could be regulated by E2FB and SnRK1. (A) TPS1 promoter-CFP (pTPS1-CFP) contract was generated and transformed in *Arabidopsis* and then crossed into *e2fb-2*, a T-DNA insertion line for E2FB (A) or in the inducible amiSNF4 line (B). Seedlings were grown on vertical plates in the presence of sucrose (1%) and in continuous light. In the case of the amiSNF4 line seedlings were grown either in the presence of 10µM ß-estradiol or in the absence of the inducer right from the beginning. Primary roots were analysed 6 days after germination. Confocal microscopy images of primary roots. CFP signal (blue) was counterstained for cell wall with propidium iodide (red).

TPS1 regulates the synthesis of trehalose-6 phosphate (T6P), an important signalling metabolite, which functions as a sensitive mediator of sucrose levels. It is suggested that T6P inhibits SnRK1 and SnRK1 represses TPS1. On the other hand, TPS1 expression was found to be up-regulated in *Arabidopsis* plants with E2F overexpression. We have suggested that the TPS1 gene could be a direct E2F target as it contains an E2F element in its first intron. A TPS1 promoter-CFP construct was made and transgenic *Arabidopsis* plants were generated in WT control in other E2F and SnRK1 transgenic lines (see Figure 5 and the summarized list of transgenic lines in Table 2). The pTPS1-CFP signal was the strongest in the vasculature of the primary root in the WT-Col, specifically in the phloem where sugar is transported, but it disappeared completely in the proximal root meristem. This pattern was changed in the T-DNA insertion *e2fb* mutant line indicating that E2FB could regulate the tissue specific expression of TPS1 (Figure 1A). Surprisingly, the pTPS-CFP signal was much weaker and a lot more diffuse in the amiSNF4 line after continuous induction of the microRNA specifically silencing the SNF4 subunit of SnRK1 in comparison to the non-induced control (Figure 5B). That indicates that SnRK1 could control the TPS1 expression, but against the expectation it is not always repression. We also generated an E2F-binding site mutant TPS1 promoter via site directed mutagenesis, and a reporter construct with CFP has already been transformed in various genetic backgrounds (see the list in Table 2).

A,

Crosses we have carried ou during this work
1.pgm X pRBR:gRBR-GFP
2.pgm X pE2FB:gE2FB-GFP
3.pgm X e2fb-2 T-DNA insertion line.
4.pgm X pAKIN10:gAKIN10-YFP
5.pgm X amiSnf4
6.pCyCD;1:gCyCD2;1-GFP X amiSnf4
7.pRBR:gRBR-GFP X amiSnf4
8.pAKIN10:gAKIN10 X amiSnf4
9.e2fb-2 X pE2FB:gE2FB-GFP

B,

Transgenic plants generated during this work
1.pTPS1-CFP/WT-Col
2.pTPS1-CFP/ pE2FB:gE2FB-GFP strong expressing line
3.pTPS1-CFP/ pE2FB:gE2FB-GFP weak expressing line
4.pTPS1-CFP/ e2fb-2 T-DNA insertion line.
5.pTPS1-CFP/ pE2FA:gE2FA-GFP strong expressing line
6.pTPS1-CFP/ pE2FA:gE2FA-GFP weak expressing line
7.pTPS1-CFP/ e2fa-1 T-DNA insertion line.
8.pTPS1-CFP/ amiSnf4
9.pTPS1-CFP/ pAKIN10:gAKIN10-YFP
11.p^{mE2F}TPS1-CFP /WT-Col
12.p^{mE2F}TPS1-CFP / pE2FB:gE2FB-GFP strong expressing line
13.p^{mE2F}TPS1-CFP / pE2FB:gE2FB-GFP weak expressing line
14.p^{mE2F}TPS1-CFP / e2fb-2 T-DNA insertion line.
15.p^{mE2F}TPS1-CFP / pE2FA:gE2FA-GFP strong expressing line
16.p^{mE2F}TPS1-CFP / pE2FA:gE2FA-GFP weak expressing line
17.p^{mE2F}TPS1-CFP / e2fa-1 T-DNA insertion line.
18.p^{mE2F}TPS1-CFP / amiSnf4
19.p^{mE2F}TPS1-CFP / pAKIN10:gAKIN10-YFP

Table 2. List of transgenic *Arabidopsis* lines generated during this work by making crosses (A) or transformations (B).

Conclusions

Based on our data together with recent development in this field RBR is in the focal point of many signalling events involving nutrients, energy and light for fine-tuning the rate of growth and proliferation. RBR is predominantly regulated on a post-translational level, mostly by phosphorylation. Previously we have shown that sucrose can stimulate RBR phosphorylation through a well-conserved cell cycle regulatory mechanism including cyclin-dependent kinases as the major player. Here we found that light can rapidly stimulate RBR phosphorylation but only in the presence of functional chloroplasts. We suggest that the energy sensor SnRK1 could be involved in this regulation but whether directly or indirectly is yet unknown. We demonstrated that SnRK1 is present in different protein complexes to regulate different processes in young developing seedlings. In nutrient limited conditions we identified II class TPS proteins as integral parts of the SnRK1 complex. In contrast to the stress-related SnRK1, RBR associated with SnRK1 in non-stressed conditions. We suggest that RBR with SnRK1 could regulate normal developmental processes such as meristem maintenance. The RBR protein was sensitive to carbon and energy starvation. We suggest that AKIN10 could regulate the protein abundance of RBR. Altogether our data indicates that RBR and SnRK1 are intimately connected in many effective ways.

Publication list

Support of the OTKA grant was acknowledged in the following scientific papers:

1. Rossana Henriques, László Bögre, Beatrix Horváth and Zoltán Magyar. Balancing act: matching growth with environment by the TOR signalling pathway. Journal of Experimental Botany. 2014 65(10):2691-701.

2. László Bögre, Rossana Henriques and Zoltán Magyar. TOR tour to auxin. EMBO Journal 2013, 32, 1069-1071.

Congress presentations with acknowledgements to the OTKA financial support

1. Márta Deli, Anikó Varga, Anita Kovács and Zoltán Magyar Kinetins switch of E2FB from activator to repressor in the *Arabidopsis* root meristem. Signalling in plant development. EMBO Conference. 2015Brno 20-24 Sept 2015.

2. Tünde Leviczky, Binish Mohammed, Márta Deli, Aladár Pettkó-Szandtner, Anita Kovács, László Bögre and Zoltán Magyar. Dual functions of E2FB transcription factor during leaf development. Signalling in plant development. EMBO Conference 2015 Brno 20-24. Sept 2015.

3. Márta Deli, Csaba Koncz and Zoltán Magyar. The *Arabidopsis* retinoblastoma-related (RBR) functions in sucrose-dependent manner. Straub Napok, Szeged Biological Research Centre, 2016.