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Overexpression of a sorghum *SnRK1βγ2* gene increases the biomass in *Setaria viridis*, but not in rice

Chudamani Sharma Prakash[1], *Lihua Wang*[2,3], *Qi Shen*[2],
Jieqin Li[2,3]*, *Yi-Hong Wang*[1]*

[1]*Department of Biology, University of Louisiana at Lafayette, Lafayette, USA*
[2]*College of Agriculture, Anhui Science and Technology University, Fengyang, Anhui, P.R. China*
[3]*Anhui Province International Joint Research Center of Forage Bio-breeding, Chuzhou, P.R. China*
**Corresponding author: lijq@ahstu.edu.cn, yihong.wang@louisiana.edu*

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Abstract: Sorghum as a C_4 crop has been shown to be both drought tolerant and photosynthetically productive. In this study, we demonstrated that sorghum *SbSnRK1βγ2* (*SbSNF4-2*), the γ subunit of the sucrose non-fermenting 1 (SNF1)/SNF1-related protein kinase 1 (SnRK1) heterotrimeric complex, increased the plant height and biomass in *Setaria viridis*, a C4 relative of sorghum, but not in rice, a C3 relative, when overexpressed driven by the maize ubiquitin promoter. However, the overexpression did not increase the tiller number in *S. viridis*, although it caused modest increases in the tiller number in both sorghum and rice. In addition, *SbSnRK1βγ2* did not affect the panicle weight in sorghum, but its overexpression doubled the panicle weight in *S. viridis* in all four evaluated transgenic lines. Overall, the overexpression of *SbSnRK1βγ2* tripled the biomass production in *S. viridis*, indicating *SbSnRK1βγ2*'s potential in any future cellulosic biofuel production and *S. viridis*' utility as an alternative genetic vehicle to functionally characterise sorghum genes.

Keywords: plant height; *SbSNF4-2*; *Sorghum bicolor*; tiller number

Plant height is among the most important biomass yield components and is regulated by hormones, such as brassinosteroids and gibberellins, in addition to unknown factors in the mapped plant height quantitative trait loci (QTLs) (Salas Fernandez et al. 2009). Those QTLs tend to be pleiotropic because the plant height is correlated with other traits mapped to the same locus (Salas Fernandez et al. 2009). For example, the plant height is highly correlated with the biomass in sorghum ($r = 0.58$) and the two QTLs were colocalised in our previous study (Upadhyaya et al. 2022). In that study, we cloned a sorghum gene (*SbSNF4-2*; *SbSnRK1βγ2* hereafter to follow the nomenclature of Broeckx et al. 2016) from the colocalised height/biomass QTL and the cloned gene encodes a γ subunit of the evolutionarily conserved AMP (adenosine monophosphate)-activated protein kinase (AMPK)/sucrose non-fermenting 1 (SNF1)/SNF1-related protein kinase 1 (SnRK1) heterotrimeric complexes (Upadhyaya et al. 2022).

Setaria viridis is the weedy relative of *S. italica* (a grain crop) with many attributes suitable as a genetic tool to study C4 photosynthesis (Li & Brutnell 2011). Using the A10.1 genotype, our lab achieved a transformation efficiency of 21.2–35.4% (Prakash et al. 2024). However, the transformation efficiency can reach over 90% with the ME034V genotype (Weiss et al. 2020). The high transformation efficiency with a sequenced small genome and fast growth also makes *S. viridis* a good alternative system to study the gene function of sorghum.

In this paper, our goal was to study the feasibility of *S. viridis* as a model system to functionally characterise sorghum genes. We transformed both

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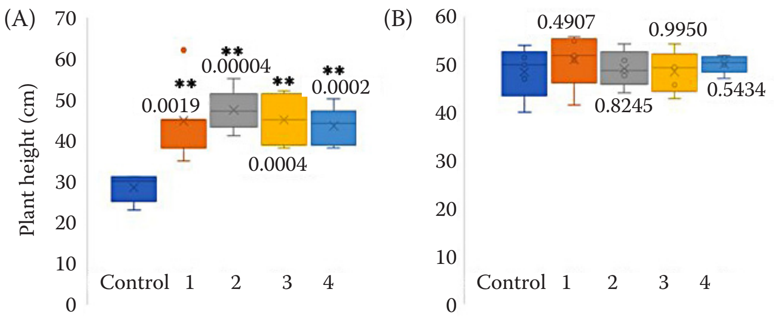

Figure 1. Plant height of transgenic *Setaria viridis* (A) and rice (B) overexpressing *SbSnRK1βγ2* compared to the control

For each box plot, the box edges represent the upper and lower quantile with the median value shown as line inside the box; 1, 2, 3, 4, indicate transgenic lines No. 1, 2, 3, and 4; **significant at $P = 0.01$

rice and *S. viridis* with the same *SbSnRK1βγ2* construct (Figure S1 in the Electronic Supplementary Material (ESM)) to evaluate the two plants' phenotypic response to the overexpression. The transgenic lines were confirmed by polymerase chain reaction (PCR) with two primers designed from the hygromycin B phosphotransferase gene (hygF: TGTAGTGTATTGACCGATTCCTTGC and hygR: GTTCGACAGCGTCTCCGACCTGAT). We used rice as a control because both *S. viridis* and sorghum are C4 plants and more closely related with each other than with rice, a C3 plant. We found that although the transgenic *S. viridis* lines showed the same increase in plant height as in sorghum and sugarcane described in Upadhyaya et al. (2022), the transgenic rice lines did not increase the plant height.

While the five control plants averaged 28.4 (ranging from 23~31) cm in height, the four transgenic lines (1, 2, 3, and 4) averaged 44.7 (35~62), 47.3 (41~55), 45 (38~52), and 43.5 (38~50) cm, respectively (Figure 1A). These corresponded to a 57.4, 66.5, 58.5, and 53.2% increase over the control, respectively, and were all significant at the $P = 0.01$ level (Figure 1A). In contrast, the four rice transgenic lines overexpressing *SbSnRK1βγ2* were similar in height to the control (Figure 1B). A representative transgenic *S. viridis* and the control plants are shown in Figure S2 in the ESM.

Transgenic *S. viridis* overexpressing *SbSnRK1βγ2* showed a similar tiller number to the control (Figure 2A) although the sorghum plants overexpressing *SbSnRK1βγ2* showed a modest increase in the tiller number (Upadhyaya et al. 2022). The transgenic rice plants also showed a modest increase in the tiller number (Figure 2B).

The primary panicles of the control and four transgenic lines averaged 0.0964 (0.053~0.117), 0.164 (0.115~ 0.256), 0.13 (0.110~0.198), 0.15 (0.089~0.229), and 0.172 (0.124~0.254) g/panicle, respectively (Figure 3A). The total panicle weight increased over the control more significantly (Figure 3B). In this case, the control and four transgenic lines averaged 1.036 (0.73~1.28), 2.394 (1.38~ 3.57), 2.342 (1.88~3.01), 1.927 (1.52~2.36), and 2.29 (1.86~2.95) g of panicles per plant, respectively (Figure 3B).

Similar to the total panicle weight above, the total dry weight (biomass) of the four transgenic lines also increased significantly over the control (Figure 3C). The five control plants averaged 1.618 (1.24~1.93) g of biomass, but the four transgenic lines averaged 5.829 (3.71~9.24), 5.778 (4.51~6.9), 5.047 (4.4~5.85), and 5.863 (4.84~7.18) g per plant, respectively. The transgenic plants produced 212~262% more dry biomass than the control.

In this study, we demonstrated that *S. viridis* can recapture most of the overexpression phenotypes in sorghum and therefore is a good alternative system to functionally characterise sorghum genes as it is very challenging to perform genetic transformation in sorghum (Raghuwanshi & Birch 2010). *SbSnRK1βγ2* was identified through association

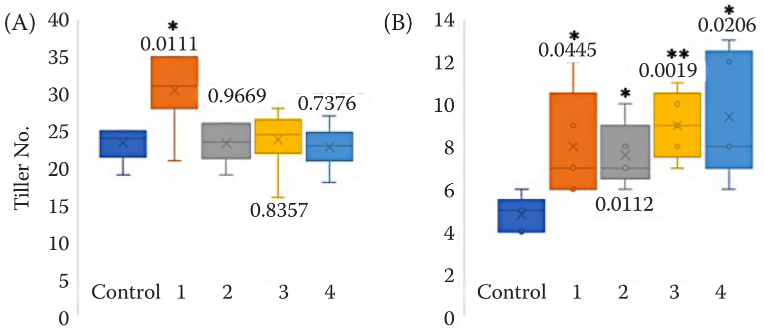

Figure 2. Tiller number of transgenic *Setaria viridis* (A) and rice (B) overexpressing *SbSnRK1βγ2* compared to the control

For each box plot, the box edges represent the upper and lower quantile with the median value shown as line inside the box; 1, 2, 3, 4, indicate transgenic lines No. 1, 2, 3, and 4; *,**significant at $P = 0.05$ and 0.01, respectively

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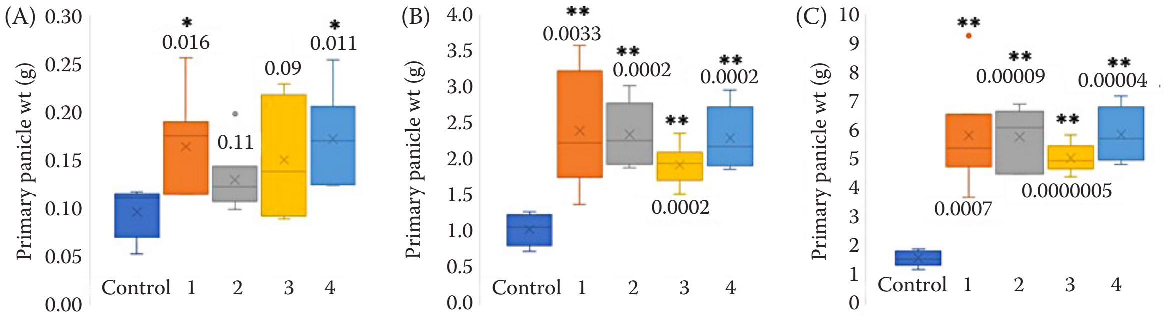

Figure 3. Performance of the transgenic *Setaria viridis* lines overexpressing *SbSnRK1βγ2* over the control: primary panicle weight (g/panicle) (A), total panicle weight (g/plant) (B), total dry weight (g/plant) (C)

For each box plot, the box edges represent the upper and lower quantile with the median value shown as line inside the box; 1, 2, 3, 4, indicate transgenic lines No. 1, 2, 3, and 4; *,**significant at $P = 0.05$ and 0.01, respectively

mapping (Upadhyaya et al. 2022) and the function of its paralogue in sorghum (*SbSNF4-1*; *SbSnRK1βγ1*; Sobic.001G005600) remains to be determined. Both *SbSnRK1βγ1* and *SbSnRK1βγ2* and their Arabidopsis orthologue *AtSNF4* (AT1G09020) contain four cystathionine β-synthetase (CBS) domains preceded by one carbohydrate-binding module (CBM) which functions as a positive regulator of the kinase activity (Ruiz-Gayosso et al. 2018). However, no other plant *SnRK1βγ* orthologues have been reported to affect the biomass. For example, in maize overexpressing *SnRK1βγ1* (*ZmAKINβγ1* or *ZmSnRK1βγ1* or *Zm00001d034896*) did not change either the shoot or root biomass weight (Li et al. 2023).

While the *SbSnRK1βγ2* overexpression increases the biomass in both sorghum (Upadhyaya et al. 2022) and *S. viridis* (Figure 3C), its effect on the rice biomass is very modest (Li & Wang, unpublished). This is because it failed to increase the plant height (Figure 1B) and the modest increase is due to the increased tiller number (Figure 2B). This phenomenon of variable expression across species is not frequently reported in the literature, but it does exist. For example, the overexpression of *OsDREB1C* driven by the 35S promoter increases the grain yield by 7.8–55.7% in rice, 17.2–23.5% in wheat, and the biomass yield by 14.2 to 35.8% in Arabidopsis (Wei et al. 2022). However, overexpressing this gene fails to increase the kernel yield in maize (Wenbin Zhou, unpublished). Further studies are needed to understand this phenomenon.

In conclusion, we have recaptured most of the *SbSnRK1βγ2* overexpression phenotypes in *S. viridis*, but not in rice, overexpressing *SbSnRK1βγ2* increases the biomass in the grasses through the increased plant height and tiller number. The fact that the overexpression of this gene fails to increase the plant height in rice warrants further investigation. One of the biotechnological applications of this gene will be to enhance the biomass yield in cellulosic biofuel production using the grasses with *SbSnRK1βγ2* or its homologues.

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