Article type: Breakthrough Report

Free Access

Received: Aug. 8, 2019; Accepted Aug. 17, 2019

Edited by Zhizhong Gong, China Agricultural University, China

Running title: Synthetic microProteins in rice

Control of flowering in rice through synthetic microProteins

Tenai Eguen[1,2], Jorge Gomez Ariza[3], Vittoria Brambilla[3], Bin Sun[1,2], Kaushal Kumar Bhati[1,2], Fabio Fornara[3] and Stephan Wenkel[1,2]*

1. Copenhagen Plant Science Centre, University of Copenhagen, Denmark
2. Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 4Au0, 1871 Frederiksberg C, Denmark
3. Department of Biosciences, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

*Correspondence: Stephan Wenkel (wenkel@plen.ku.dk)

Summary

Photoperiod-dependent flowering in rice is regulated by HEADING DATE 1 (Hd1) which acts as both an activator and repressor of flowering in a daylength-dependent manner. To investigate the use of microProteins as a tool to modify rice sensitivity to the photoperiod, we designed a synthetic Hd1 microProtein (Hd1miP) capable of interacting with Hd1 protein, and overexpressed it in rice. Transgenic *OX-Hd1miP* plants flowered significantly earlier than wild type plants when grown in non-inductive long day conditions.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jipb.12865.

Our results show the potential of microProteins to serve as powerful tools for modulating crop traits and unraveling protein function.

INTRODUCTION

The timing of the transition from vegetative to reproductive growth in plants is essential to their reproductive success (Turck et al. 2008; Song et al. 2015). In *Arabidopsis thaliana*, the B-box zinc finger transcription factor CONSTANS (CO) positively regulates the florigen gene *FLOWERING LOCUS T* (*FT*) to activate flowering in long days (Putterill et al. 1995; Suárez-López et al. 2001; Brambilla and Fornara 2017; Shim et al. 2017). Flowering in rice, a facultative short day plant is modulated by the CO homolog HEADING DATE 1 (Hd1), which regulates the expression of the florigens *HEADING DATE 3A* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1)* in short day (SD) and long day conditions (LD) (Yano et al. 2000; Kojima et al. 2002; Hayama et al. 2003; Doi et al. 2004; Corbesier et al. 2007; Tamaki et al. 2007; Komiya et al. 2008; Shrestha et al. 2014; Zhang et al. 2017; Gnesutta et al. 2018). B-box proteins are a conserved group of proteins that are involved in growth and development, they contain highly conserved B-box domains that mediate protein-protein interactions within and outside the B-box protein family (Gangappa and Botto 2014). In *Arabidopsis*, two B-box containing microProteins, microProtein1a (miP1a) and microProtein1b (miP1b), are also involved the regulation of CO to control flowering time (Graeff et al. 2016). MicroProteins are small proteins that interact with larger proteins to modulate protein activity (Eguen et al. 2015). Phylogenetic analysis have shown however that miP1a/b-type microProteins are specific to the dicotyledonous

lineage of plants and are not present in monocots (Graeff et al. 2016). To explore the possibility of using synthetic microProteins to change the cycle length of cereal crops we created a synthetic Hd1 microProtein to interfere with the endogenous function of CO homolog, Hd1, in rice. To achieve this, we used the B-Box domains of Hd1 and expressed it as an individual FLAG-tagged fusion protein in transgenic rice plants. The synthetic Hd1miP microProtein is thus similar to miP1a/b type microProteins that naturally exist in dicotyledonous plants but it is also special since it lacks the TOPLESS-interaction motif (Graeff et al. 2016).

RESULTS AND DISCUSSION

In order to test Hd1miP effect in plants, the *Hd1* and *Hd1miP* forms (Figure 1A) were overexpressed in WT Nipponbare plants. The protein expression of OX-*Hd1miP* and *OX-Hd1* was measured and the three overexpressors of *Hd1miP* and the highest overexpressor of *OX-Hd1* were selected for further experiments (Figure 1B). To determine if a synthetic Hd1 microProtein (Hd1miP) and Hd1 protein can interact, their interaction was tested in yeast. Hd1miP fused to the GAL4 binding domain (BD) and Hd1 fused to the GAL4 activation domain (AD) were transformed into yeast. Yeast growth was observed on media lacking leucine, histidine and tryptophan (-L/-H/-W) in the presence of 10 mM 3-aminotriazole, which is evidential of Hd1 and Hd1miP interaction (Figure 1C). The interaction of Hd1 and Hd1miP was further verified *in vitro* by co-immunoprecipitation. Fusions of Hd1 to the maltose binding protein tag (MBP-Hd1) and fusions of Hd1miP to the glutathion-S-transferase tag (GST-Hd1miP) were expressed under the inducible T7

promoter in *Escherichia coli* BL21 cells. As a negative control, enhanced green fluorescent protein fused to MBP-tag (MBP-eGFP) was also expressed in *E. coli*. After cell lysis, soluble protein fractions of either GST-Hd1miP and MBP-Hd1 or GST-Hd1miP and MBP-eGFP were mixed and incubated with gluthathione sepharose 4B resin. After precipitation and washing, immune complexes were released by boiling in SDS-loading buffer and separated by SDS-PAGE. Hd1 was observed to interact with the Hd1miP whereas no interaction was observed between eGFP and Hd1miP (Figure S1). This further confirms specific interaction between Hd1 and Hd1miP.

To investigate the effect of the overexpression *Hd1miP* on flowering, the heading dates of WT, *OX-Hd1miPs*, *OX-Hd1* and *hd1-1* plants were determined in SD (10 h light/14 h dark), LD (14.5 h light /9.5 h dark) and strong long day (SLD - 16.5 h light/ 7.5 h dark) conditions. In SD, no significant difference was observed between the lines (Figure 1D). In LD, *OX-Hd1miP-1 and OX-Hd1miP-2* showed 11% and 17% earlier heading dates than WT; *hd1-1* showed 34% earlier heading date. *OX-Hd1miP-W* plants, which had the lowest Hd1miP protein expression, showed late heading dates similar to WT (Figure 1D, E). In SLD, *OX-Hd1miP-1, OX-Hd1miP-2* and *hd1-1* plants showed 34%, 32% and 49% earlier heading dates than WT respectively; *OX-Hd1miP-W* plants showed late heading dates similar to WT and *OX-Hd1* showed 34% later heading dates than WT (Figure 1D). Taken together, the results show that the overexpression of Hd1miP results in early flowering in LD and SLD and the effect on flowering time is dependent on the abundance of Hd1miP protein. The flowering phenotypes of *OX-Hd1miP-1* and *OX-Hd1miP-2* reveal a microProtein type inhibitory mechanism by which

Hd1miP can inhibit the repressor activity of Hd1 in LD thereby giving rise to earlier flowering plants.

Hd1miP may function by transcriptionally or post-translationally regulating Hd1. To determine the effects of the overexpression of *Hd1miP* on the endogenous levels of *Hd1* in LD, the expression level of *Hd1* was quantified by qPCR using primers that recognize the 3' untranslated region (3' UTR) of *Hd1*. The *Hd1* levels in *OX-Hd1miP-1* and *OX-Hd1miP-W* were similar to WT suggesting that the presence of the synthetic Hd1miP does not affect endogenous *Hd1* levels. Conversely, *OX-Hd1miP-2* lines showed endogenous *Hd1* levels that were much lower than in WT, suggesting a possible suppression of *Hd1* expression in *OX-Hd1miP-2.* No expression of *Hd1* was detected in *hd1-1* plants (Figure 1F). Attempts to correlate *Hd1* gene expression in the different *OX-Hd1miPs* lines with their corresponding flowering phenotype revealed that the regulation of *Hd1* expression levels is not the determining factor in the early flowering phenotype of *OX-Hd1miPs*. A more fitting correlation is that of the protein expression profile and the flowering phenotype which shows that the abundance of Hd1miP proteins positively correlates with the early flowering phenotype, thus, supporting the idea that Hd1 is post-translationally regulated (Figure 1B, D). To analyze the expression of the B-box region of Hd1, *Hd1 B-Box* primers were used to quantify *Hd1* levels. The levels of *Hd1* were higher in all *Hd1miP* overexpressor lines than in WT and no *Hd1* expression was observed in *hd1* mutant plants (Figure 1G).

Hd1 suppresses the expression of the florigens *Hd3a* and *RFT1* in LD. The expression profile of florigens *Hd3a* and *RFT1* was analyzed in the different lines to obtain additional information on the molecular mechanisms underlying the flowering time phenotype. The *hd1-1* plants showed higher levels of *Hd3a* than WT due to the absence of the Hd1 repression. In *OX-Hd1miP* plants, the levels of *Hd3a* were similar to WT (Figure 1H). The expression of *RFT1* was higher in all *Hd1miP* overexpressors than in WT and *hd1-1* (Figure 1I). This indicates that although *OX-Hd1miP* and *hd1-1* result in early flowering in LD, they differ in the regulation of *Hd3a* and *RFT1*. Interestingly, the regulation of *RFT1* in *OX-Hd1miP* plants in LD resembles that observed in *Hd3a* RNAi mutants in SD (Komiya et al. 2008).

Hd1 also plays a role in grain yield (Zhang et al. 2016). To determine the viability and productivity of *OX-Hd1miP* plants in LD, the panicle characteristics were measured 35 days after the first panicle emergence (Figure 1J–L; Table 1).The grain width and grain length of *OX-Hd1miPs* was similar to WT with the exception of *OX-Hd1miP-1* which showed slightly longer grains and *hd1-1* plants which showed slightly wider grains (Figure 1J, K). Measurements of the moisture content revealed *hd1-1* plants to have 160% increased moisture content, *OX-Hd1miP1* and *OX-Hd1miP-2* had 56% increased moisture content, while *OX-Hd1miP-W* showed similar moisture content to WT (Figure 1l). This suggests that Hd1 may also be involved in the control of the moisture content in rice grains but it does not have a strong effect on grain size.

The number of panicles produced by *OX-Hd1miPs*, *OX-Hd1*, *hd1-1* and WT was similar. The panicles produced were further classified based on the maturity of the panicles. Greater than 50% of the WT panicles were mature. In *hd1-1* plants, 39% of the panicles were mature while 38% and 36% were mature in *OX-Hd1miP-1* and *OX-Hd1miP-2* respectively *(*Table 1). This suggests that Hd1 levels and activity may also play a role in the maturity rate of the panicles. The delayed maturity of the grains may be due to their higher moisture content.

The *OX-Hd1miP-1* and *OX-Hd1miP-2* plants showed an average spikelet fertility of 31% and 36% which is less than the 63% spikelet fertility in the WT; *hd1-1 plants* had an average spikelet fertility of 37%. When the spikelet fertility of only mature panicles was calculated there was approximately 100% increase in spikelet fertility in both the *OX-Hd1miPs* and *hd1-1* lines compared to the 40% increase in WT. The average total number of grains produced in the *OX-Hd1miP* and *hd1-1* lines was also less than WT plants (Table 1). Hd1 seems to also affect the fertility rate and the number of grains produced irrespective of Hd1 being inhibited at a transcriptional (*hd1-1*) or at a post-translational level (*OX-Hd1miP*). Extending the harvest time beyond 35 days may significantly increase the yield of *OX-Hd1miP* and *hd1-1* plants.

Our findings demonstrate how a synthetic microProtein-based system is effective at changing flowering time in rice (Figure 2). The inhibition of the long day repression mechanism of rice might have beneficial implications in the expansion of rice cultivation in equatorial regions and areas of northern latitudes. Synthetic microProteins are proving to be a useful tool in targeting

and inhibiting proteins in a more specific manner with the reduced likelihood of an off-target effect (Bhati et al. 2018; Dolde et al. 2018). Furthermore, synthetic microProteins can also aid in deciphering what processes are dependent on the activity of the inhibited target protein. However, using microProteins as a tool involves knowing the interaction domain of the protein of interest and the ability of the microProteins to form heterodimers with the target. As is observed in OX-*Hd1miP*, microProteins as a regulatory tool can be extended to crop plants the phenotypes of which are reflective of the molecular pathways that they are involved in.

ACKNOLWLEDGEMENTS

We thank marcel Tutor Ale (DTU) for advice on measuring yield traits. The work was funded by the European Research Council (grant no. 336295) to S.W. and through Copenhagen Plant Science Centre.

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Figures

Figure 1. Synthetic *Hd1 microProtein* transgenic plants heading dates, expression profiles and yield

(**A**) Schematic diagram of *Hd1* and *Hd1miP*. (**B**) Protein expression profile of Hd1 and Hd1miP; LC-loading control. (**C**) Interaction of Hd1miP and Hd1 was tested using a yeast two-hybrid assay. Yeast growth was observed in serial dilutions on non-selective media lacking leucine and tryptophan (SD-L-W). Positive interactors showed growth on selective media lacking leucine, tryptophan, and histidine (SD-L-W-H) supplemented with 10mM 3-aminotriazole (3-AT). (**D**) The heading date counts of plants in SD, LD and SLD. Significant difference: *P≤0.05, **P≤0.01, ***P≤0.001, and **** P≤0.0001. (**E**) Picture of representative rice plants grown in LD. *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1* lines. The expression profiles of the different genes were measured by quantitative RT-PCR performed on material harvested from plants grown for 70 days in long day condition (14.5 h light/9.5 h dark). (**F, G**) The expression of *Hd1* was analyzed in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1* by using primers amplifying in the 3'UTR of *Hd1* (**F**) and the B-Box domain respectively (**G**). (**H**) The expression of *Hd3a* was analyzed in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1*. (**I**) The expression of *RFT1* was analyzed in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1*. (**J**) Grain width was measured in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1*. (**K**) Grain length was measured in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1*. (**L**)

Moisture content was measured in WT, *hd1-1*, *OX-Hd1miP-W*, *OX-Hd1miP-1, OX-Hd1miP-2* and *OX-Hd1*. The values of the grain measurements are given as the mean ± *SD*. Significant difference: **P*≤0.05, ***P*≤0.01, ****P*≤0.001, and **** *P*≤0.0001 measured.

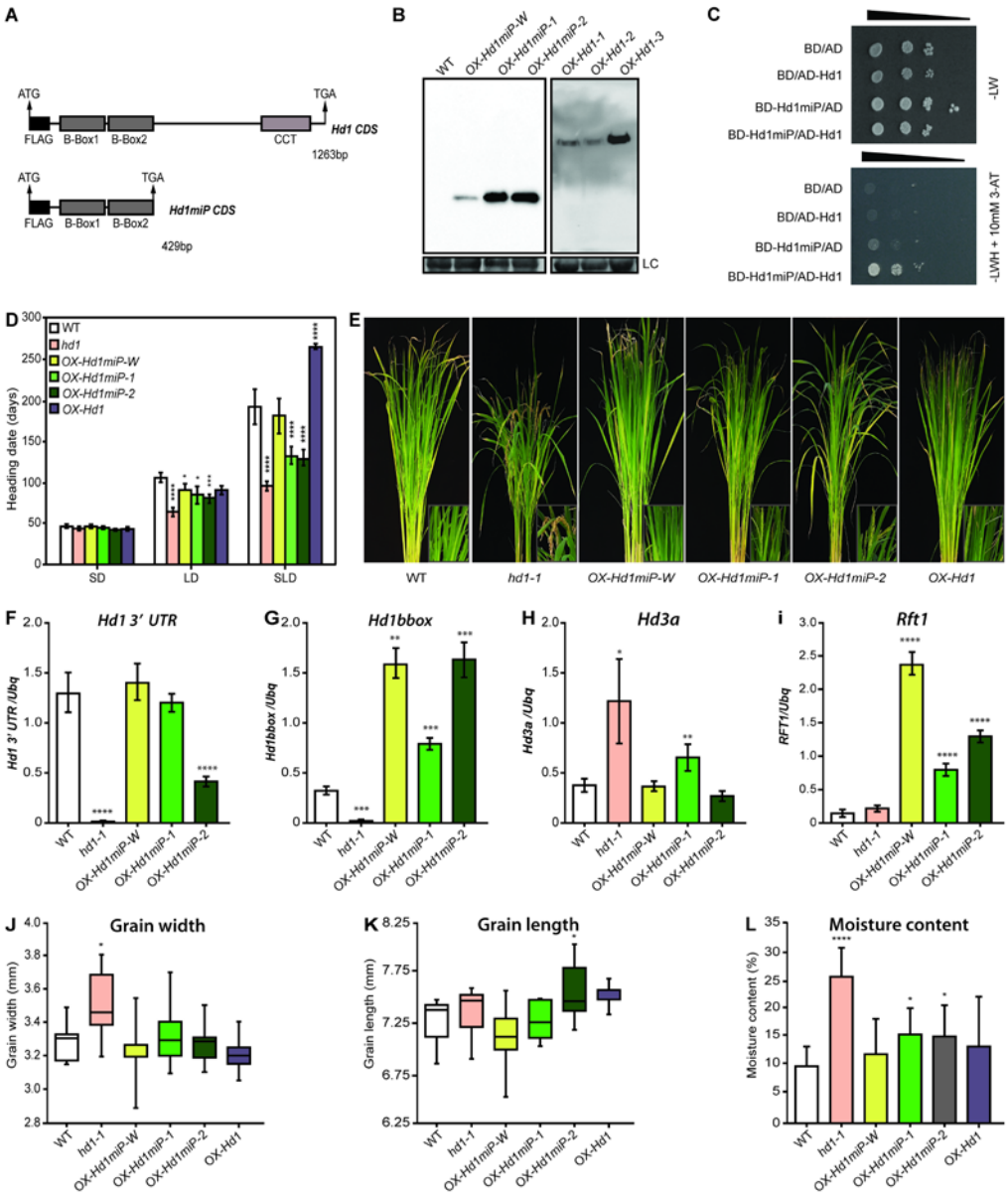

Figure 2. Hypothetical model how synthetic microProteins affect Hd1 protein activity

Left: Flowering is promoted by Hd3a and RFT1. Ehd1 induces *RFT1* regardless of photoperiod and enhances *Hd3a* in response to short photoperiod. Hd1 acts as a repressor both *Ehd1* and *Hd3a* when exposed to long photoperiods. In response to short photoperiods Hd1 activates *Hd3a*. Right: In the presence of the synthetic microProtein (Hd1miP), *Hd3a* expression is increased regardless of the photoperiod which results in early flowering in both short day (SD) and long day (LD) conditions. We also observed increased expression of *RFT1* which could be an indirect effect.

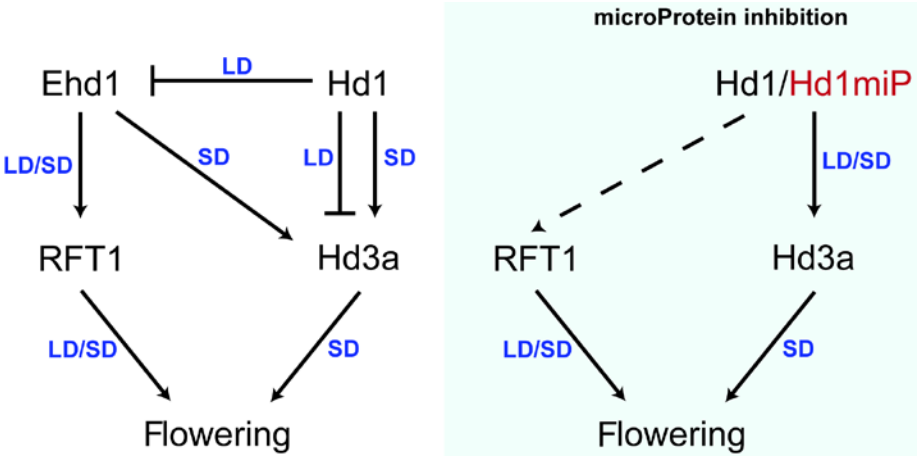

Table 1. **Qualitative analysis of panicle characteristics after 35-days**

Panicle Characteristics	WT	hd1-1	OX-Hd1miP-W	OX-Hd1miP-1	OX-Hd1miP-2	OX-Hd1
Ave. Immature Panicles/plant	4 ± 3.16	6.38 ± 3.42	4.42 ± 3.45	5.25 ± 3.02	7.13 ± 2.55	3.67 ± 4.57
Ave. Semi-Mature Panicles/plant	1.85 ± 1.72	0.88 ± 0.99	0.71 ± 0.75	1.38 ± 1.38	1.25 ± 1.22	0
Ave. Mature Panicles/plant	7 ± 1.92	3.63 ± 1.18	5.85 ± 2.34	3.25 ± 0.98	4.63 ± 1.27	8.33 ± 3.43
Ave. of total Panicles/plant	12.88 ± 3.04	10.88 ± 3.68	11 ± 2.16	9.88 ± 2.90	13 ± 2.33	12 ± 5.80
Mature panicles (%)	58.66 ± 25.35	39.28 ± 25.86	53.24 ± 25.81	37.83 ± 26.07	35.60 ± 7.53	69.44 ± 29.29
Immature Panicles (%)	28.10 ± 16.74	53.18 ± 25.49*	40.25 ± 24.87	48.72 ± 23.64	53.94 ± 10.93**	24.44 ± 29.29
Ave. Number of grains/plant	957 ± 273.16	483 ± 212.11 **	730 ± 169.79	553 ± 191.88**	686 ± 208.60*	638 ± 504.44*
Spikelet Fertility (SF)	63.25 ± 15.59	37.04 ± 14.55**	58.61 ± 18.25	30.46 ± 15.71***	36.07 ± 12.18**	56.39 ± 6.82
SF Mature panicles	88.16 ± 5.47	76.08 ± 8.83**	86.52 ± 9.03	58.39 ± 25.40**	76.83 ± 8.64**	70.57± 18.71

The data was expressed as the mean ± *SD* (n = 8 plants). *Represents significant difference compared to WT (Student's *t*-test *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$).