

ARTICLE ADDENDUM

## A novel trimeric complex in plant cells that contributes to the lamina inclination of rice

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### ABSTRACT

The architecture determining grain production in rice is mainly affected by factors such as lamina angle, tillering, plant height and panicle morphology. In particular, leaf angle, the degree of bending between the leaf blade and leaf sheath directly affects crop architecture and grain yields. Balancing activities between the 2 antagonistic groups of proteins, atypical helix-loop-helix (HLH) and basic HLH (bHLH) proteins have been regarded as one of the major molecular machineries regulating lamina angles through the control of cell elongation in the lamina joint of rice. Recently, formation of a complex consisting of atypical HLH protein, OsBUL1, a KxDL motif-containing protein, LO9-177 and a bHLH protein, OsBC1 has been reported to play a positive role in lamina inclination of rice unraveling a novel layer of cell elongation control in rice lamina joint. Here, we demonstrate a trimeric complex formation in rice cells by a combination of BiFC and FRET-FLIM assays.

### ARTICLE HISTORY

Received 23 November 2016  
Revised 13 December 2016  
Accepted 13 December 2016

### KEYWORDS

Bimolecular fluorescence complementation (BiFC); fluorescence resonance energy transfer (FRET)-fluorescence lifetime imaging (FLIM); leaf angle; rice architecture; trimeric protein complex

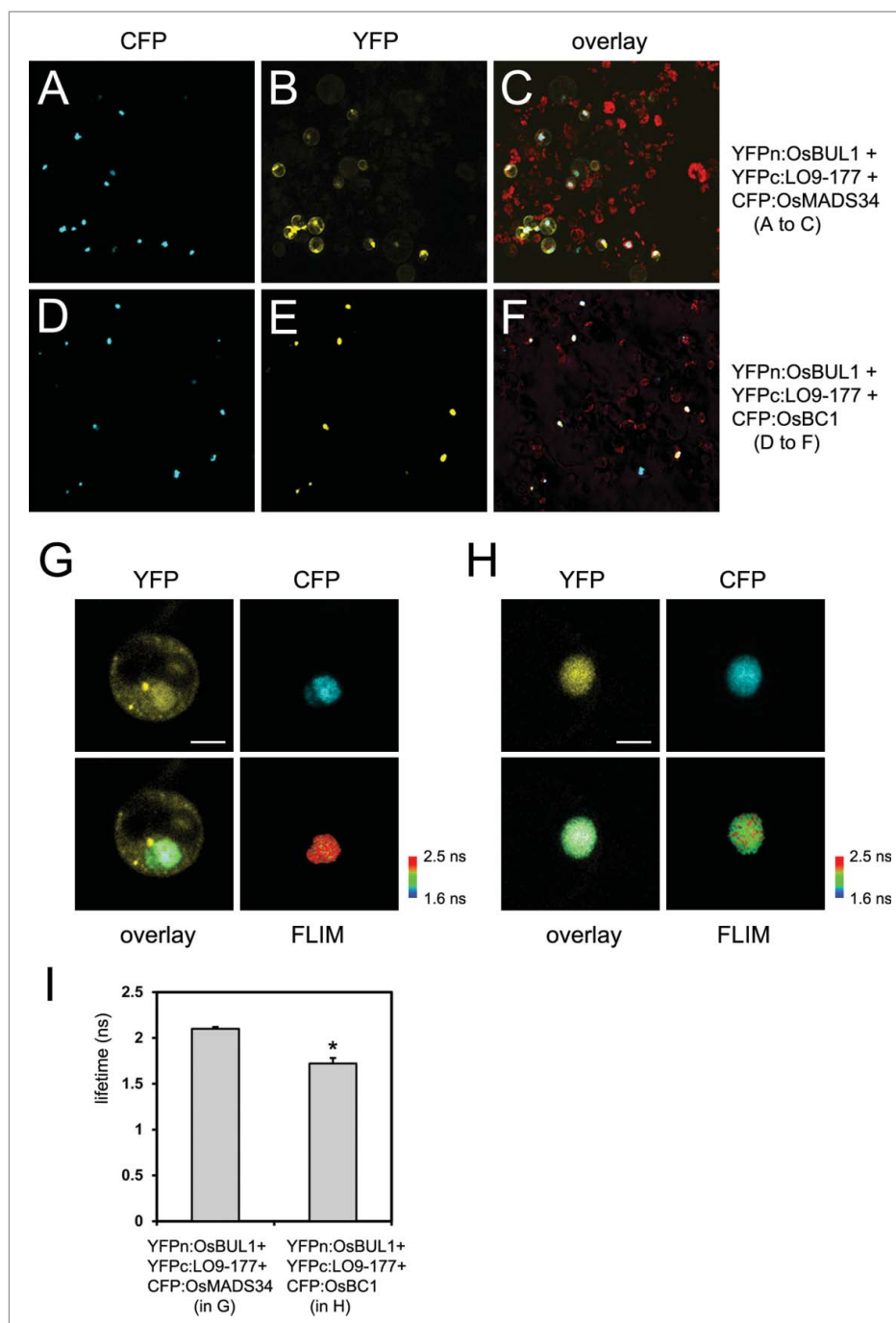
Rice is one of the most important food crops and feeds more than half of the world population.<sup>1</sup> Plant architecture, a complex of the key agronomic traits that determines grain yield, is a primary target of artificial selection for rice domestication. Yield-related plant architecture includes tillering pattern, plant height, leaf angle and so on.<sup>1,2</sup> ‘The Green Revolution’ is a successful example of developing high-yield rice and wheat with shorter and sturdier stems. Researchers have tried to increase yield by dense planting plants with erect leaves.<sup>2,3</sup> In rice, erect leaves have a higher leaf area index that increases photosynthetic carbon assimilation rates through increased light capture and nitrogen use efficiency.<sup>2,3</sup> Especially, the angle of rice lamina inclination is an important trait for the determination of rice architecture. A rice leaf is composed of a leaf sheath, leaf blade, and a lamina joint (area between leaf blade and leaf sheath, also called the collar) with auricles and ligules.<sup>4</sup>

Previously, it was shown that brassinosteroid (BR) is an important phytohormone for controlling lamina inclination implicated in rice architecture and grain yield.<sup>5,6,7,8</sup> In particular, controlling the balance of activities between the 2 groups of antagonistic proteins, atypical helix-loop-helix (HLH) and basic HLH (bHLH) proteins has been regarded as a key step in determination of rice lamina inclination at the molecular level. *BRASSINOSTEROID UPREGULATED1 (BUI)*,<sup>9</sup> *INCREASED LAMINA INCLINATION1 (IL1)*<sup>10</sup> and *Oryza sativa BUI-like1 (OsBUL1)*<sup>11</sup> genes upregulated by brassinolide (BL) treatment encode rice atypical HLH proteins and overexpression of those genes caused increased lamina inclination with enlarged grain size whereas ectopic expression of *IL1 BINDING bHLH1 (OsIBH1)*<sup>10</sup> encoding a bHLH protein produced erect leaves. Of note, reduction of *OsIBH1* transcript levels by BL treatment was also reported. Thus, BR regulation of lamina inclination is likely due to the

antagonizing HLH-bHLH transcription factors acting in the BR signaling pathways.<sup>10,12</sup> Moreover, in *Arabidopsis*, a similar observation was also reported: *PACLOBUTRAZOL RESISTANCE1 (PRE1)*,<sup>13</sup> *KIDARI*<sup>14</sup> and *ACTIVATION-TAGGED Bri1-SUPPRESSOR1 (ATBS1)*<sup>15</sup> encode atypical HLH proteins and *Arabidopsis* IBH1<sup>10</sup> and ATBS1-INTERACTING FACTORS (AIFs)<sup>15,16</sup> are members of the bHLH family. However, it was recently reported that the bHLH domain of IBH1 was diverged from that of typical bHLH proteins since it lacks amino acids essential for binding to the E-box and G-box in the basic motif.<sup>17</sup> Thus, a triantagonistic model through the balance of PRE1, IBH1 and newly identified IBH1-interacting bHLH proteins, ACTIVATORS FOR CELL ELONGATION (ACEs) has been suggested for cell elongation regulation in *Arabidopsis*.<sup>17</sup>

In this system, ACE bHLH transcription factors activate the expression of enzyme genes for cell elongation by interacting with their promoter regions. IBH1 negatively regulates cell elongation by interacting with the ACEs thus interfering with their DNA binding. PRE1 interacts with IBH1 and counteracts the ability of IBH1 to affect ACEs. Thus, PRE1 restores the transcriptional activity of ACEs, resulting in cell elongation.

Even though it is not yet obvious whether the triantagonistic model can be applied to other plant species, recently a novel layer of cell elongation regulation through the HLH-bHLH system has been reported in rice.<sup>11</sup> A trimeric complex composed of OsBUL1, LO9-177 and OsBUL1 COMPLEX1 (OsBC1) was shown to positively affect lamina inclination through cell elongation. For the complex formation, an atypical HLH protein, OsBUL1 interacted with a typical bHLH transcription factor, OsBC1 only in the presence of LO9-177, a KxDL motif-



**Figure 1.** Protein localization and interaction. (A–C) CFP:OsMADS34, YFPn:OsBUL1 and YFPc:LO9–177 were introduced into rice protoplasts. CFP signals were exclusively detected in the nucleus (A) while fluorescent signals from reconstituted YFP are in the cytoplasm as well as the nucleus (B). (C) Merged image of CFP and YFP signals with auto-fluorescence from chloroplasts. (D–F) CFP:OsBC1, YFPn:OsBUL1 and YFPc:LO9–177 were introduced into rice protoplasts. YFP signals from YFPn:OsBUL1 and YFPc:LO9–177 as well as CFP signals were only found in the nucleus (D, E) suggesting the formation of a trimeric complex in rice cells. (F) Merged image of CFP and YFP signals with auto-fluorescence from chloroplasts. FRET-FLIM images in the nucleus containing YFPn:OsBUL1, YFPc:LO9–177 and CFP:OsMADS34 (G) and YFPn:OsBUL1, YFPc:LO9–177 and CFP:OsBC1 (H). (G–I) FRET-FLIM analyses show significant changes in the lifetime of CFP from the CFP:OsBC1 fusion when OsBUL1 and LO9–177 were co-transformed compared with the control, CFP:OsMADS34 was transformed together with OsBUL1 and LO9–177 (\*, Student's *t* test:  $P < 0.01$ ). ns, nanoseconds. Scale Bars, 10  $\mu\text{m}$  in (G, H). Image and data acquisition was obtained with a Leica TCS SP5, combined with a PicoHarp 300 TCSPC Module and a Ti:Sapphire multiphoton laser (Coherent). The samples were excited with a 840 nm pulsed laser (80 MHz). The emission was recorded from 470 nm to 510 nm in  $512 \times 512$  pixel images with at least 500 counts/pixel. The fluorescence lifetime measurements were analyzed using the PicoQuant SymphoTime Software (ver. 5.3.2.2). For each nucleus average fluorescence decay profiles were plotted and lifetimes were estimated by fitting the data with a double-exponential decay function.

containing protein that is able to interact directly with OsBUL1 and OsBC1.<sup>11,18</sup>

Since the trimeric complex formation was shown previously by *in vitro* pull-down assays and the yeast system, the assembly of the trimeric complex in rice cells was examined here by a combination of bimolecular fluorescence complementation (BiFC) and

fluorescence resonance energy transfer (FRET)-fluorescence lifetime imaging (FLIM) assays.<sup>19</sup> Each N-terminal and C-terminal half of the yellow fluorescent protein (YFP)<sup>20</sup> was fused to OsBUL1 and LO9–177, respectively for BiFC, and OsBC1 was fused to the cyan fluorescent protein (CFP).<sup>21</sup> Those 3 constructs were introduced into rice protoplasts simultaneously.<sup>22</sup> A plasmid for the

CFP:OsMADS34 fusion protein was also introduced into rice pro-toplasts together with YFPn:OsBUL1 and YFPc:LO9–177 plasmids as a negative control for FRET-FLIM. OsMADS34 is a rice MADS-domain transcription factor that interacts with neither OsBUL1 nor LO9–177 in yeast systems (data not shown).<sup>23</sup>

Of note, we could observe the YFP signals preferentially from the nucleus when we introduced YFPn:OsBUL1 and YFPc:LO9–177 together with CFP:OsBC1, which is distinct from the results either of the combination YFPn:OsBUL1 and YFPc:LO9–177<sup>11</sup> or YFPn:OsBUL1 and YFPc:LO9–177 together with CFP:OsMADS34 suggesting the possibility of trimeric complex formation as was shown in yeast systems and *in vitro* pull-down assays (Fig. 1A–F). Next, we performed FRET-FLIM experiments<sup>19</sup> to test whether physical interactions between the reconstituted YFP by OsBUL1 and LO9–177 interaction and the CFP from the CFP:OsBC1 fusion are available and detected significantly shorter lifetime of the CFP fluorophore compared with controls that co-express YFPn:OsBUL1, YFPc:LO9–177 and CFP:OsMADS34 in the nuclei where YFP and CFP fusion proteins are co-localized (Fig. 1G–I). Therefore, we demonstrated here that formation of a trimeric complex comprised of OsBUL1, LO9–177 and OsBC1 occurs in rice cells, as well.

Proper cellular or developmental processes in plants demand precise, robust and efficient regulation of gene expression. Recently, several reports have been published showing that such a sophisticated control for gene expression is exerted by trimeric transcriptional complexes: Microproteins such as B-box 30 (BBX30) and BBX31 interact with flowering activator, CONSTANS (BBX1) and additionally engage in a large protein complex involving the co-repressor protein TOPLESS resulting in delayed flowering by reduced *FLOWERING LOCUS T (FT)* expression.<sup>24</sup> In addition, trichome-promoting genes encode the R2R3 MYB transcription factors such as GLABRA1 (GL1) and MYB23, the bHLH factors including GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) and the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1) while a class of 6 single-repeat MYB-related transcription factors such as TRIPTYCHON (TRY) and CAPRICE (CPC) are trichome inhibitors.<sup>25</sup> Trichome activators form a R2R3 MYB/bHLH/WD40 trimeric complex, which is counteracted by the inhibitors through competition with R2R3MYB for binding to bHLH proteins.<sup>25</sup>

Components of the OsBUL1 transcriptional activator complex such as OsBUL1 and OsBC1 are able to interact with OsIBH1, a negative regulator of cell elongation.<sup>11</sup> Thus, competition may occur between LO9–177 and OsIBH1 for binding to OsBUL1 and/or OsBC1 suggesting that various cellular and developmental processes are controlled by a complex transcriptional network involving different groups of proteins.

## Disclosure of potential conflicts of interest

No conflict of interest declared.

## Acknowledgments

The author would like to thank the core facility of the Institute of Cellular and Organismic Biology, Academia Sinica for the technical support for FRET-FLIM, and Ms. Miranda Loney for help with English editing.

## Funding

This work was supported by a core grant from the Biotechnology Center in Southern Taiwan (BCST) of the Agricultural Biotechnology Research Center (ABRC), Academia Sinica, Taiwan.

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