Brief Communication

Overexpression of ZmSPL12 confers enhanced lodging resistance through transcriptional regulation of D1 in maize

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Introduction of the semi-dwarf genes (sd1 in rice, RhtB1b and RhtD1b in wheat) has drastically increased lodging resistance and grain yields in these two important crops since the 1960s, resulting in the so called ‘Green Revolution’ (Hedden, 2003). Maize (Zea mays L.) is one of the most widely cultivated cereal crops in the world and increasing planting density is an effective strategy to increase grain yield (Duvick, 2005). However, high planting density could trigger shade avoidance response and cause increased plant height and ear height, prone to lodging and yield loss. Thus, reducing plant/ear height and increasing lodging resistance is a continuous goal for maize breeders.

‘Green Revolution’ genes in rice or wheat are both either involved in GA biosynthesis or signalling. Previous studies have identified a number of maize dwarf mutants that are defective in GA biosynthesis or signalling, such as anther ear1, dwarf plant1 (d1), dwarf3, dwarf8 and dwarf9 (Lavivit et al., 2010; Teng et al., 2013). However, these mutants could not be used in breeding because of pleiotropic detrimental effects, such as dwarfishm, small ear size, and yield losses. Thus, identification of semi-dwarf genes is desirable for genetic improvement of lodging resistance in maize.

D1, encoding a GA 3-oxidase (ZmGA3ox2) catalysing the final step of bioactive GA synthesis, is a candidate gene for qPH3.1, a major QTL for plant height in maize (Teng et al., 2013). To identify candidate transcription factors (TFs) that negatively regulate D1 expression in maize, we first performed WGCNA co-expression network analysis of D1 using the published transcriptomic data (Stelpflug et al., 2016). We initially identified 67 TFs showing negative expression correlation with that of D1 in the shoot apical meristem (SAM) and internodes (cut-off value ≤ −0.8). Among them, we found 10 candidate TFs that potentially bind to the D1 promoter (~3 kb upstream of the translation initiation site) using plantpan 3.0 (Chow et al., 2019), including ZmARF18 (Zm00001d014377), ZmSPL12 (Zm00001d015410), ZmGN1(Zm00001d007842), ZmbZIP104 (Zm00001d015421), ZmMYB77 (Zm00001d036362), ZmbHLH128 (Zm00001d054038), ZmNAC123 (Zm00001d035084), ZmMADS73 (Zm00001d018142), ZmbHLH55 (Zm00001d012067) and ZmbHLH117 (Zm00001d024783) (Figure 1a).

We next selected top six TFs with the highest correlation, including ZmARF18, ZmSPL12, ZmGN1, ZmbZIP104, ZmMYB77 and ZmbHLH128, and tested their binding to D1 promoter. Yeast one-hybrid assay showed that only ZmSPL12 could bind to D1 promoter (Figure 1b). Transient expression assay showed that ZmSPL12 strongly repressed the expression of the pD1::LUC reporter gene (Figure 1c). Quantitative reverse transcription PCR (RT-qPCR) assay revealed that ZmSPL12 was mainly expressed in seedling at V2 stage, SAM and internodes at the V8 and V10 stages (Figure 1d). Subcellular localization assay revealed that ZmSPL20 protein was exclusively localized to the nucleus (Figure 1e).

To investigate the role of ZmSPL12, we generated ZmSPL12 knockout plants using the CRISPR/Cas9 technology. Two independent lines (ko#1 and ko#2) with frame-shift mutations were selected and used for phenotypic investigation. Both ko#1 and ko#2 plants exhibited significantly higher plant height and ear height (Figure 1f). While two independent transgenic ZmSPL12 overexpression lines (ZmSPL12-OE, #499 and #500) showed reduced plant height and ear height, compared with the non-transgenic control plants, and that the degree of reduction in plant height is negatively correlated with the expression levels of ZmSPL12 (Figure 1g). As expected, the expression level of D1 was decreased significantly in the ZmSPL12-OE plants (Figure 1h). Histological observation revealed that internode cells in the ZmSPL12-OE plants were significantly shorter than those of control plants (Figure 1i), indicating that the shorter internode in the ZmSPL12-OE plants is mainly caused by decreased cell length. Moreover, measurement of stalk strength showed that the ZmSPL12-OE plants were significantly stronger than that of the control plants (Figure 1j).

To confirm the effect of ZmSPL12 on plant height is caused by altered GA levels, we measured endogenous bioactive GA levels, including GAs, GA20, GA3 and GA4 in the internodes. The results showed that the levels of these GAs were all significantly decreased in ZmSPL12-OE plants (Figure 1k). In addition,
ZmSPL12 regulates maize lodging resistance
Overexpression ZmSPL12 enhances lodging resistance and grain yield. (a) Heatmap illustrates the negative correlation of expression between ten TF genes and D1 in the SAM and internodes. (b) Yeast one-hybrid assay shows direct binding of ZmSPL12 to the D1 promoter. (c) Transient expression assay in N. benthamiana leaves shows repressing of D1 expression by ZmSPL12. (d) RT-qPCR analysis shows preferential expression of ZmSPL12 in stem, seedling, SAM and different internodes. (e) ZmSPL12 protein is localized to the nucleus. Pro-35S::mRFPMH22 (Xiao et al., 2009) is used as a nucleus marker. (f) ZmSPL12 knockout lines exhibit lower plant stature than their corresponding control plants. (g) ZmSPL12-OE lines (#499 and #500) show reduced plant height and ear height compared with the corresponding non-transgenic control plants (CK). (h) The expression level of D1 was significantly decreased in the ZmSPL12-OE plants. (i) Longitudinal section of the 13th internode of #500 plants and CK plants. (j) Increased stalk strength of the ZmSPL12-OE compared with CK. (k) Reduced endogenous bioactive GAs contents in the shoot of ZmSPL12-OE plants at the V9 stage. (l) Exogenous GA3 treatment largely restores the plant height phenotype of the ZmSPL12-OE (#500) plants. (m) ZmSPL12-OE plants show moderately decreased plant stature under three different planting densities. (n) Overexpression ZmSPL12 enhances maize grain yield under high planting densities in the field in Hainan. (o) Overexpression ZmSPL12 enhances maize lodging resistance under high planting densities. (p, q) Plant height and ear height of the improved Chang7-2 and improved PH6WC carrying the ZmSPL12-OE transgene (#500) decrease significantly compared with the original Chang7-2 and PH6WC. The Tubbins (Zm00001d006651) is used as a reference in RT-qPCR detection. Error bars represent ± SD, n = 24, 10, 3 in panel f, g and k; n = 3 replications in panel m, n and o. Asterisks indicate significant differences by Student’s t-test, * indicates P < 0.05, ** indicates P < 0.01, *** indicates P < 0.001. Different letters denote significant differences (P < 0.05) from Duncan’s multiple range tests. HN: Hainan (18°N, 108°E), LF: Langfang (39°N, 116°E).

Conflict of interest
The authors declare no conflict of interests.

Author contributions
HW and BW conceived and designed the project. BZ conducted the experiments. YZ, YL, HW, DX, YX and ZZ participated in some experiments. CL analysed the data. BZ and MX wrote the manuscript. HW revised the manuscript.

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