Overexpression of a Rice TIFY Gene Increases Grain Size through Enhanced Accumulation of Carbohydrates in the Stem

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Screening of rice full-length cDNA overexpressing (FOX) lines allowed the identification of a TIFY gene, TIFY11b, as a growth-promoting gene whose overexpression increased plant height and seed size. The grains of TIFY11b-overexpressing plants exceeded those of non-transformants in length, width and thickness, resulting in 9–21% increases in grain weight. The increase was achieved by overexpressing the gene in the whole plant body, but not by seed-restricted expression, indicating that seed enlargement is attributable to overexpression in vegetative organs such as the leaf. The whole-body overexpressing plants developed longer leaves along with higher levels of starch and sucrose in the leaf sheath and culm at the heading stage than the non-transformants. Although overexpression of TIFY11b did not alter the photosynthetic rate per leaf area before and after heading, it caused an accumulation of higher levels of the carbohydrate assimilate, probably due to increased photosynthesis per plant, suggesting that the increase in grain size and weight is attained by enhanced accumulation and translocation of the carbohydrate in the culms and leaf sheaths of the transgenic plants. Thus, TIFY11b is a novel grain-size increasing gene.

Key words: full-length cDNA overexpressing gene hunting system (FOX hunting system); grain size; rice (Oryza sativa L.); transient starch accumulation; TIFY/JAZ protein

Grain size is an important determinant of yield in cereal crops. A series of genes regulating grain size have been identified by QTL analysis. Among them, GS3, encoding a transmembrane protein, regulates grain length and weight,13 while GW2, a gene for a RING-type ubiquitin E3 ligase, and GW5/SW5, for a novel nuclear protein, govern grain width and weight.2–4 These genes are suitable for breeding high yield rice varieties. In combination with genes that control grain number such as Gn1a,5 AP01,6,7 and OsSPL14,8,9 they are expected to confer a sink ability on the plant.

Another approach to identifying genes that fortify yield is screening of transgenic plants that allow overexpression of arbitrary genes. Overexpression of cyclin genes that regulate cell division promotes growth, resulting in increased plant height10 and enhanced root growth.11 However, in terms of cereal grain yield, a limited number of growth-promoting genes are available.

In an effort to identify genes regulating growth and grain yield in rice, we previously employed the full-length cDNA overexpressing gene (FOX) hunting system, in which an individual rice cDNA was ectopically overexpressed by a maize ubiquitin promoter.12–14 Extensive screening of 5,500 independent FOX lines made possible the identification of lines harboring a single TIFY/ZIM-domain gene, TIFY11b, which showed increased plant height.12 TIFY11b is a member of the Arabidopsis JA ZIM-domain (JAZ) gene family.15,16 Arabidopsis JAZ proteins are nuclear proteins that act as negative regulators of the response to a plant stress-related hormone, jasmonate (JA), by repressing key transcription factors governing JA signaling such as MYC2.17–19 When a bioactive JA derivative, JA-isoleucine conjugate (JA-Ile), is perceived by an ubiquitin E3 ligase, COI1, JAZ proteins are selectively ubiquitinated and recruited for rapid degradation by the 26S proteasome. Then MYC2 liberated from tethering by JAZ proteins drives the transcription of JA-responsive genes showing responses such as inhibition of root growth and activation of defense.20 In contrast, the physiological function of TIFY/JAZ genes in rice remains largely unknown. Recently, we noticed that TIFY11b-overexpressing rice plants produced large grains. Here, we report on rice TIFY11b as a novel grain size-regulating gene the overexpression of which increases grain size and weight through enhanced accumulation of transient carbohydrate reserves in the leaf and culm at the heading stage.

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Abbreviations: FOX hunting system, full-length cDNA overexpressing gene hunting system; JA, jasmonate; GA, gibberellin
Materials and Methods

Plant materials and growth conditions. Seeds of wild-type and transgenic rice (Oryza sativa L., cultivar Nipponbare) were sterilized by an antifogger solution containing 2.5% available chlorine for 30 min and germinated for 2 weeks on Murashige and Skoog (MS) agar medium without and with hygromycin (50 μg/mL) respectively. The resulting plants were transferred to soil and grown in plant incubators (model FLJ-2000, Eyela, Tokyo) under a sodium lamp (a cycle of 12 h light, 27°C/12 h dark, 22°C). Six plants were grown in a plastic container (15 x 10 x 6 cm) filled with 700 mL of rice nursery culture soil containing 0.16 g each of nitrogen, phosphate, and potassium, and each plant was restricted to the main culm by removal of the tillers. Approximately 15 d before heading, 3 g of fertilizer containing 0.18 g cDNA fragment (AK067971), which was excised with TIFY11b from pFLCI, 21) into the compatible empty vectors were introduced into and control gene (software. The expression level, normalized to that of the endogenous light, 27) was measured using a portable photosynthesis system, LI-6400XT (Li-Cor, Lincoln, NE). The conditions in the leaf chamber were made the same as those in the plant incubators (550 μmol m−2 s−1 photosynthetic photon flux density, 370 μmol mol−1 CO2 concentration, 27°C, approximatively 70% relative humidity). Net photosynthetic rates were expressed as rates of CO2 uptake. Measurements were conducted on the top expanded mature leaf before heading or the flag leaf after heading. After the leaf chamber was closed, data were recorded in a stable state.

Results

Overexpression of TIFY11b in the whole plant body, but not endosperm-restricted overexpression, increased grain size and weight

Our previous screening of rice FOX lines identified lines expressing a gene for protein containing TIFY/ZIM domain, TIFY11b, which showed enhanced growth, including increased plant height. 23) Upon repeated rounds of culture, we noticed that the TIFY11b-overexpressing lines produced large grains (Fig. 1A). The transgenic lines expressing 31 to 121-fold higher levels of the TIFY11b transcript as determined for the leaves of 2-week-old seedlings (Fig. 1B) yielded significantly larger grains (on average, 7.0%, 7.3%, and 3.1% increases in length, width, and thickness respectively), resulting in a 9 to 21% increase in grain weight as compared to the non-transformant, wild-type (WT) grains (Fig. 1C–F).

To dissect organs (the sink endosperm and/or source leaf) in which the enhanced expression of TIFY11b contributes critically to enlargement of the grain, we examined to determine whether endosperm-restricted overexpression of TIFY11b can produce grains of increased size. Several independent transgenic plants harboring the TIFY11b transgene driven by the promoter of an endosperm-specific prolamin gene (Os03g0766100), whose expression was barely detected in vegetative organs according to a previous promoter assay 28) and a comprehensive gene expression profile database (RiceXPro database; http://ricexpro.dna.affrc.go.jp/), 29) accumulated the transcript in the developing endosperm to levels comparable to those in plants with systemic overexpression due to the ubiquitin promoter (Fig. 2A), and most of them bore numbers of ripened grains similar to the systemic overexpressing and control plants (Fig. 2C). However, these seed-specific expression lines did not exhibit increases in grain weight, while all the systemic lines did (Fig. 2B), suggesting that the expression of TIFY11b in vegetative organs other than the developing endosperm, most likely the source leaf, is required for increases in grain weight.

The TIFY11b-overexpressing plants produced longer leaves containing much more starch and sucrose at the heading stage

Since grain filling relies largely on the remobilization of carbohydrates temporarily stored in source organs
such as the leaf and culm to the panicle, leaf size and amounts of carbohydrate in leaves and culms were measured at the heading stage. Mature leaves (the 4th leaf from the top frag leaf) of TIFY11b-overexpressing lines were 1.09 to 1.18-fold longer than corresponding leaves of WT plants (Fig. 3A). Microscopic observation of the leaf surface revealed that it was filled with cells of similar sizes among the transgenic lines and WT plants (Fig. 3B, C), suggesting that the elongation caused by the overexpression of TIFY11b was due to an increase in the number, not the size, of the cells.

The culms of TIFY11b-overexpressing lines contained more and larger starch granules than those of WT plants at the heading stage as observed at the 2nd internode (Fig. 4A). Quantification of carbohydrates confirmed that overexpression of TIFY11b increased starch and sucrose contents in the culm and leaf sheath 1.12 to 1.29-fold and 2.08 to 2.43-fold respectively (Fig. 4C, D). Taking account of the increased biomass fresh weight of culm and leaf sheath (Fig. 4B), overexpression of TIFY11b resulted in 1.55 to 1.78-fold and 2.56 to 3.14-fold increases in the overall starch and sucrose reserve per plant respectively (Fig. 4E, F). Thus the TIFY11b-overexpressing lines produced greater amounts of source organ biomass that was also more enriched with carbohydrate reserves before heading than did the WT plants.

The photosynthetic rate in the source leaf was not altered by overexpression of TIFY11b

Another crucial factor governing grain filling is photosynthesis in the source leaf. The photosynthetic rate after heading directly affects the accumulation of starch in grain, which is prerequisite for an increase in grain weight, and that before heading, which determines the amount of starch that accumulates in the culms and leaves, also indirectly contributes to the final amount of starch in grain through translocation of photosynthates. In order to estimate the contribution of photosynthesis to the increase in grain size and weight in the TIFY11b-overexpressing lines, photosynthetic rates in source leaves were examined before and after heading by measuring the rate of CO₂ uptake. However, the rate per leaf area in the TIFY11b-expressing lines, which produced grains of increased size and weight, was not significantly different from that in the WT plants before or after heading (Fig. 5).

Discussion

We found that overexpression of a member of the rice TIFY gene family, TIFY11b, resulted in increases in grain size and weight (Fig. 1) and that expression of the gene in source organs other than the sink, ripening seeds, was required for grain enlargement (Fig. 2). Since, along with grain weight, the biomass of photosynthetic leaf organs (Fig. 3) and the accumulation of starch and sucrose in the leaf and culm (Fig. 4) increased at the heading stage in the TIFY11b-overexpressing plants, TIFY11b is a novel gene whose expression contributes to an increase in grain size and weight by enhancing the amounts of remobilized carbohydrate reserves nourishing ripening grains. Although the photosynthetic rate per unit of leaf area did not differ significantly between the
TIFY11b-overexpressing lines and the WT plants around the heading stage (Fig. 5), an increase in the area of the leaf (a longer leaf), which actively conducts photosynthesis, contributed to a temporary accumulation of carbohydrates in leaf and culm in the transgenic line. Generally, the carbohydrate that accumulates in the culm and leaf sheath before heading and is transferred to the developing grains in the course of ripening accounts for up to 38% of the carbohydrate in the mature grains32) and approximately 30% of rice grain yield.30,31,33) Therefore, enhanced accumulation of carbohydrates in the culm and leaf sheath prior to heading would cause increases in grain weight in TIFY11b-overexpressing lines. Among grain weight/size-regulating genes, GS3 (a transmembrane protein), GW2 (a RING-type ubiquitin E3 ligase), and GW5/SW5 (a nuclear protein), which have been identified by QTL analyses, are negative regulators, loss of which produces larger grains, mainly due to increases in length, width, and weight, respectively,1–4) whereas the expression of TIFY11b positively regulates grain weight by evenly promoting all dimensions, length, width, and thickness. Recently, another gene, GS5, encoding a serine carboxypeptidase, was found to regulate grain width and weight positively.34) However, none of these, except for TIFY11b, have been found to accumulate increased amount of carbohydrates in the stem at heading.

Arabidopsis plants overexpressing a TIFY gene, JAZ9, have been reported to show enhanced growth with elongated petioles and hypocotyls, and knock-down of coi1, which encodes a TIFY/JAZ-targeting ubiquitin E3 ligase, in rice showed increased plant height and grain length.35) Such phenotypes of growth promotion, which are reminiscent of gibberellin (GA)’s actions, have been explained by titration and inhibition of DELLA protein, a key repressor of the response to GA that can bind physically to TIFY/JAZ proteins,35,36) by accumulated TIFY/JAZ proteins, which allows activation of the GA-driven growth programs. In TIFY11b-overexpressing rice, such masking of the growth-inhibiting effect of DELLA by overexpressed TIFY11b protein might increase sensitivity to endogenous GA, thus increasing plant height12) and grain size (Fig. 1).
Another Arabidopsis TIFY gene, ZIM, containing a zinc-finger domain, also elongated the hypocotyls and petioles when overexpressed, confirming the positive role of TIFY genes in growth enhancement.

Plant size is determined by the number and the size of cells. The epidermis of mature leaves of the TIFY11b overexpressing rice was covered with cells similar in size to that of the non-transformant, WT plants (Fig. 3), indicating that the elongated leaf consists of an increased number of cells generated by increased rounds of cell division. This is in clear contrast to the internodes of coi1 knock-down rice and the hypocotyls of ZIM-overexpressing Arabidopsis, in which elongated cells were observed. This discrepancy suggests that the downstream targets and affected pathways vary and that the outcome depends on the type of TIFY member expressed dominantly. One group of TIFY genes, including Arabidopsis ZIM and unidentified rice members whose endogenous levels are regulated by coi1, might modulate the enlargement of cells, probably via GA signaling, while another group, exemplified by rice TIFY11b, might control cell proliferation. Thus overexpression of TIFY11b might promote growth by accelerating cell division. It is well documented that exogenously applied JA represses the progression of the cell cycle and inhibits the growth of tobacco and Arabidopsis calli. In one study, upon repeated wound stress, plants were stunted by endogenously accumulated JA and showed reductions in cell division.
and cyclin gene expression, whereas Arabidopsis mutants compromised as to JA biosynthesis (aoa or perception (coi1) as well as the jai3 mutant expressing a truncated form of JAIZ3, which is resistant to JA-induced destruction by F-box protein COI1, remained large truncated form of JAZ3, which is resistant to JA-induced perception (mutants compromised as to JA biosynthesis (increased number of cells consisting of the endosperm in the whole-plant TIFY11b-overexpressing line (Hakata et al., manuscript in preparation), but for the observed increases in grain weight, expression of TIFY11b in the vegetative organs is critical.

In this study, we identified TIFY11b as a novel gene whose overexpression increases grain size and weight by enhancing the accumulation of carbohydrate in the culm and leaf prior to heading. Although the grain yield in cases of combined introgression with genes that increase biomass of cereal crops.

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