Analysis of gamma ray-induced sweet sorghum mutants

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September 2014

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Chapter 1

General Introduction

Sorghum (*Sorghum bicolor* L. Moench*),* is an annual or short-term perennial plant with culms reaching up to about 4 m or more in height (http://www.fao.org/ag/agp/agpc/doc/gbase/data/pf000319.htm). It belongs to the grass family (*Poaceae*) which includes rice, wheat and corn, and is believed to have originated from Africa, particularly in Ethiopia and surrounding countries (Reddy *et al.*, 2006; Paterson *et al.*, 2009). Just like rice, sorghum usually matures in about 4 months or less. However, compared to rice and corn (C3 and moderate C4, respectively), sorghum is an extreme C4 grass and is much more efficient in terms of photosynthesis and water use efficiency, making it highly adaptable to semi-arid or arid environments (Xin *et al.*, 2008, 2009; Blomstedt *et al.*, 2012).

The above-mentioned characteristics of sorghum speak of its great potential as a crop. In these times when the world's available resources and food supplies are hardly enough for the ever-growing human population, it should be wise to utilize sorghum and further breed it to produce varieties that can provide for existing global demands Aside from food, feed and fiber, one of such demands is the production of biofuels to address the worsening problem of global warming and climate change that could threaten the existence of mankind in the long run. In view of this scenario, a special cultivar of sorghum has caught the interest of ecologists— sweet sorghum (Grooms, 2008; Kim and Day, 2011; Wortmann and Regassa, 2011; Yu *et al.*, 2012; van der Weijde *et al.*, 2013)

Sweet sorghum has long been known in the U.S. since the 1850s. For a long time, it was cultivated mainly for the production of syrup or sugar (Hunter and Anderson, 1997). This is because sweet sorghum accumulates 10–25% sugar in the culm juice near the time of grain maturity, with glucose and fructose as the predominant

reducing sugars and sucrose as the predominant disaccharide (Ritter *et al.*, 2007). This special characteristic of sweet sorghum makes it suitable for biorefineries for the production of bioethanol and other materials (Yu *et al.*, 2012). Although sugarcane and corn starch have long been used to produce bioethanol in large scale, their biggest drawback is that they compete with food supplies, dramatically affecting the prices of related commodities (Salas Fernandez *et al.*, 2009). In the case of sweet sorghum, such dilemma hardly applies since sorghum can be grown even in limiting environments not usually devoted to the cultivation of food crops, and that bioethanol can be produced from almost all of its parts— cellulosic biomass, grains, or culm juice.

Despite the good attributes of sweet sorghum, there remain some aspects that need to be improved to make it more suitable for bioethanol production. For example, it should be important to develop lines with higher sugar content. Also, since other parts of sweet sorghum can be processed into bioethanol, improvements to grain yield, plant height and tillering should be welcome developments. In terms of grain production, sweet sorghums generally have lower grain yields compared to modern grain sorghums. And as for plant height, sweet sorghums are also generally taller (Ritter *et al.*, 2007), which make them prone to lodging in times of strong winds and typhoons. Such scenario makes it hard to harvest the crop even with the use of machines, not to mention the possibility of untimely fermentation of sugars and spoilage of starch, which negatively influence the amount and quality of grains or bioethanol that can be produced. In view of these circumstances, the development of semi-dwarf lines of sweet sorghum (ideally with thicker culms) should be one of the attractive solutions. However, as considerable amount of biomass is inevitably lost to semi-dwarfism, such plants ideally must have additional traits such as higher tillering, sweeter culms, or earlier

maturity. Having these traits would also make them suitable parentals for hybrid breeding to produce robust F_1 lines with excellent qualities.

Many sweet sorghum varieties of today have been derived from lines developed through conventional breeding carried out by public breeding programs during the early to mid-1900s in the U.S. (Ritter, *et al.*, 2007). However, such process proved to be slow and tedious as several generations are required to produce stable plants. Now, with the advent of newer techniques, several options have become available for crop breeders to improve sorghum in a significantly shorter span of time. One of these is gamma ray mutagenesis (see Chapter 2). This method can produce an array of mutants by inducing random physical breaks (single or double strand) or deletions in DNA strands (Henner *et al.*, 1982; Ordonio *et al.*, 2014). By selecting mutants based on desired parameters, candidate plants can be isolated for use in basic science (e.g. isolation of novel genes and functional analysis) or for breeding by DNA marker-assisted selection (MAS), using the existing sorghum genome sequence as a reference tool.

Sorghum (Btx-623 variety) is the first *Saccharinae* and C4 grass to be fully sequenced by the shotgun sequencing technique (Paterson *et al.*, 2009; Sasaki and Antonio, 2009). Prior to this, the main criteria for its selection as a model was its ease of sequencing owing to its smaller genome (2n=20; ~730 Mb) relative to other C4 plants like maize (2.6 Gb) (Paterson *et al*., 2009), *Miscanthus* species (4.4- 6.8 Gb) (Karlen, 2014), and sugarcane (10 Gb) (Le Cunff *et al.*, 2008). Recently, because of the demands of the times due to global warming, sorghum was hailed as a Flagship Plant of the Joint Genome Institute (JGI) of the U.S. Department of Energy (DOE) for its potential as a major biofuel crop and cellulosic feedstock, eventually leading to the improvement of existing sorghum genomic resources, particularly the entire gene space of the current

draft version of the sorghum genome sequence (http://www.phytozome.net/sorghum_er.php).

The precise genome sequence of sorghum is without doubt, a valuable tool for sorghum research as it serves as a repository for the wealth of genetic information about sorghum genes. As genes are always associated with a particular phenotype, it should be important to demonstrate their biological functions in conjunction with forward or reverse genetics as in the case of rice and *Arabidopsis* where numerous mutants had been instrumental in identifying novel genes and their corresponding functions (see Chapter 3, Introduction).

To date, only a limited number of sorghum mutants with known causal genes are reported in the literature (see Chapter 3, Introduction), and this reflects the poor availability of established mutant libraries. Also, any existing libraries solely dealt with grain sorghums (Xin *et al.*, 2008). This manuscript, thus, attempts to put priority on sweet sorghum genetic research. Particularly, I focused on two objectives: (1) construction of a mutant library of sweet sorghum containing mutants of various phenotypes ranging from mild to severe; and (2) elucidation of the causal genes of some of the identified height-related morphological mutants, the mechanism(s) involved, and their implications for agriculture.

In Chapter 2, I describe the use of a forward genetics approach employing gamma-ray mutagenesis to produce a mutant population of sweet sorghum in an attempt to construct an online database. More than 5,400 M_2 lines were screened based on their morphology and a total of 669 independent mutant lines were classified into several phenotypic categories.

In Chapter 3, I present the analysis of the causal genes of some height-related

mutants consisting of five severe dwarf mutants (*bdw1*, *bdw2, bdw3-1, bdw3-2* and *bdw4*) with bent culms and dark green leaves, three slender/hyper-elongated mutants, one severe dwarf mutant with twisted and irregular leaves, and one semi-dwarf mutant. The severe dwarf mutants with bent culms were found to be mutated in four GA-biosynthetic genes, namely, *Copalyl diphosphate synthase 1* (*SbCPS1*), *Kaurene synthase 1* (*SbKS1*), *Kaurene oxidase 1* (*SbKO1*), and *Kaurenoic acid oxidase 1* (*SbKAO1*). On the other hand, the three slender mutants were found to have mutations in *Slender 1* (*SbSLR1*), which encodes a repressor protein for GA signaling. Further, the severe dwarf mutant with twisted leaves was found to have a mutation in *SbBR6ox* involved in brassinosteroid (BR) biosynthesis. Lastly, the semi-dwarf mutant had a mutation in *Dw3,* a gene that encodes a phosphoglycoprotein involved in auxin transport, the loss of function of which was instrumental for the production of many semi-dwarf cultivars of sorghum in the past (Multani *et al.*, 2003).

References

Blomstedt, C.K., Gleadow, R.M., O'Donnell, N., Naur, P., Jensen, K., Laursen, T., Olsen, C.E., Stuart, P., Hamill, J.D., Møller, B.L., Neale, A.D. (2012). A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. Plant Biotechnol. J. **10**: 54-66.

Le Cunff, L., Garsmeur, O., Raboin, L.M., Pauquet, J., Telismart, H., Selvi, A., Grivet, L., Philippe, R., Begum, D., Deu, M., Costet, L., Wing, R., Glaszmann, J.C. and D'Hont, A. (2008). Diploid/polyploid syntenic shuttle mapping and haplotype-specific chromosome walking toward a rust resistance gene (*Bru1*) in highly polyploid sugarcane (2n approximately 12x approximately 115). Genetics **180**: 649–660.

Grooms, L. (2008). Sweet energy crop: Sweet sorghum biomass and stem juice to produce ethanol. Biofuels J. 38.

Henner, W.D., Grunberg, S.M. and Haseltine, W. A. (1982). Sites and structure of gamma radiation-induced DNA strand breaks. J. Biol. Chem. **257**: 11750–4.

http://www.fao.org/ag/agp/agpc/doc/gbase/data/pf000319.htm

http://www.phytozome.net/sorghum_er.php

Hunter, E. and Anderson, I. (1997). Sweet Sorghum. Hort. Rev. **21**: 73–104.

Karlen, D.L. (Ed.). (2014). *Cellulosic Energy Cropping Systems*. Wiley.

Kim, M. and Day, D.F. (2011). Composition of sugar cane, energy cane, and sweet sorghum suitable for ethanol production at Louisiana sugar mills. J. Ind. Microbiol. Biotechnol. **38**: 803–807.

Multani, D.S., Briggs, S.P., Chamberlin, M. a, Blakeslee, J.J., Murphy, A.S. and Johal, G.S. (2003). Loss of an MDR transporter in compact stalks of maize *br2* and sorghum *dw3* mutants. Science **302**: 81-84.

Ordonio, R.L., Ito, Y., Hatakeyama, A., Ohmae-Shinohara, K., Kasuga, S., Tokunaga, T., Mizuno, H., Kitano, H., Matsuoka, M. and Sazuka, T. (2014). Gibberellin deficiency pleiotropically induces culm bending in sorghum: an insight into sorghum semi-dwarf breeding. Sci. Rep. **4**: 5287.

Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A.K., Chapman, J., Feltus, F.A., Gowik, U., Grigoriev, I. V, Lyons, E., Maher, C. a, Martis, M.,

Narechania, A., Otillar, R.P., Penning, B.W., Salamov, A. a, Wang, Y., Zhang, L., Carpita, N.C., Freeling, M., Gingle, A.R., Hash, C.T., Keller, B., Klein, P., Kresovich, S., McCann, M.C., Ming, R., Peterson, D.G., Mehboob-ur-Rahman, Ware, D., Westhoff, P., Mayer, K.F.X., Messing, J. and Rokhsar, D.S. (2009). The *Sorghum bicolor* genome and the diversification of grasses. Nature **457**: 551–556.

Reddy, B.V.S., Ramesh, S. and Reddy, S. (2006). Sorghum genetic resources, cytogenetics, and umprovement. In *Cereals*: 309–363.

Ritter, K.B., McIntyre, C.L., Godwin, I.D., Jordan, D.R. and Chapman, S.C. (2007). An assessment of the genetic relationship between sweet and grain sorghums, within *Sorghum bicolor* ssp. *bicolor* (L.) Moench, using AFLP markers. Euphytica **157**: 161–176.

Salas Fernandez, M.G., Becraft, P.W., Yin, Y. and Lübberstedt, T. (2009). From dwarves to giants? Plant height manipulation for biomass yield. Trends Plant Sci. **14**: 454–461.

Sasaki, T. and Antonio, B. (2009). Sorghum in sequence. Nature **457**: 547–548.

van der Weijde, T., Alvim Kamei, C.L., Torres, A.F., Vermerris, W., Dolstra, O., Visser, R.G.F. and Trindade, L.M. (2013). The potential of C4 grasses for cellulosic biofuel production. Front. Plant Sci. **4**: 107.

Wortmann, C.S. and Regassa, T. (2011). Sweet sorghum as a bioenergy crop for the US great plains. In *economic effects of biofuel production*: 225–240. dos Santos Bernardes, M.A. (Ed). InTech Publications.

Xin, Z., Wang, M.L., Barkley, N.A., Burow, G., Franks, C., Pederson, G. and Burke, J. (2008). Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. BMC Plant Biol. **8**: 103.

Xin, Z., Wang, M.L., Burow, G. and Burke, J. (2009). An induced sorghum mutant population suitable for bioenergy research. BioEnergy Res. **2**: 10–16.

Yu, J., Zhang, T., Zhong, J., Zhang, X. and Tan, T. (2012). Biorefinery of sweet sorghum stem. Biotechnol. Adv. **30**: 811–816.

Chapter 2

Towards the establishment of an online database of gamma ray-induced sweet sorghum mutants

Introduction

In nature, plants need to adapt to changes in their environment in order to successfully propagate. Such adaptation coincides with some advantageous mutations that spontaneously occur in their genomes over a long period of time, leading to the evolution of different species each endowed with a specific set of characteristics. Today, this diversity is seen as an important tool for plant breeding as specific plants can be used as donors of agronomically important traits such as those relating to yield, nutrient value, drought tolerance, lodging resistance, pest and disease resistance, and others. In conventional breeding, such donors are sexually crossed with other varieties to produce segregating populations which are systematically screened to ensure that only the progenies with favorable traits are advanced to succeeding generations. It also involves back-crossing the progenies with any of the parents (http://www.csiro.au/Outcomes/Food-and-Agriculture/What-is-the-difference-between-conventional-and-GM-plant-breeding.aspx) to produce near isogenic lines (NILs). The entire process runs for several years until a stable line or variety is produced. However, with the advent of artificial mutation induction techniques, modern plant breeders have not only become able to address the slow process of producing new varieties, they also found an alternative method of broadening a crop's natural genetic diversity.

Mutation induction employs three categories of mutagens: physical (e.g., gamma rays or heavy ion beam) (Bhaskara Rao and Reddi, 1975), chemical (e.g., ethyl methane sulphonate (EMS), nitrosomethylurea (NMU) and diethyl sulfate (DES)) (Komorisono *et al.*, 2005; Saito *et al.*, 2011; Rao *et al.*, 2012), and insertional elements (e.g., tissue culture-induced Tos17 retrotransposon, and transgenic Ac/Ds and T-DNA techniques) (Hirochika, 2001; Miyao *et al.*, 2007; Lo *et al.*, 2008; Qu *et al.*, 2009)). Of these,

physical and chemical agents afford the advantage of stable mutations suitable for crop varietal development. For instance, gamma ray was reported to produce direct mutant varieties that do not go through the otherwise lengthy and laborious process of conventional breeding (http://www-naweb.iaea.org/nafa/pbg/crp/d2-effects-mutagenic.html). It is also known to generate both mild and severe phenotypes, depending on the extent of damage incurred by the plant genome, which usually come in the form of DNA deletions ranging from 1 bp up to more than 10 kb in length (Henner *et al.*, 1982; Asencion, 2005; Ordonio *et al.*, 2014). Given the random nature of gamma ray's deleterious effect on individual genes in the genetic repertoire, the resulting mutant population can provide a library of mutants that can be used for gene isolation, functional analysis, or molecular plant breeding (van Harten, 1998; Henikoff and Comai, 2003; Till *et al.*, 2003; Ahloowalia *et al.*, 2004; Henikoff *et al.*, 2004).

In contrast to rice and *Arabidopsis*, only a few reports on the use of gamma ray for sorghum mutagenesis are available. These include the use of gamma ray for improving or studying nutritional quality such as the reduction of kafirin content to make sorghum highly digestible (Mehlo *et al.*, 2013); drought and soil acidity tolerance (Hanna, 1982; Human *et al.*, 2011); allelopathic potential (Alsaadawi and Dayan, 2009); agronomical traits (Bhaskara Rao and Reddi, 1975; Hanna, 1982; Hayat *et al.*, 1990; Larik *et al.*, 2009) and heterosis and combining ability (Kenga *et al.*, 2005). It was also used for the isolation of some morphological mutants (Orange Leaf, Zebra Leaf, Midget Plant and Slender Plant) from the cultivar Experimental 3 (Haensel *et al.*, 1963). On the molecular genetics front, reports about the use of gamma-ray to produce mutant libraries intended for advanced gene research (forward genetics) are lacking.

In this chapter, I describe the use of gamma ray for the mutagenesis of SIL-05

(sweet sorghum) for the purpose of generating an online mutant database of sweet sorghum. In the case of rice, the availability of mutant libraries made way for the discovery of many novel genes and their underlying mechanisms (Noguchi *et al.*, 1999; Bouquin, 2003; Sakamoto *et al.*, 2004; Komorisono *et al.*, 2005). Thus, doing the same approach on sweet sorghum should also help advance sorghum genetic research and crop improvement.

Materials and Methods

Plant materials and mutagenesis

Sorghum bicolor cv. SIL-05 (sweet sorghum) (Figure 1) was used for mutant library construction by subjecting a large number of its seeds to about 200 Gy of gamma-ray irradiation from a Cobalt-60 source. After exposure, the resulting M_1 lines were sown in seedling trays and grown inside the greenhouse at 25℃ for more than 3 weeks before planting in the field. At the heading stage, panicles of more than 5,400 M_1 lines were covered with envelopes to prevent outcrossing. Finally, the M_2 seeds were collected per panicle in small envelopes, grouped per line and used in the succeeding mutant screening and segregation analyses.

Phenotypic screening of the M_2 generation and database construction

M_2 lines were planted to 15-20 plants per line in plastic seedling trays and grown for about 1 month inside the greenhouse at 25℃. Mutants were screened by checking for mutant phenotypes and their segregation patterns. Identified segregating lines, along with other normal-looking lines were transplanted in the field. Phenotypic screening and segregation analysis were also carried out during the reproductive stage. All data on the mutant lines and their observed characteristics were organized for the construction of a database of morphological mutants.

Results

In order to produce a diverse set of mutants with sweet sorghum background, mature SIL-05 seeds were irradiated with gamma ray and then sown. During the screening of the M_2 population, the following objectives were borne in mind: (1) isolation of morphological mutants with severe phenotypes that maybe useful for basic science because of their novelty, and (2) isolation of mutants with mild phenotypes that maybe useful for sweet sorghum breeding (e.g. semi-dwarf, lodging resistant phenotype).

At the seedling stage, exactly 566 out of the 5466 lines screened showed at least 1 or more phenotypic mutations per plant (entire blue circle in Figure 2). As some plants had multiple traits, a total of 1,046 phenotypic mutations were observed at the seedling stage (Table 1 and Figure 3). These were classified into 9 categories, namely, germination (late germination), growth (weak growth, early growth, etc.), leaf color (albino, pale green, yellow, etc.), leaf morphology (broad, narrow, short, etc.), plant height (extremely dwarf, severe dwarf, semi-dwarf and slender mutants), disease response (target spot-like), tillering (high tillering), culm morphology (thick culm), and others (abnormal gravitropism and abnormal plastochron). Examples of some of the mutants are shown in Figure 4. Among the phenotypic categories, plant height had the most number of phenotypic mutations, amounting to 69% of all the mutations observed (Figure 3). Under this category, severe dwarf was the most numerous (337 plants). The next category in terms of size was leaf morphology, although it had a much lower degree of occurrence (11%) than plant height (Figure 3).

Out of the 566 independent mutant lines from the seedling stage, 215 were further found to possess discernable mutant phenotypes at the heading stage (entire

brown circle in Figure 2). Also at this stage, another 106 independent lines that were previously normal at the seedling stage showed some mutant phenotypes. This brought the total of isolated independent lines in this study to 672. As some of these lines possessed multiple mutant traits, a total of 829 phenotypic mutations were observed at the reproductive stage (Table 2 and Figure 5). They fall under 10 categories, which included 2 new ones namely, heading date (early and late heading) and panicle morphology (short, long, long rachis, etc.), under which 95 and 39 mutations were observed, respectively. Photos of typical mutants are shown in Figure 6. As expected, plant height still had the most number of phenotypic mutations, amounting to 41% of the total phenotypic mutations (Figure 5). At this stage, plants with semi-dwarfism, most of them showing no adverse morphological effects, were found to be the most numerous. The next category in terms of size was culm morphology, which was only 21%. These results clearly indicate that plant height-related mutations are the main effects of gamma ray in sorghum.

Discussion

Construction of a mutant database is important for advanced sorghum genetic research. In this study, various mutants of different phenotypes ranging from severe to mild were isolated from the gamma ray-induced mutant library/population of sweet sorghum. From the seedling stage to the reproductive stage, a total of 11 phenotypic categories were noted. After analyzing the percentage of occurrence of phenotypic mutations, majority of the mutants at both the seedling and vegetative stages were found to exhibit height-related defects, especially dwarfism, which suggests that the gene network that controls plant height in sorghum is highly prone to disruption by gamma ray. In the case of rice and *Arabidopsis*, many genes linked to dwarfism, along with their mechanisms have been elucidated by studying mutants with loss-of-function mutations (Clouse *et al.*, 1996; Sato *et al.*, 1999; Ikeda *et al.*, 2001; Bouquin, 2003; Takeda *et al.*, 2003; Aya *et al.*, 2014). As for sorghum, using the generated mutant population in this study as a tool, isolation of many height-related genes could also be possible. Actually, genetic analyses of some of the height-related mutants isolated in this study are presented in Chapter 3.

Sweet sorghum has always been left behind by grain sorghums in terms of research priorities given to them. In fact, the existing sorghum genome sequence available since 2009 is derived from a grain sorghum variety, Btx-623. The data from this study is thus an important contribution to sweet sorghum research. Such data will be uploaded online in the form of a database to provide such information as accession/line numbers, phenotypes, gamma ray level used, mutated genes, the specific mutation sites, and others.

Another important use for the generated population is to serve as source of new parentals for sweet sorghum breeding geared towards biorefinery use. This is especially true with the isolated semi-dwarf mutants at the reproductive stage. Actually, one of these mutants, namely, S09P-2101 (later changed to *dw3-sd3*), had been genetically analyzed in Chapter 3. Characterization of these mutants in terms of agromorphological traits especially sugar content should also be necessary.

Taken altogether, the above observations show that gamma ray is a useful tool for inducing mutations in sweet sorghum. The wide range of generated mutants is not only attractive for basic research but also for breeding purposes.

Summary

Seeds of sweet sorghum cv. SIL-05 were mutagenized using approximately 200 Gy of gamma-ray irradiation. Phenotypic screening was carried out using 5,466 lines (M_2), of which, a total of 672 independent lines showed at least one phenotypic mutation at the seedling and reproductive stages in such categories as germination, growth, leaf color, leaf morphology, plant height, disease response, tillering, culm morphology, heading date, panicle morphology and others. Among these categories, plant height was the largest. The data from this study will be uploaded online to serve as a valuable reference on sweet sorghum mutants.

References

Ahloowalia, B.S., Maluszynski, M. and Nichterlein, K. (2004). Global impact of mutation-derived varieties. Euphytica **135**: 187–204.

Alsaadawi, I.S. and Dayan, F.E. (2009). Potentials and prospects of sorghum allelopathy in agroecosystems. Allelopath. J. **24**: 255–270.

Asencion, A.B. (2005). *Mutation breeding manual*. Philippine Nuclear Research Institute Press.

Aya, K., Hobo, T., Sato-Izawa, K., Ueguchi-Tanaka, M., Kitano, H. and Matsuoka, M. (2014). A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. Plant Cell Physiol. **55**: 897–912.

Bhaskara Rao, E.V.V. and Reddi, V.R. (1975). A radiation-induced highly productive mutant in sorghum. Radiat. Bot. **15**: 29–32.

Bouquin, T. (2003). The *Arabidopsis lue1* mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. J. Cell Sci. **116**: 791–801.

Clouse, S.D., Langford, M. and McMorris, T.C. (1996). A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. **111**: 671–678.

Haensel, H.D., Ross, J.G. and Huang., C.C. (1963). Irradiation-induced mutations in a colchicine-ractive genotype in sorghum. Crop Sci. **3**: 242–245.

Hanna, W.W. (1982). Mutation breeding of pearl millet and sorghum. Mutat. Breed. Rev. **1**: 1–13.

Hayat, K., Sadiq, M., Hashim, M. and Ilahi, M.F. (1990). Gamma radiation induced variation in some genetic parameters of sorghum varieties. Gomal Univ. J. Res. **10**: 75–81.

Henikoff, S. and Comai, L. (2003). Single-nucleotide mutations for plant functional genomics. Annu. Rev. Plant Biol. **54**: 375–401.

Henikoff, S., Till, B.J. and Comai, L. (2004). TILLING. Traditional mutagenesis meets functional genomics. Plant Physiol. **135**: 630–636.

Henner, W.D., Grunberg, S.M. and Haseltine, W. A. (1982). Sites and structure of gamma radiation-induced DNA strand breaks. J. Biol. Chem. **257**: 11750–4.

Hirochika, H. (2001). Contribution of the *Tos17* retrotransposon to rice functional genomics Hirohiko Hirochika. Curr. Opin. Plant Biol. **4**: 118–122.

Human, S., Andreani, S. and Indriatama, W.M. (2011). Stability test for sorghum mutant lines derived from induced mutations with gamma-ray irradiation. Atom Indones. **37**: 102–106.

Ikeda, A, Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M. and Yamaguchi, J. (2001). *slender* rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. Plant Cell **13**: 999–1010.

Kenga, R., Alabi, S.O. and Gupta, S.C. (2005). Heterosis and combining ability for grain yield and its components in induced sorghum mutants. African Crop Sci. J. **13**: 143–152.

Komorisono, M., Ueguchi-Tanaka, M., Aichi, I., Hasegawa, Y., Ashikari, M., Kitano, H., Matsuoka, M. and Sazuka, T. (2005). Analysis of the rice mutant *dwarf and gladius leaf 1*. Aberrant katanin-mediated microtubule organization causes up-regulation of gibberellin biosynthetic genes independently of gibberellin signaling. Plant Physiol. **138**: 1982–1993.

Larik, A.S., Memon, S. and Soomro, Z.A. (2009). Radiation induced polygenic mutations in *Sorghum bicolor* L.. J. Agric. Res. **47**: 11–19.

Lo, S.-F., Yang, S.-Y., Chen, K.-T., Hsing, Y.-I., Zeevaart, J. a D., Chen, L.-J. and Yu, S.-M. (2008). A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. Plant Cell **20**: 2603–2618.

Mehlo, L., Mbambo, Z., Bado, S., Lin, J., Moagi, S.M., Buthelezi, S., Stoychev, S. and Chikwamba, R. (2013). Induced protein polymorphisms and nutritional quality of gamma irradiation mutants of sorghum. Mutat. Res. **749**: 66–72.

Miyao, A., Iwasaki, Y., Kitano, H., Itoh, J., Maekawa, M., Murata, K., Yatou, O., Nagato, Y. and Hirochika, H. (2007). A large-scale collection of phenotypic data describing an insertional mutant population to facilitate functional analysis of rice genes. Plant Mol. Biol. **63**: 625–635.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, a, Yoshida, S., Li, J. and Chory, J. (1999). Arabidopsis *det2* is defective in the conversion of (24*R*)-24-methylcholest-4-En-3-one to (24*R*)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. Plant Physiol. **120**: 833–840.

Ordonio, R.L., Ito, Y., Hatakeyama, A., Ohmae-Shinohara, K., Kasuga, S., Tokunaga, T., Mizuno, H., Kitano, H., Matsuoka, M. and Sazuka, T. (2014).

Gibberellin deficiency pleiotropically induces culm bending in sorghum: an insight into sorghum semi-dwarf breeding. Sci. Rep. **4**: 5287.

Qu, S., Jeon, J.-S., Ouwerkerk, P.B.F., Bellizzi, M., Leach, J., Ronald, P. and Wang, G.-L. (2009). Construction and application of efficient *Ac-Ds* transposon tagging vectors in rice. J. Integr. Plant Biol. **51**: 982–92.

Rao, P.S., Deshpande, S., Blümmel, M., Reddy, B.V.S. and Hash, T. (2012). Characterization of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). Eur. J. Plant Sci. Biotechnol. **6**: 71–75.

Saito, T., Ariizumi, T., Okabe, Y., Asamizu, E., Hiwasa-Tanase, K., Fukuda, N., Mizoguchi, T., Yamazaki, Y., Aoki, K. and Ezura, H. (2011). TOMATOMA: a novel tomato mutant database distributing Micro-Tom mutant collections. Plant Cell Physiol. **52**: 283–296.

Sakamoto, T., Miura, K., Itoh, H., Tatsumi, T. and Ueguchi-Tanaka, M. (2004). An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol. **134**: 1642–1653.

Sato, Y., Sentoku, N., Miura, Y., Hirochika, H., Kitano, H. and Matsuoka, M. (1999). Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. EMBO J. **18**: 992–1002.

Takeda, T., Suwa, Y., Suzuki, M., Kitano, H., Ueguchi-Tanaka, M., Ashikari, M., Matsuoka, M. and Ueguchi, C. (2003). The *OsTB1* gene negatively regulates lateral branching in rice. Plant J. **33**: 513–520.

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C., Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L. and Henikoff, S. (2003). Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. **13**: 524–530.

van Harten, A.M. (1998). *Mutation breeding theory and practical applications*. Cambridge University Press.

Tables and Figures

Figure 1. Sorghum bicolor cv. SIL-05 (sweet sorghum) used for gamma-ray mutagenesis in this study. Gross morphology of SIL-05 at the reproductive stage. Scale bar = 1 m.

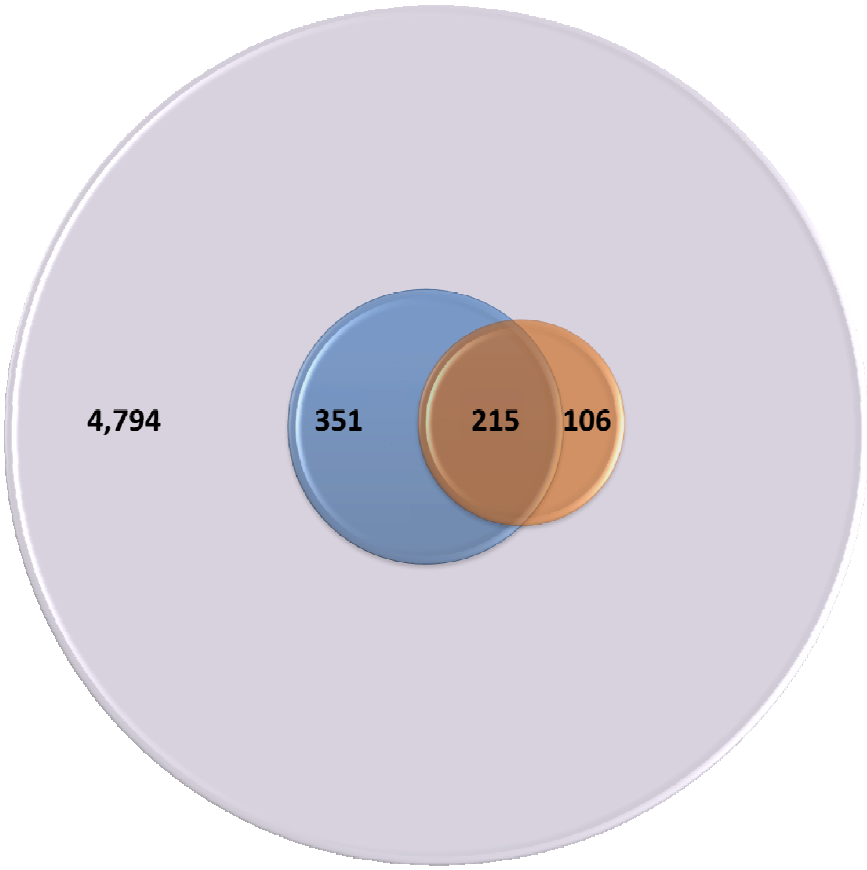

Figure 2. Venn diagram showing the gamma ray-treated population consisting of 5,466 independent M_2 lines phenotypically analyzed in this study. Gray circle represents the entire mutant population treated with ~200 Gy of gamma ray. Blue and brown circles represent the lines with morphological mutations at the seedling stage and reproductive stage, respectively.

Table 1. Cumulative tally of phenotypic mutations observed at the seedling stage.

Category	Phenotype	No. of occurrences
germination	late germination	23
growth	weak growth	22
	early growth	7
	twin shoot	1
leaf color	albino	7
	pale green leaf	22
	yellow leaf	3
	dark-green leaf	29
	virescent leaf	21
	abnormal chlorophyll	2
	purple leaf	1
leaf morphology	broad leaf	7
	narrow leaf	36
	short leaf blade	3
	long leaf blade	4
	short leaf sheath	6
	droopy leaf	7
	erect leaf	3
	rolled leaf	4
	spiral leaf	6
	wavy leaf	16
	brittle	1
	soft leaf	2
	straw leaf blade	3
	withering	4
	malformed leaf	9
plant height	extremely dwarf	139
	severe dwarf	337
	semi dwarf	237
	slender	4
disease response	target spot-like	7
	leaf blight	34
	spl/lesion mimic	6
tillering	high tillering	20
culm morphology	thick culm	8
others	abnormal gravitropism	2
	abnormal plastochron	3
		1046

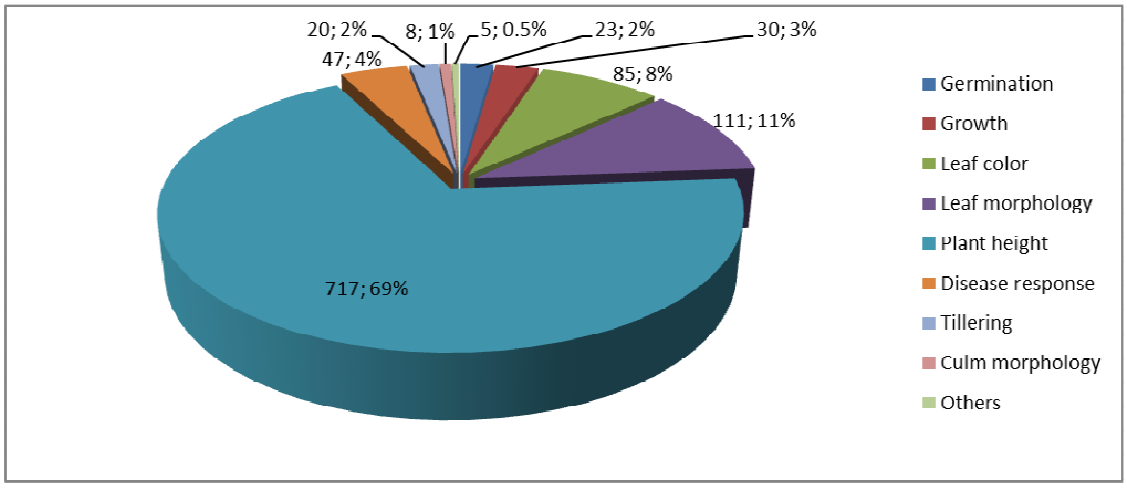

Figure 3. Breakdown of the 1,046 phenotypic mutations observed at the seedling stage. The category, Plant height, had the most number of occurrences among the 566 independent lines observed at the seedling stage.

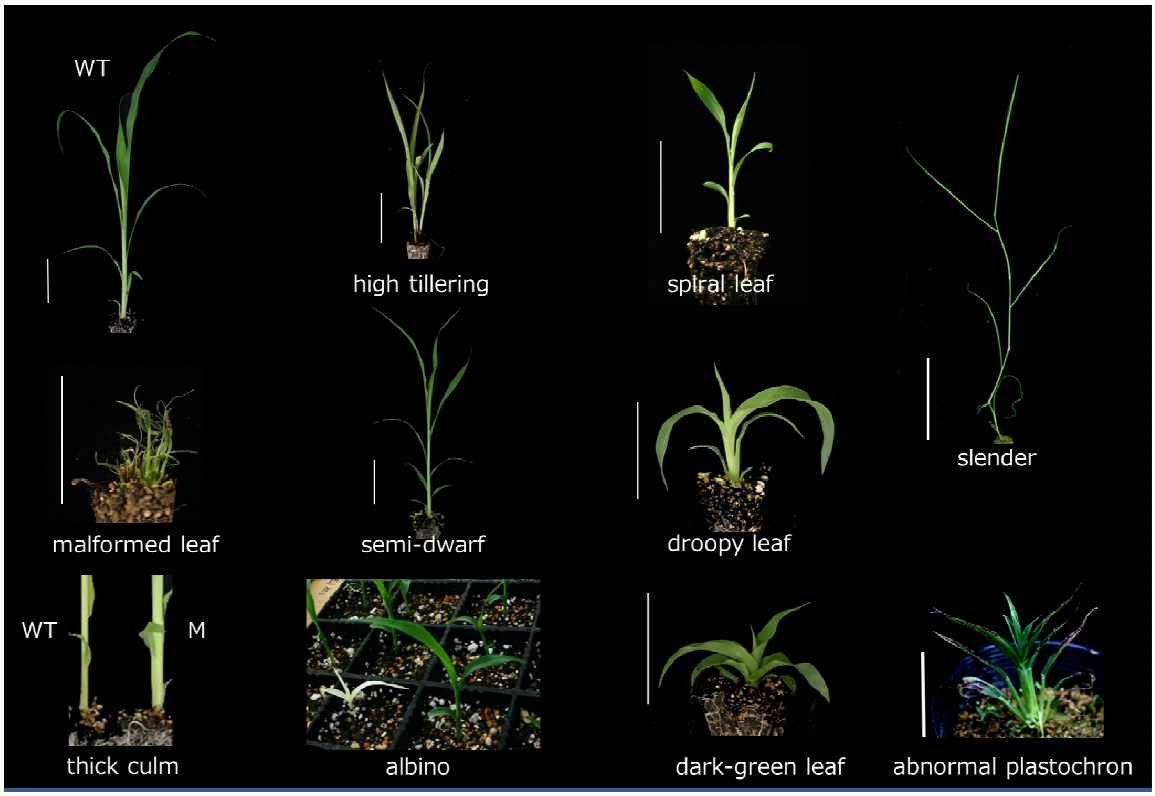

Figure 4. Typical mutants isolated at the seedling stage. Scale bars = 5 cm.

Table 2. Cumulative tally of phenotypic mutations observed at the reproductive stage.

Category	Phenotype	No. of occurrences
growth	weak growth	27
leaf color	pale green leaf	5
	yellow leaf	4
	dark-green leaf	5
	virescent leaf	5
	stripe leaf	1
leaf morphology	wide leaf	18
	narrow leaf	30
	short leaf blade	5
	erect leaf	6
	droopy leaf	1
	rolled leaf	2
	wavy leaf	2
	soft leaf	1
plant height	extremely dwarf	5
	severe dwarf	112
	semi dwarf	185
	long culm	41
disease response	spl/lesion mimic	5
tillering	high tillering	54
	branching from upper internodes	4
culm morphology	slender culm	94
	thick culm	65
	pithy	2
	short upper internode	2
	short lower internode	1
	incomplete panicle exertion	5
	many internodes	1
	bending culm	5
heading date	early heading	56
	late heading	39
panicle morphology	short panicle	14
	long panicle	5
	long rachis	3
	dense panicle	3
	sparse panicle	5
	shattering	4
	abnormal panicle shape	1
	abnormal flower	1
	multiple panicles	1
	lax panicle	2
others	chimera-like	2
		829

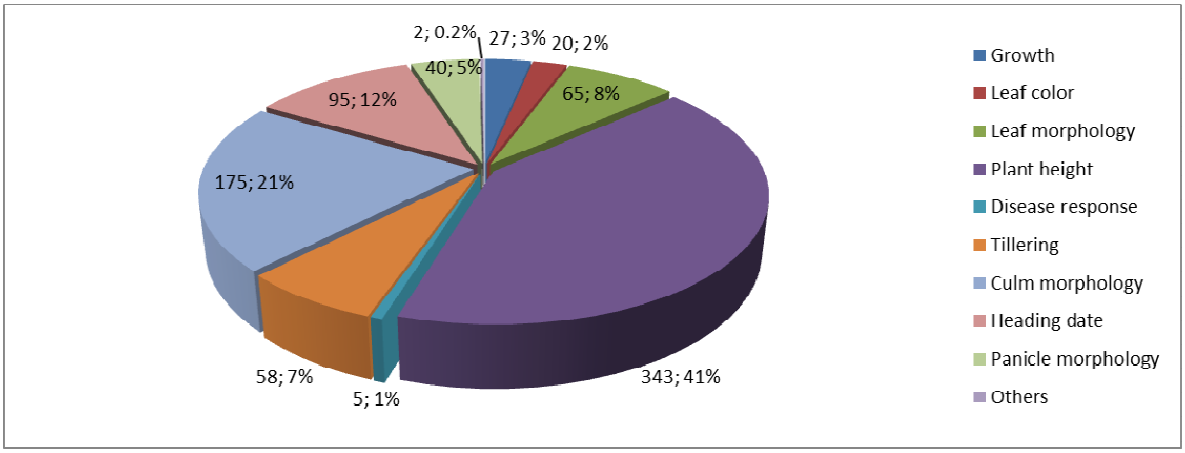

Figure 5. Breakdown of the 829 phenotypic mutations observed at the reproductive stage. The category, Plant height, had the most number of occurrences among the 322 independent lines observed at the reproductive stage.

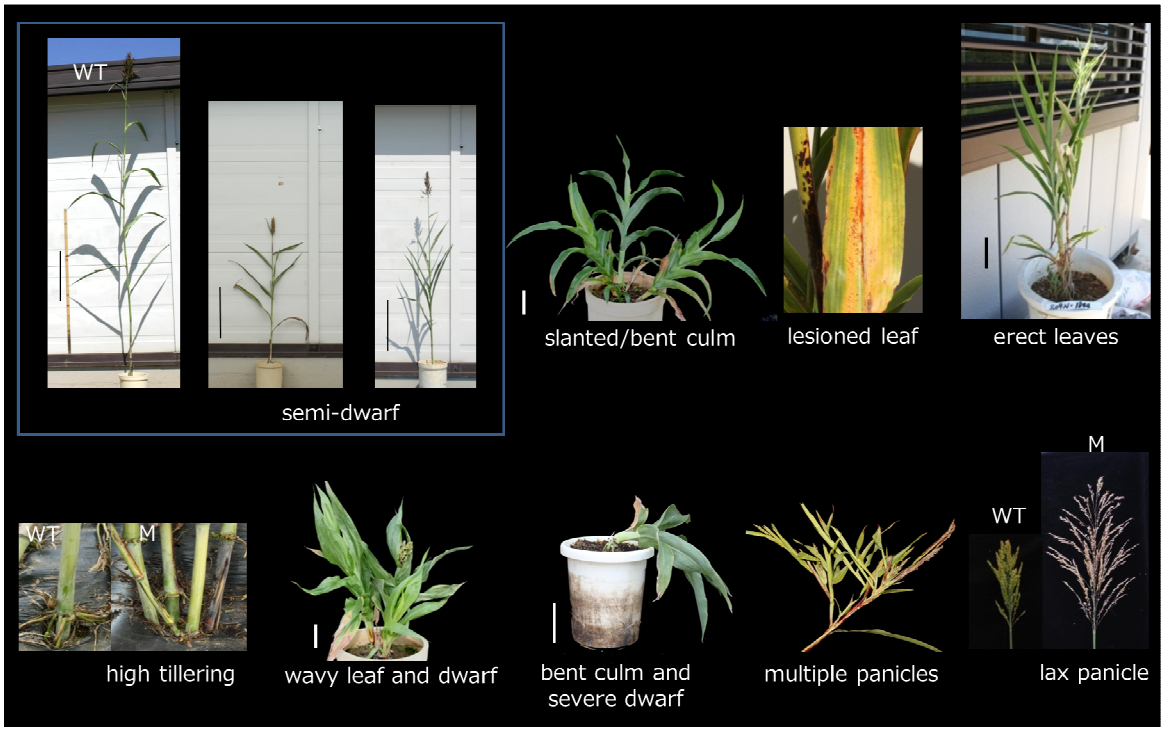

Figure 6. Typical mutants isolated at the reproductive stage. Scale bars inside blue box = 50 cm. Scale bars outside = 5 cm.

Chapter 3

Mutant Analysis: Identifying the causal genes of height-related mutants of sweet sorghum and their implications for agriculture

Introduction

Genetic mutations are often accompanied by changes in phenotype and such changes reveal the biological function of affected genes. This is the reason why mutants have been regarded as powerful tools for genetic research in rice, *Arabidopsis*, and other species. As for sorghum, there are currently just a few reported mutants in the literature. These include the naturally occurring mutants like *candystripe* (*y-cs*) (Zanta *et al.*, 1994), *ma3* (*phytochrome B*) (Childs *et al.*, 1997), *maturity 1* (*ma1*) (*PRR37*) (Murphy *et al.*, 2011), *dwarf 3* (*dw3*) (*pgp1/br2*, *dw3-sd1* and *dw3-sd2*) (Multani *et al.*, 2003; Barrero Farfan *et al.*, 2012) and *ma6* (*ghd7*) (Murphy *et al.*, 2014). Other known sorghum mutants were products of different mutagenic techniques which include the low lignin *brown midrib* (*bmr*) mutants that are defective in lignin-biosynthetic genes such as *caffeic acid-O-methyltransferase* (*COMT*) (*bmr12*, *bmr18* and *bmr26*), *cinnamyl-alcohol dehydrogenase* (*CAD*) (*bmr6*), and *4-coumarate: coenzyme A ligase* (*4CL*) (*bmr2*) (Bout and Vermerris, 2003; Vogler *et al.*, 2009; Rao *et al.*, 2012; Saballos *et al.*, 2012); the low toxicity cyanide-free *dhurrinase 2* (Krothapalli *et al.*, 2013), *totally cyanide deficient1* (*tcd1*) and *elevated cyanide potential1* (*ecp1*) mutants (Blomstedt *et al.*, 2012); the herbicide-resistant mutant with an amino acid substitution in sorghum acetohydroxyacid synthase (AHAS) protein subunit (Uriarte *et al.*, 2013); the mutants defective in Phytochrome A (PhyA) and myoinositol kinase I (MIKI) (low phytic acid) identified through TILLING (Xin *et al.*, 2008); and the *highly digestible high-lysine* (*hdhl*) mutant with reduced accumulation of the main storage protein of sorghum, kafirin (Wu *et al.*, 2013).

As mentioned in Chapter 2, height-related mutations occurred with the highest

frequency in the gamma ray-induced mutant population of sweet sorghum in this study. This chapter thus focuses on sorghum mutants with height-related traits, namely, dwarfism and slenderness.

Dwarfism in plants is brought about by an irregularity in one or more of the various growth-related mechanisms, and may involve physical defects in some cellular growth processes, or problems in the production and action of phytohormones. So far, aberrant cellular division or elongation has already been reported to cause dwarfism in some mutants such as the rice *d6* (Sato *et al.*, 1999), *bent uppermost internode 1* (*bui1*) (Yang *et al.*, 2011), *jmj703* (Cui *et al.,* 2013) and *gladius leaf* (*dgl-1*) (Komorisono *et al.,* 2005), and the *Arabidopsis lue1* (Bouquin *et al*., 2003). However, the relationship between dwarfism and phytohormones has been more widely studied using various kinds of plant species. In the case of auxin, some of the reported dwarf mutants include the maize *brachytic 2* (*br2*) (Multani *et al.,* 2003; Knöller *et al*., 2010) and the sorghum *dwarf 3* (*dw3*) (Multani *et al.,* 2003) which both have semi-dwarfism due to a defect in auxin transport; the rice *small organ 1* (*smos1*), which has a defective transcription factor that disrupts auxin signaling (Aya *et al.,* 2014), and the auxin-deficient *tdd1* (Sazuka *et al.,* 2009); and the *Arabidopsis sax1* which shows high sensitivity to auxin (Ephritikhine *et al*., 1999), and *bud1* which has a defect in auxin metabolism (Dai *et al*., 2003). Another growth-promoting hormone is brassinosteroid (BR). Mutants that have a defect in BR biosynthesis and signaling show severe dwarf phenotypes as in the case of the *Arabidopsis* BR-deficient *de-etiolated 2* (*det2*) (Noguchi *et al.,* 1999) and *seuss-1* (*seu-1*) (Nole-Wilson *et al*., 2010), and the brassinosteroid-insensitive *bri1* (Clouse *et al*., 1996); the rice BR signaling mutants, *d61-4* (Nakamura *et al.,* 2006) and *erect leaf 1* (*elf1-1*) (Sakamoto *et al*., 2013), and the BR-deficient *ebisu dwarf* (*d2*) (Hong *et al.,*

2003); and the tomato *br6ox* (BR biosynthesis) mutant "Micro-Tom", a commercial cultivar (Marti *et al*., 2006). BR-related mutations can also give rise to semi-dwarfism, and among them, a barley mutant, *uzu*, was used for producing a lodging-resistant variety (Chono *et al.,* 2003). Recently, strigolactones (SLs), a group of newly identified plant hormones that control plant shoot branching, have also been implicated for dwarfism in high-tillering dwarf mutants as in the case of the SL-deficient *d10* and the SL-insensitive *d53* and *d14* mutants of rice (Arite *et al.,* 2009; Jiang *et al.,* 2013).

Among the phytohormones, gibberellin (GA) is the most well known to be involved in controlling stem elongation, and a deficiency or insensitivity to GA could easily result in severe dwarfism as reported in many different kinds of plant species such as the rice mutants independently mutated in any of the six GA-biosynthetic enzymes, *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), GA20-oxidase (GA20ox), and GA3-oxidase (GA3ox) (Sakamoto *et al*., 2004); the pea GA-deficient *na-1* (Davidson *et al*., 2003); the *Arabidopsis* GA-insensitive *short internodes* (*shi*) (Fridborg *et al*., 1999) and *ga-insensitive* (*gai*) (Peng *et al.*, 1997), and the GA-deficient *ga1 – 3* mutants (Sun and Kamiya, 1994); the barley GA-deficient *grd2c* mutant (Wolbang *et al.,* 2007) and the GA-insensitive *sln1* mutants (Chandler *et al.*, 2002); and the potato *andigena* (*adg*) mutant (Bamberg and Hanneman, 1991). GA-related dwarfism can also be caused by viral agents that lower the levels of active GA in plants, as in the case of rice dwarf virus (Zhu *et al.*, 2005) and rice tungro bacilliform virus (RTBV) infection (Petruccelli *et al.*, 2001). Across different species, GA-related mutants with severe dwarfism show several consensus phenotypes such as small dark-green leaves, delayed germination, defective flowering, reduced seed production and male sterility (Bamberg

and Hanneman, 1991; Hedden and Phillips, 2000; Sakamoto *et al*., 2004; Fleet and Sun, 2005; Tanimoto, 2005; Wang and Li, 2005; Chhun *et al.,* 2007; Lo *et al.,* 2008), whereas, semi-dwarf mutants only show mild height reduction with no adverse effects on overall morphology or agronomical traits. This is the reason why GA-related mutants with semi-dwarfism were widely used in the history of crop production. Actually, the rice *semi-dwarf1* (*sd1*) and wheat *Reduced height-1* (*Rht-1*) mutants became the highlights of the Green Revolution, which avoided the imminent food shortage of the mid-20th century (Hedden, 2003). Later on, the *SD1* gene was characterized as a GA20-oxidase (Sasaki *et al.,* 2002; Spielmeyer *et al*., 2002), which catalyzes the penultimate step of GA biosynthesis, while the *Rht1-1* was found to encode a constitutively active repressor of GA signaling (Peng *et al.,* 1999). Further, another GA-deficient mutant defective in KO, *Tan-ginbozu*/*d35*, also contributed to rice crop productivity due to its lodging resistance (Itoh *et al.,* 2004).

Aside from dwarfism, another type of height-related defect is the abnormal increase in height of plants. This phenomenon is also reported to be mainly due to GA-related anomalies, as in the case of the rice *slr1* mutant (Ikeda *et al.*, 2001; Ueguchi-Tanaka *et al.*, 2005, 2007), which has a defective GA signaling repressor, DELLA protein, and in the case of the classical "bakanae" disease of rice (Yabuta and Sumiki, 1938) caused by *Gibberella fujikuroi*.

In contrast to the other plant species above, there are still no other reports of height-related mechanisms (including those relating to GA) in sorghum aside from the auxin-related semi-dwarf *dw3* mutants mentioned earlier. Until now, even the underlying mechanisms behind the major dwarfing QTL genes (*dw1*, *dw2* and *dw4*) used in the history of sorghum breeding have remained unknown (Wang *et al*., 2012;

Morris *et al.,* 2013; Thurber *et al*., 2013). Aside from these classical dwarfing genes, increasing the number of available dwarfing mechanisms for sorghum should be important not only for breeding but also for basic research.

Here, I present the isolation of 5 GA-biosynthetic severe dwarf mutants (*bdw1-bdw4*) that show identical unusual bent culms, 3 GA-signaling slender (constitutive) mutants (*slr1-1 – 2*), 1 BR-biosynthetic severe dwarf mutant (*br6ox*) and 1 auxin-related semi-dwarf mutant (*dw3-sd3*) from our gamma ray-induced sweet sorghum mutant population. Aside from the direct contribution of these mutants for sorghum genetic research, the results also reveal some important findings that are believed to have significant implications for sorghum breeding.

Materials and Methods

Plant materials

All identified mutants, including *bdw1–bdw4*, *slr1-1*, *slr1-2*, *br6ox*, *dw3-sd3* and WT plants for analysis were grown in pots inside a temperature-controlled greenhouse under ambient lighting conditions.

DNA isolation

Total genomic DNA isolation was done using the modified CTAB (hexadecyltrimethylammonium bromide) method (Murray and Thompson, 1980). Plant tissues were thoroughly ground using mortar and pestle with the aid of liquid nitrogen and quickly transferred into Eppendorf tubes containing 0.4 ml of 2x CTAB extraction buffer, followed by mixing. The tubes were incubated at 65ºC for 30 min and then added with 0.4 ml of chloroform-isoamyl solution (24:1), followed by centrifugation at 15,000 rpm for 15 min. Afterwards, supernatant was transferred into a new Eppendorf tube, mixed with an equal volume of isopropanol (~360 µl) and then centrifuged at 15,000 rpm for 20 min. Liquid portion was discarded and pellet was rinsed by adding 0.5 ml of 70% ethanol. Tubes were centrifuged at 15,000 rpm for 10 min and the ethanol was discarded. After vacuum-drying the tubes for 5 min, 50 µl of TE solution containing 100 ng/µl of RNAse A was added to dissolve the pellet. DNA concentration was quantified using Beckman DU 640 Spectrophotometer by aliquoting 1 µl of the extracted DNA with 99 µl of sterilized Milli-Q (Millipore) water.

BLAST, primer design, PCR and DNA sequencing

Amino acid sequences of known GA- and BR-biosynthetic genes and auxin-related genes of rice were used as queries for the TBLASTN program (http://www.phytozome.net/search.php?show=blastandorg=Org_Sbicolor_v2.1) to identify homologous sequences in the sorghum genome. PCR analysis of the GA-related genes such as *SbCPS1* (*Sobic.001G248600*), *SbKS1* (*Sobic.006G211500*), *SbKO1* (*Sobic.010G172700*), *SbKAO1* (*Sobic.010G007700*), *SbGA20ox2* (*Sobic.003G379500*), *SbGA3ox2* (*Sobic.003G045900*), and *SbSLR1*(*Sobic.001G120900*); BR-related genes such as *SbBR6ox/SbBRD1* (*Sobic.001G172400*), *SbD2* (*Sobic.003G030600*), *SbD11* (*Sobic.006G114600*) and *SbBRI1/D61* (*Sobic.003G277900*); and auxin-related *SbDw3* (*Sobic.007G163800*), was done on concerned mutants and the WT using the PCR primers listed in Table 5.

PCR solution constituted of sterilized Milli-Q water, 15.15 µl; 10x PCR buffer, 3 µl; 2 mM dNTPs, 3.75 µl; DMSO, 1.5 µl; taq polymerase, 0.6 µl; 10 pmol forward and reverse primer, 1.5 µl each; and 40 ng/µl genomic DNA template, 3µl. For amplification, GeneAmp PCR System 9700 was employed. Cycling conditions were varied based on the requirement of each specific primer sets.

PCR products were run on 1.5% agarose gel. Afterwards, desired bands were cut out and eluted using the Wizard SV Gel and PCR Clean-up System kit (Promega).

One-sided PCR solution consisted of 0.25x reaction buffer (Applied Biosystems), 4 µl; sterilized Milli-Q water, 2.84 µl; 10-pmol primer, 0.16 µl; and eluted DNA, 3 µl. Cycling condition was set at: 25 cycles of 96°C, 10 sec; 50°C, 5 sec; and 60°C, 4 min; and 25°C holding temperature. After PCR, 10 µl of isopropanol was added into each well, followed by pipet-mixing (~10x) and centrifugation at 3,000 rpm for 30 min. The

plate was then inverted and spun at 300 rpm for 30 sec. The wells were briefly rinsed with 200 µl of 75% ethanol, inverted and spun at 300 rpm for 30 sec and then vacuum-dried for 5 min.

Prior to sequencing, 20 µl of deionized formamide was added into each well, followed by pipet-mixing (~10 times). The samples were denatured at 95°C for 3 min on a thermal cycler and then quickly put on ice while shielding from light. DNA sequencing was done with the ABI Prism 310-10 sequencer following the manufacturer's protocol.

Clustal analysis and phylogenetic tree construction

Deduced amino acid sequences of sorghum CPS1, KS1, KO1 and KAO1, SLR1, BR6ox and Dw3 were compared with those of other species by aligning with ClustalW (http://www.genome.jp/tools/clustalw/) using default settings (slow/accurate) and then manually adjusted to optimize alignments. The unrooted phylogenetic tree with branch length (N-J) tree file output was used to create a phylogenetic tree with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) using default settings for the rectangular tree layout.

Total RNA isolation, cDNA synthesis and semi-quantitative RT-PCR analysis

Total RNA was isolated using a modified Trizol method (Chomczynski and Sacchi, 1987). For cDNA synthesis, Omniscript RT Kit (Qiagen) was used. Prior to cDNA synthesis, a master mix consisting of 10x buffer RT, 2 µl; 5 mM dNTPs, 2 µl ; 10 µM oligo-dT primer, 1µl; 40 units/µl RNAse inhibitor, 0.25µl; and Omniscript reverse transcriptase, 1 µl, was prepared. Afterwards, 1 µg RNA and RNAse-free water were

mixed to a total volume of 13.75 µl in PCR tubes. This was followed by incubation at 65ºC for 5 min and then placed on ice. Exactly 6.25 µl of the master mix was dispensed into each well. PCR cycling condition was set at: 37ºC, 60 min; 93ºC, 5 min; and 20ºC, infinite. cDNA concentration was determined with a Beckman DU 640 Spectrophotometer. All the cDNA per well was diluted at once to 40 ng/µl for use in semi-quantitative RT-PCR. Semi-quantitative RT-PCR was carried out as in standard PCR above. Primers used were designed to span two succeeding exons to be sure that the amplified bands originate from nothing else but the cDNA, in case of genomic DNA contamination. Cycling conditions were optimized and adjusted as required by each specific primer sets and cDNA samples.

Microscopic analysis

A culm longitudinal section of the *bdw3-1* mutant was made with a cryotome. For staining, hematoxylin and eosin stains were used. Photographs were taken using an Olympus photomicroscope connected to a CCD camera. Resulting photographs were stitched to create a panoramic image (Photostitch, Canon). In order to understand the underlying cause of the bending in the *bdw3-1* mutant, both the upper and lower sides of the fifth internode, where the greatest degree of bending took place, were analyzed by checking the length and number of cells in 5 cell files within a 500-µm long region (orange rectangles, Figure 7D) and then estimating the total number of cells in the internode.

Gravitropism test

Seeds of the *bdw1* and *bdw3-1* mutants (as representatives of the phenotypically identical *bdw* mutants) and WT were disinfected with 50x antiformin for 30 min and rinsed 5 times with milliQ water followed by soaking in 5x benlate solution overnight at 4ºC and then sowed on MS medium with 0.9% agar in plastic rectangular culture plates. The plates were prepared as follows: After autoclaving at 120ºC for 15 min, the medium was poured into sterile plastic rectangular culture plates up to the brim and then allowed to solidify. As soon as it was hard, a rectangular piece of agar, about 7.5 cm long, was cut away from one end of the plate to make room for seed sowing and seedling growth. Seeds were sown aseptically and grown under lighted condition at 25ºC up to 4 days after germination (DAG). Afterwards, the plates were rotated 90º and examined 4 days after rotation (DAR). Plate imaging was done by using a flatbed digital scanner. Also, bent *bdw1* plants at the vegetative stage were rotated 90 degrees following the direction of culm bending and compared their response to the WT.

Genome mapping of the bdw3 locus and sequence analysis

To carry out gene mapping, heterozygous siblings of the *bdw3-1* mutant (sweet sorghum (SIL-05) background) were crossed with bmr-6 (grain sorghum cultivar) to produce F_1 plants that were progeny-tested to obtain the desired F_2 population for analysis. Segregation ratio of the tall and dwarf phenotypes was observed and analyzed by chi-square test.

About 1,500 Simple Sequence Repeats (SSR) markers (Yonemaru *et al.,* 2009) were screened and 162 polymorphic markers were selected. For rough mapping of the candidate region, 16 F_2 seedlings with tall phenotype were analyzed using the SSR

markers. For fine mapping, the analysis was expanded to a total of 249 F_2 tall plants.

Southern blotting analysis

DNA gel blot analysis was done to analyze the *SbCPS1* and *SbKO1*genes of *bdw1, bdw3-1* and WT using 1 μg genomic DNAs following a previously described protocol (Miura *et al*., 2009). Primers used to amplify the probes are listed in Table 5.

Agrobacterium-mediated transformation

To examine the bioactivities of the identified sorghum GA-related genes, 11,287, 6,938, 7,628, 5,909 and 4,800 of sorghum genomic DNA fragments containing entire *SbCPS1*, *SbKS1*, *SbKO1*, *SbKAO1* and *SbSLR1* genes along with their native promoters, respectively, were cloned into a pCAMBIA1300 binary vector. On the other hand, 5991 bp of *SbBR6ox*/*SbBRD1* genomic DNA was cloned into pCAMBIA 1380 vector. The chimeric plasmids were respectively introduced into rice *oscps1-1*, *osks1-1*, *osko2-1*, *oskao-1*, T-65 and T2-177 through *Agrobacterium*-mediated transformation (Sakamoto *et al*., 2004; Hiei and Komari, 2008). The transformants were screened on selection medium with hygromycin and the regenerated plants were documented.

Exogenous gibberellin treatment, uniconazole and brassinolide treatments

For GA treatment, two-week old *bdw3-1* seedlings were transplanted into pots. A set of three pots (with holes in the bottom) were placed in a square plastic vat containing a designated concentration of GA_3 (0, 1 x 10^{-8}, 1 x 10^{-7}, 1 x 10^{-6}, 1 x 10^{-5} and 1 x 10^{-4} M) and were grown continuously in the solution for four months inside the greenhouse. For uniconazole treatment, a set of five 4-week-old WT plants (SIL-05)

were grown continuously for 2 months in a vat containing specified concentrations of uniconazole (0, 1 x 10^{-7}, 3 x 10^{-7}, 1 x 10^{-6}, 3 x 10^{-6} and 1 x 10^{-5} M). In both GA and uniconazole experiments, the vats were regularly checked and augmented with water whenever necessary to guard against evaporation and fluctuation in the concentration of the original solution until the completion of the experiment. Measurement of plant height was done with a rope by tracing the contour of the main culm from the base to the longest tip. Culm angle was taken by measuring the angle formed by the culm against the vertical axis. For BL treatment, the BR-related mutant was sprayed with 0.25 ppm (5.22 x 10-7 M) BL (Maity and Bera, 2009) every day for 3 weeks.

DNA and RNA-seq analysis by Next Generation Sequencing (NGS)

Two isogenic varieties, tall white sooner milo (*Dw1Dw1*) and dwarf white milo (*dw1dw1*) carrying gain-of-function and loss-of-function alleles of *Dw1*, respectively, were utilized for genomic DNA sequence analysis using NGS. Sequencing was performed at Hokkaido System Science Co. Ltd. (Sapporo, Japan). Library for sequencing was prepared using TruSeq DNA/RNA Sample Prep Kit (Illumina, Inc.) according to the manufacturer's protocol. The resultant library was sequenced (2 x 101 cycles, paired-end) on HiSeq 2500/2000 instrument using TruSeq PE Cluster Kit and TruSeq SBS Kit (Illumina, Inc.) to obtain approximately 40 million raw sequence reads for analysis. Sequences were mapped to the BTx623 sorghum reference genome (Paterson *et al.,* 2009) by using BWA version 0.6.1-r104 (http://bio-bwa.sourceforge.net/), and SNPs were called with the SAMtools version 0.1.18 (http://samtools.sourceforge.net/). The two varieties were also subjected to RNA-seq analysis by using elongating internode total RNAs. Total RNAs were utilized for RNA-

seq experiment as previously described (Mizuno *et al.,* 2012).

Results

I. Analysis of GA-deficient severe dwarf mutants

During the process of screening for dwarf phenotypes, several unusual dwarf mutants, which developed bent culms were isolated. Succeeding analyses revealed that they were mutated in four different loci, hence, they were named *bending dwarf* (*bdw*)*1* - *bdw4*, with *bdw3* having two different alleles (*bdw3-1* and -*2*). At the seedling stage, the bending culm phenotype was not evident in the mutants (Figure 7A) but severe inhibition of internode elongation was observed (Figure 7B). At about three and a half months after sowing, the bent culm phenotype was clearly observed (Figure 7C). The mutants bent in a prostrate manner with leaves extending from the sides of the culm (rightmost in Figure 7C).

In order to study the underlying reason for the bending, histological analysis of the *bdw3-1* culm (Figure 7D) was carried out. The cell length at the upper side of the bent region was significantly shorter than that of the lower side (Figure 7D, E), suggesting that there is a faster rate of cell proliferation at the upper side. Actually, the cell number at the upper side was more than two-folds higher than that at the lower side (Figure 7F). These observations suggest that the bending was due to a difference in cell proliferation rates between the upper and lower sides of the bent culm.

The bdw mutants have reduced gravitropic response.

As the gravitropic perception of the mutants might be defective, the response of the *bdw1* culm to gravity at two stages, seedling and vegetative (Figure 8) was directly examined. The aerial part of *bdw1* at the seedling stage responded positively to

gravistimulation, but with a much weaker response compared to the WT (Figure 8A), possibly due to its naturally inhibited culm elongation. Such weak gravitropic response was also observed at the late vegetative stage (Figure 8B). Aside from the culm, the roots of *bdw1* also responded positively but much more slowly to gravistimulation at the seedling stage (Figure 8A), as observed in another *bdw* mutant, *bdw3-1* (Figure 8C), strongly suggesting that the *bdw* mutants have diminished ability to respond to gravity.

A loss of function of GA-biosynthetic genes causes the bending dwarf phenotype.

To further characterize the *bdw* mutants, positional cloning of the *BDW3* gene was carried out. F_2 plants derived from the cross between *bdw3-1* and the cultivar bmr-6 were used. These plants segregated into two phenotypic groups, tall and dwarf, with a segregation ratio of 3:1 ($P<0.05$), respectively. By genotyping 249 F_2 plants, the *BDW3* locus was narrowed down to approximately 4.0-Mb region between the markers Sb5370 and SSR5_5423 (Figure 9A). This region contains 283 genes annotated in the sorghum genomic DNA sequence database (http://www.phytozome.net/), including *Sobic.010G172700* (Table 3), a gene which is homologous to the rice *ent-kaurene oxidase* (*KO*) that encodes a GA-biosynthetic enzyme (Sakamoto *et al*., 2004) (Figure 10). In rice and *Arabidopsis*, the loss of function of KO causes a severe dwarf phenotype with small dark-green leaves (Helliwell *et al.,* 1998; Sakamoto *et al*., 2004), which was also evident in *bdw3-1* except that it had an added bent culm trait (Figure 7C). The deduced amino acid sequence of *Sobic.010G172700* showed a high similarity to the entire sequence of rice KO (67%) and also to that of *Arabidopsis* KO (56%) (Figure 11) (Helliwell *et al.,* 1998; Sakamoto *et al*., 2004). Possible mutation in the genomic DNA sequence encompassing the *SbKO* gene was probed by using PCR, the

result of which showed no PCR products from the genome of *bdw3-1* (upright red triangles, Figure 9B), whereas the same primers produced PCR products from the WT genome at the same condition. By using primers covering the flanking sequences of the *SbKO1* gene, sequences that yielded PCR products (upright blue triangles KO1, KO2, KO18 and KO19, Figure 9B) were successfully located, showing the deletion around the *SbKO1* gene to be at about 45 kb (KO3 to KO17). The same experiment was also performed on another *bdw3* mutant, *bdw3-2*, and found that it contained a shorter deletion (~17 kb) involving all portions of its *SbKO1* gene (inverted red or blue triangles in Figure 9B). A genomic DNA blotting analysis using the promoter sequence of the *SbKO1* gene as probe also confirmed the large deletion in the corresponding region of the *bdw3-1* genome (Figure 12C, D).

The above results suggest that mutation in the *SbKO1* gene could be the reason for the severe dwarfism in the *bdw3* mutants. To determine whether such loss of function of GA-biosynthetic genes is behind the phenotype of all the *bdw* mutants, the sequences of the other GA-biosynthetic genes (*CPS*, *KS*, *KAO*, *GA20ox*, and *GA3ox*) in the remaining mutants were examined. As expected, the genome of *bdw1* contained an approximately 16-kb deletion involving the entire genomic region of *SbCPS1* (*Sobic.001G248600*) (Figure 9C; Figure 12A, B). That of *bdw2* had a one-nucleotide deletion in the exon 3 of *SbKS1* (*Sobic.006G211500*) (Figure 9D), while *bdw4* contained a five-nucleotide deletion in the exon 2 of *SbKAO1* (*Sobic.010G007700*) (Figure 9E). These genes encode the enzymes indicated in Figure 10, and their amino acid alignment with orthologous proteins and the corresponding phylogenetic tree are presented in Figures 13-15. Overall, these findings demonstrate that all of the five *bdw* mutants have defects in GA-biosynthetic genes.

To confirm that the concerned GA-biosynthetic genes in sorghum really function *in vivo*, they were used to transform corresponding GA-deficient mutants of rice, namely, *oscps1-1, osks1-1, osko2-1* and *oskao-1* (Sakamoto *et al*., 2004). A 7,545-bp sorghum genomic DNA fragment containing the entire *SbKO1* sequence was introduced into rice *osko2-1* via *Agrobacterium tumefaciens*-mediated transformation and found that it completely rescued the severe dwarfism of the mutant (Figure 16). Similarly, introduction of *SbCPS1*, *SbKS1*, and *SbKAO1* genes rescued the dwarfism of corresponding rice mutants (Figure 16). These observations confirmed that the isolated sorghum genes have biological activity in rice, and therefore the dwarf phenotype of the *bdw* mutants should be caused by the loss of function of these genes.

GA rescues the bending culm phenotype, whereas, uniconazole induces bending in WT plants.

The results described above indicate that loss of function of GA-biosynthetic genes causes dwarfism accompanied by unusual culm bending in the mutants. GA deficiency was further confirmed to cause the culm bending by using two approaches. First, *bdw3-1* plants were treated with different concentrations of GA_3 for 4 months. Both the dwarf phenotype and the bending culm phenotype were rescued in a dose-dependent manner by GA_3, and were almost normal at 10^{-4} M GA_3 (Figure 17). At 10^{-6} M GA_3, the dwarfism was partially rescued to about 60% but the culm remained at a significantly bent state (Figure 17). Secondly, WT plants were treated with various concentrations of uniconazole (Figure 18), an inhibitor of GA biosynthesis. As a result, treatment with 10^{-5} M uniconazole induced WT to bend in a similar manner observed in the mutants (Figure 18A). At 3 x 10^{-6} M uniconazole, the plant height was reduced to

about 25% while the bending was significantly induced from 0 to about 5 degrees against the vertical axis. These results show that the alleviation of the dwarf and bent phenotype of the mutants by GA was exactly opposite to the effect of uniconazole on WT plants, and that dwarfism and bending are tightly correlated (Figure 17A, 18A).

II. Analysis of slender GA signaling mutants

Three lines from the mutant population, namely, S09P-2030, S09P-2002 and S09N-0009 were observed to exhibit a slender or hyper-elongated phenotype (Figure 19). Since the GA-deficient severe dwarf mutants above showed phenotypic resemblance to similar mutants of rice and that such resemblance also coincided with the same type of genetic mutations, a phenotypic approach to determining the causal genes of the 3 sorghum slender mutants was used. By careful examination, the hyper-elongated morphology of the mutants generally resembled that of the reported slender mutant of rice (*slr1*) (Ikeda *et al.*, 2001; Ueguchi-Tanaka *et al.*, 2005; Ueguchi-Tanaka *et al.*, 2007), except for some culm twisting and ability to produce panicles albeit abnormal (Figure 20). The slender phenotype of the mutants was clearly observed even at an early stage because of their rapid elongation relative to the WT.

To confirm that the mutants have the same mutation as the rice *slr* mutants, their *SbSLR1* genes were analyzed by DNA sequencing. As a result, two mutants were found to have a 2-bp deletion at the SAW domain-coding region of the *SbSLR1* gene, resulting in a frame shift (Figure 21A). The third mutant also had a 2-bp deletion that overlapped by 1 bp from the earlier allele.

To demonstrate the biological function of the sorghum *SLR1* gene, it was used to transform a tall variety of rice, T65, by *Agrobacterium tumefaciens*-mediated

transformation (Figure 22). As a result, the *SbSLR1* transformants showed a significant decrease in height compared to the vector control, consistent with the role of the encoded DELLA protein as a repressor of GA action.

III. Analysis of the severe dwarf brassinosteroid (BR) biosynthetic mutant

Some of the severe dwarf mutants identified from the gamma ray-induced mutant population showed twisted and irregular leaves (broad, thin and rough lamina) (Figure 23A) that generally resembled known brassinosteroid (BR)-related mutants of rice. One of them, S09N-1794 had an abnormal skotomorphogenesis when grown in the dark which is a strong indication of a BR-related defect (Hong *et al.*, 2002) (Figure 23B). Furthermore, brassinolide (BL) treatment significantly increased its height relative to the untreated plant (Figure 23C), suggesting its deficiency in BR. To confirm this, different BR-biosynthetic genes were sequenced in the mutant, and among these, *BR6ox*, which encodes an enzyme involved in several steps in BR synthesis (Zhao and Li., 2012) and exists as a single copy in sorghum, was found to have a premature stop codon in its exon 1 (Figure 24). The biological function of the gene was also confirmed by complementation of the T2-177 mutant of rice with WT *BR6ox* gene from sorghum through *Agrobacterium*-mediated transformation (Figure 25).

IV. Analysis of the semi-dwarf auxin-related mutant

In contrast to the severe mutants mentioned above, some mutants in the gamma ray-induced mutant population had mild reduction in height with no adverse morphological side-effects, making them good candidates for lodging resistance breeding purposes. Phenotypic screening of such mutants revealed a line (S09P-2101)

that had brachytic culm characteristics similar to a previously reported agriculturally important *dw3* mutant of sorghum (Figure 26). This led to the assumption that it could also be mutated in *Dw3*. After sequencing the concerned gene of the mutant, a 5-bp deletion at its first exon was pinpointed (Figure 27). The significance of this mutation is presented in the discussion.

Discussion

In this chapter, several height-related mutants isolated during the large-scale screening were genetically analyzed. Majority of these include gibberellin-related mutants such as the five mutants with abnormal culm bending (*bdw1*, *2*, *3-1*, *3-2*, and *4*) and three *slr* plants (2 *slr1-1* and 1 *slr1-2*), while 1 BR-biosynthetic (*br6ox*/*brd1*) and 1 auxin-related (*dw3-sd3*) mutant were also found. Here, their significance for research and their implications for agriculture are discussed.

Each of the five *bdw* mutants was confirmed to have a defect in a specific GA-biosynthetic gene, namely, *SbCPS1*, *SbKS1*, *SbKO1* and *SbKAO1* (Figure 9)— the genes involved in the early part of GA biosynthesis— and such mutations were found not only to induce dwarfism but also culm bending (Figure 7). Curiously, although previous studies in maize, rice, and *Arabidopsis* have shown that loss-of-function mutations in GA-biosynthetic genes result in the suppression of internode elongation and dwarfism (Fujioka *et al.,* 1988; Helliwell *et al.,* 1998; Sakamoto *et al*., 2004), there was no mention of culm bending or GA being involved in such a phenomenon. In barley, however, the loss of function of GA3ox1, which is involved in the last step of GA biosynthesis, resulted not only in dwarfism but also prostrate growth in the *grd2c* mutant (Wolbang *et al.,* 2007). Also in wheat, a strong allele of *Rht-B1, Rht-B1c,* induced severe dwarfism that correlated with an increase in tiller angle (Wu *et al.,* 2011). These indicate that culm bending (prostrate growth) is one of the pleiotropic effects of GA-related gene mutations and such event appears to be species-dependent. The conclusion in this study that GA deficiency causes culm bending in sorghum was derived from three experimental evidences. First, the independent loss-of-function mutants defective in four different GA-biosynthetic genes were found to have bent

culms (Figure 7, 9). Second, the bent culm phenotype was reverted by exogenous GA treatment in a dose-dependent manner (Figure 17). Lastly, uniconazole treatment of WT mimicked the bending of the mutants also in a dose-dependent manner (Figure 18).

Although the exact molecular mechanism controlling bending in sorghum under GA deficiency is still unclear, there was a strong indication that the bending in the *bdw3-1* mutants was due to the asymmetric growth of culm internodes (Figure 7D, F), which could be partially due to the reduced gravitropic response of the mutants as compared to the WT (Figure 8). A comparable situation was observed in the GA-deficient barley *grd2c* mutant (Wolbang *et al.,* 2007), which showed a slower pulvinar gravitropic response than the WT, and to a greater extent, to *sln1c*, a GA-hypersensitive mutant. The evidence indicated that gravity-induced auxin asymmetry leads to an asymmetry of GA distribution, and that GAs play a role in facilitating faster gravitropic response by inducing rapid cell elongation on the lower side of the pulvini, following an asymmetric localization of auxin which serves as an initial trigger for bending. Further, just like the *bdw* mutants, the barley *grd2c* mutant was also reported to have a prostrate growth habit and GA appears to be affecting the gravitropic set-point angle (GSA) of its lateral shoots (tillers) (Wolbang *et al.,* 2007). Recently, using pea and *Arabidopsis*, GSA values were found to be dynamically specified by auxin throughout development by regulating the magnitude of the anti-gravitropic offset component via TIR1/AFB-Aux/IAA-ARF-dependent auxin signaling within the gravity-sensing cells of the root and shoot (Roychoudhry *et al*., 2013), suggesting that the bending in the *bdw* mutants could also be auxin-related. Our results show that GA deficiency triggers prostrate growth habit in sorghum in the presence of auxin, which is consistent with the case of

barley *grd2c* (Wolbang *et al.,* 2007), strongly indicating that GA also has a crucial role in controlling the GSA of the culm, at least in these species.

Mutations involving partial GA-deficiency and semi-dominant GA-insensitivity have long been harnessed in breeding programs to induce the favorable semi-dwarf trait made famous by the Green Revolution rice (*sd1*) and wheat (*Rht-B1b* and *Rht-D1b*), respectively (Sasaki *et al.,* 2002; Peng *et al.,* 1999). In such instances, semi-dwarfism led to increased lodging resistance and high yield. However, the present study strongly suggests that the use of GA-related genes for sorghum breeding is a big challenge because of the bending side-effect linked with GA deficiency. Actually, results presented in Figures 17 and 18 demonstrated that even semi-dwarf sorghum plants show a potentially destabilizing (e.g. lodging-prone) culm curvature under reduced GA levels. Our assumptions are also being upheld by the fact that there are still no reports of GA-related genes used for sorghum semi-dwarf breeding to date.

So far, it is widely known that four major classical dwarfing genes (*dw1-dw4*) had been introgressed into elite varieties in the early history of sorghum breeding (Quinby and Karper, 1954; Hadley, 1957; Quinby, 1974; Smith and Frederiksen, 2000; Klein *et al.,* 2008). Among these dwarfing genes, only the causal gene of *dw3* has been identified, namely, a gene encoding a phosphoglycoprotein involved in auxin transport (Multani *et al*., 2003). Because the locations of *dw1*, *dw2* and *dw4* have also been identified (Brown *et al*., 2008; Wang *et al*., 2012; Morris *et al.,* 2013; Thurber *et al*., 2013) (Table 4), they were compared against the loci of GA-related genes (Figure 28 and Table 3) and found that the loci of *dw2*, *dw4* and as expected, *dw3*, do not coincide with mapped positions of known GA-related genes. On the other hand, *dw1* is in close proximity to a *GA2-oxidase*-like gene (Figure 28), *Sobic.009G230800* (referred to as

GA2ox5 in Table 3), which was previously discussed by two independent groups to be the possible causal gene of *dw1* (Wang *et al*., 2012; Morris *et al.,* 2013). However, based on the experiments above, it is unlikely that a *GA2ox* is behind the phenotype of *dw1* because in that case, bending would become inevitable. Since GA2ox is involved in GA catabolism, its effect on sorghum should be similar to that of a GA synthesis inhibitor, uniconazole. To investigate whether *dw1* is the same as *GA2ox5*, the DNA sequence and expression level of the gene in a tall (*Dw1Dw1*) and dwarf (*dw1dw1*) isogenic sorghum cultivar carrying gain- and loss-of-function alleles of *Dw1*, respectively, were directly compared. As expected, there was no difference in the genome sequence and expression level of *GA2ox5* between the two cultivars (Figure 29). This result supports the above idea that a *GA2ox* is unlikely to be the causal gene of *dw1*.

As mentioned earlier, in wheat, the use of a strong allele of *Rht-B1* caused severe dwarfism and bending (Wu *et al.,* 2011), and such traits were further aggravated by overexpression of a GA-inactivating enzyme, GA2ox (Hedden and Phillips, 2000; Appleford *et al.,* 2007). In contrast, the weak *Rht-B1* mutation used in the Green Revolution only resulted in semi-dwarfism, with no obvious bending (Peng *et al.,* 1999). On the other hand, the Green Revolution rice and even the severe dwarf rice mutants carrying null alleles for GA-biosynthetic or signaling genes do not show culm bending at all (Sasaki *et al.,* 2002; Sakamoto *et al*., 2004; Ueguchi-Tanaka *et al.,* 2005). These observations suggest that, because of the exceptionally tight relationship between GA deficiency-induced dwarfism and bending in sorghum, the use of GA-related mutations to induce semi-dwarfism, without compromising the straightness and lodging resistance of the culm, may not be possible. Taken altogether, the results strongly suggest that the classical dwarfing genes that have been utilized for sorghum breeding in the past may

not be related to GA. As in the case of *dw3* (Multani *et al.,* 2003), the isolation and characterization of *dw1*, along with those of *dw2* and *dw4* will reveal novel mechanism(s) for semi-dwarfism in sorghum. Once identified, these genes can also be exploited in rice and other crops as additional or alternative options for lodging resistance breeding.

Just like the *bdw* mutants above, the *slr* mutants isolated in this study do not have applicability for agriculture because of their severe morphology. Nevertheless, as no other sorghum mutants with similar characteristics have been reported to date, they constitute an important addition to basic science, particularly in the study of SLR-mediated GA signaling in sorghum. In this case, the mutants had a deletion in *SbSLR1* (Figure 21), which exists as a single copy in sorghum, resulting in the loss of function of the encoded SLR1 or DELLA repressor protein, in turn causing constitutive hyper-elongation in the mutants. Such situation is also true with rice (Ikeda *et al.*, 2001; Ueguchi-Tanaka *et al.*, 2005; Ueguchi-Tanaka *et al.,* 2007). Further, both the case of sorghum and rice indicate the importance of the SAW domain-coding region of *SLR1* gene for the repressive function of the DELLA protein against the expression of GA-regulated downstream genes. This, along with the result of the complementation test, clearly suggests that the *SLR* genes of the two species are highly similar in terms of function. Curiously, however, the *sbslr1* mutants showed unexpected crooked or twisted culm and panicle stalk (Figure 20) not observed in rice, showing that the sorghum culm has a high tendency to show anomalies not only under GA deficiency (Figure 7) but also in the extreme opposite condition.

Next, the BR-deficient mutant presented here is the first of its kind in sorghum, as no similar mutants have been reported yet. In contrast to the *bdw* mutants, it can

produce panicles and a few fertile seeds (data not shown), indicating that BR may have no or little role in panicle formation in sorghum, whereas, it has a major role in determining plant height, proper leaf morphology and grain number. The mutant was found to respond to 0.25 ppm (5.22×10^{-7} M) of BL (Figure 23) applied through spray. However, in contrast to the fast effect of GA on GA-deficient plants, it took two weeks before any visible effects of BL could be seen, which may be explained by further experiments. Another important aspect to be checked is the crosstalk or interaction of BR with other hormones such as GA and auxin in the mutant, which can be done through the production of double or triple mutants.

Overall, the GA- and BR-related mutants in this study showed a tight correspondence with reported mutants of rice in terms of phenotype and the type of gene mutation. This suggests that GA- and BR-related genes in these related species share the same mechanism in maintaining proper plant morphology, whereas they may induce pleiotropic effects that are only observed in sorghum.

Lastly, the semi-dwarf mutant with novel *dw3* allele (*dw3-sd3*) is a good candidate for breeding purposes. As mentioned in Chapter 2, semi-dwarfism is one of the ideal traits that may need to be incorporated into sweet sorghum to address the problem of lodging especially in typhoon-prone areas. In rice and wheat, this was made possible by GA-related mutations (Peng *et al.,* 1999; Sasaki *et al.*, 2002; Ueguchi-Tanaka *et al.,* 2007). However, since GA does not seem to be applicable for sorghum as shown by the results in this study, finding better and realistic alternatives to promote semi-dwarfism in sorghum should be important. To date, only the classical dwarfing genes (*dw1 – dw4*) are available for breeding semi-dwarf varieties. Further, the *dw3* mutation was unstable due to an 882-bp duplication mutation in exon 5 (two abutting

arrows in Figure 27) that result in unequal crossing-over during meiosis, resulting in the appearance of revertants. In 2003 however, Multani *et al.* reported the isolation of *dw3-sd1* which has multiple mutations in exon 5 but did not have the duplication and thus, a stable line. This was followed by another stable mutant, *dw3-sd2* (Barrero Farfan *et al.*, 2012), which has a 6-bp deletion in exon 5. In this study, the *dw3* allele isolated also do not have the duplication (Figure 27), hence, it is the so far, the third stable allele of *dw3* (*dw3-sd3*) that have been isolated. However, this is the only stable line with a sweet sorghum background, which makes it a promising line for sweet sorghum breeding.

In view of the above results, this study showed the usefulness of gamma ray for the systematic mutagenesis of sweet sorghum. All the agromorphological and genetic data obtained here will be uploaded in the form of a database that will serve as a useful reference for other scientists worldwide who are interested in sorghum.

Summary

In this chapter, I described the genetic characterization of 5 GA-related severe dwarf mutants (*bdw1- bdw4*), 3 slender mutants (*slr1-1 – 2*), 1 BR-related severe dwarf mutant (*br6ox*) and 1 auxin-related semi-dwarf mutant (*dw3*). The 5 GA-deficient mutants exhibited severe dwarf phenotypes and had unusually bent stems. Each of them, with the exception of *bdw3-1* and *2* were independently mutated in specific GA-biosynthetic genes. The 3 mutants with hyper-elongated (slender) phenotype all showed a mutation at the SAW domain-coding region of the *SbSLR1* gene. The severe dwarf BR-related mutant has a base substitution that resulted in a premature stop codon in exon 1. And lastly, the semi-dwarf mutant was found to have a new stable allele of *dw3*, *dw3-sd3*, which is due to a 5-bp deletion in exon 1.

References

Appleford, N.E.J., Wilkinson, M.D., Ma, Q., Evans, D.J., Stone, M.C., Pearce, S.P., Powers, S.J., Thomas, S.G., Jones, H.D., Phillips, A.L., Hedden, P. and Lenton, J.R. (2007). Decreased shoot stature and grain α-amylase activity following ectopic expression of a gibberellin 2-oxidase gene in transgenic wheat. J. Exp. Bot. **58**: 3213-3226.

Arite, T., Umehara, M., Ishikawa, S., Hanada, A., Maekawa, M., Yamaguchi, S. and Kyozuka, J. (2009). *d14*, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. Plant Cell Physiol. **50**: 1416-1424.

Aya, K., Hobo, T., Sato-Izawa, K., Ueguchi-Tanaka, M., Kitano, H. and Matsuoka, M. (2014). A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. Plant Cell Physiol. **55**: 897–912.

Bamberg, J.B. and Hanneman, R.E. (1991). Characterization of a new gibberellin related dwarfing locus in potato (*Solanum tuberosum* L.). Am. Potato J. **68**: 45–52.

Barrero Farfan, I.D., Bergsma, B.R., Johal, G. and Tuinstra, M.R. (2012). A stable allele in sorghum and a molecular marker to facilitate selection. Crop Sci. **52**: 2063.

Blomstedt, C.K., Gleadow, R.M., O'Donnell, N., Naur, P., Jensen, K., Laursen, T., Olsen, C.E., Stuart, P., Hamill, J.D., Møller, B.L., Neale, A.D. (2012). A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. Plant Biotechnol. J. **10**: 54-66.

Bouquin, T. (2003). The *Arabidopsis lue1* mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. J. Cell Sci. **116**: 791–801.

Bout, S. and Vermerris, W. (2003). A candidate-gene approach to clone the sorghum *Brown midrib* gene encoding caffeic acid *O*-methyltransferase. Mol. Genet. Genomics **269**: 205–214.

Brown, P.J., Rooney, W.L., Franks, C. and Kresovich, S. (2008). Efficient mapping of plant height quantitative trait loci in a sorghum association population with introgressed dwarfing genes. Genetics **180**: 629-637.

Chandler, P.M., Marion-Poll, A., Ellis, M. and Gubler, F. (2002). Mutants at the *Slender1* locus of barley cv Himalaya. molecular and physiological characterization. Plant Physiol. **129**: 181–190.

Chhun, T., Aya, K., Asano, K., Yamamoto, E., Morinaka, Y., Watanabe, M., Kitano, H., Ashikari, M., Matsuoka, M. and Ueguchi-Tanaka, M. (2007). Gibberellin regulates pollen viability and pollen tube growth in rice. Plant Cell **19**: 3876-3888.

Childs, K.L., Miller, F.R., Cordonnier-Pratt, M.M., Pratt, L.H., Morgan, P.W. and Mullet, J.E. (1997). The sorghum photoperiod sensitivity gene, *Ma3*, encodes a phytochrome B. Plant Physiol. **113**: 611–619.

Chomczynski, P. and Sacchi, P. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**: 156–159.

Chono, M., Honda, I., Zeniya, H., Yoneyama, K., Saisho, D., Takeda, K., Takatsuto, S., Hoshino, T. and Watanabe, Y. (2003). A semidwarf phenotype of barley uzu results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. Plant Physiol. **133**: 1209–1219.

Clouse, S.D., Langford, M. and McMorris, T.C. (1996). A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. **111**: 671–678.

Cui, X., Jin, P., Cui, X., Gu, L., Lu, Z., Xue, Y., Wei, L., Qi, J., Song, X., Luo, M., An, G. and Cao, X. (2013). Control of transposon activity by a histone H3K4 demethylase in rice. Proc. Natl. Acad. Sci. U. S. A. **110**: 1953-1958.

Dai, Y., Fu, Z. and Li, J. (2003). Isolation and characterization of an *Arabidopsis* bush and dwarf mutant. Acta Bot. Sin. **45**: 621–625.

Davidson, S.E., Elliott, R.C., Helliwell, C.A., Poole, A.T. and Reid, J.B. (2003). The pea gene *NA* encodes *ent*-kaurenoic acid oxidase. Plant Physiol. **131**: 335–344.

Ephritikhine, G., Fellner, M., Vannini, C., Lapous, D. and Barbier-Brygoo, H. (1999). The *sax1* dwarf mutant of *Arabidopsis* thaliana shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. Plant J. **18**: 303-314.

Fleet, C.M. and Sun, T. (2005). A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr. Opin. Plant Biol. **8**: 77–85.

Fridborg, I., Kuusk, S., Moritz, T. and Sundberg, E. (1999). The Arabidopsis dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell **11**: 1019-1031.

Fujioka, S., Yamane, H., Spray, C.R., Gaskin, P., Macmillan, J., Phinney, B. and Takahashi, N. (1988). Qualitative and quantitative analyses of gibberellins in

vegetative shoots of normal, *dwarf-1, dwarf-2, dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol. **88**: 1367–1372.

Hadley, H.H. (1957). An analysis of variation in height in sorghum. Agron. J. **49**, 144‑147.

Hedden, P. (2003). The genes of the Green Revolution. Trends in Genet. **19**: 5–9.

Hedden, P. and Phillips, a L. (2000). Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci. **5**: 523-530.

Helliwell, C.A., Sheldon, C.C., Olive, M.R., Walker, A.R., Zeevaart, J.A., Peacock, W.J. and Dennis, E.S. (1998). Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*. Proc. Natl. Acad. Sci. U. S. A. **95**: 9019-9024.

Hiei, Y. and Komari, T. (2008) *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. Nat. Protoc. **3**: 824–834.

Hong, Z., Ueguchi-Tanaka, M., Umemura, K., Uozu, S., Fujioka, S., Takatsuto, S., Yoshida, S., Ashikari, M., Kitano, H. and mats. (2003). A rice brassinosteroid-deficient mutant, *ebisu dwarf (d2)*, is caused by a loss of function of a new member of cytochrome P450. Plant Cell **15**: 2900–2910.

http://bio-bwa.sourceforge.net/

http://samtools.sourceforge.net/

http://tree.bio.ed.ac.uk/software/figtree/

http://www.genome.jp/tools/clustalw/

http://www.phytozome.net/search.php?show=blastandorg=Org_Sbicolor_v2.1

Ikeda, A, Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M. and Yamaguchi, J. (2001). *slender* rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. Plant Cell **13**: 999–1010.

Itoh, H., Tatsumi, T., Sakamoto, T., Otomo, K., Toyomasu, T., Kitano, H., Ashikari, M., Ichihara, S. and Matsuoka, M. (2004). A rice semi-dwarf gene, *Tan-Ginbozu (D35)*, encodes the gibberellin biosynthesis enzyme, *ent*-kaurene oxidase. Plant Mol. Biol. **54**: 533-547.

Jiang, L., Liu, X., Xiong, G., Liu, H., Chen, F., Wang, L., Meng, X., Liu, G., Yu, H., Yuan, Y., Yi, W., Zhao, L., Ma, H., He, Y., Wu, Z., Melcher, K., Qian, Q., Xu, H.E., Wang, Y. and Li, J. (2013). DWARF 53 acts as a repressor of strigolactone signalling in rice. Nature **504**: 401-405.

Klein, R.R., Mullet, J.E., Jordan, D.R., Miller, F.R., Rooney, W.L., Menz, M. a., Franks, C.D. and Klein, P.E. (2008). Effect of tropical sorghum conversion and inbred development on genome diversity as revealed by high-resolution genotyping. Crop Sci. **48**: S12-S26.

Knöller, A.S., Blakeslee, J.J., Richards, E.L., Peer, W.A. and Murphy, A.S. (2010). Brachytic2/ZmABCB1 functions in IAA export from intercalary meristems. J. Exp. Bot. **61**: 3689–3696.

Komorisono, M., Ueguchi-Tanaka, M., Aichi, I., Hasegawa, Y., Ashikari, M., Kitano, H., Matsuoka, M. and Sazuka, T. (2005). Analysis of the rice mutant *dwarf and gladius leaf 1*. Aberrant katanin-mediated microtubule organization causes up-regulation of gibberellin biosynthetic genes independently of gibberellin signaling. Plant Physiol. **138**: 1982–1993.

Krothapalli, K., Buescher, E.M., Li, X., Brown, E., Chapple, C., Dilkes, B.P. and Tuinstra, M.R. (2013). Forward genetics by genome sequencing reveals that rapid cyanide release deters insect herbivory of *Sorghum bicolor*. Genetics **195**: 309–318.

Lo, S.-F., Yang, S.-Y., Chen, K.-T., Hsing, Y.-I., Zeevaart, J. A. D., Chen, L.-J. and Yu, S.-M. (2008). A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. Plant Cell **20**: 2603-2618.

Maity, U. and Bera, A.K. (2009). Effect of exogenous application of brasinolide and salicylic acid on certain physiological and biochemical aspects of green gram (Vigna radiata L. Wilczek). Indian J. Agric. Res. **43**: 194–199.

Martí, E., Gisbert, C., Bishop, G.J., Dixon, M.S. and García-Martínez, J.L. (2006). Genetic and physiological characterization of tomato cv. Micro-Tom. J. Exp. Bot. **57**: 2037-2047.

Miura, K., Agetsuma, M., Kitano, H., Yoshimura, A., Matsuoka, M., Jacobsen, S.E. and Ashikari, M. (2009). A metastable *DWARF1* epigenetic mutant affecting plant stature in rice. Proc. Natl. Acad. Sci. U. S. A. **106**: 11218-11223.

Mizuno, H., Kawahigashi, H., Kawahara, Y., Kanamori, H., Ogata, J., Minami, H., Itoh, T. and Matsumoto, T. (2012). Global transcriptome analysis reveals distinct expression among duplicated genes during sorghum-*Bipolaris sorghicola* interaction. BMC Plant Biol. **12**: 121.

Morris, G.P., Ramu, P., Deshpande, S.P., Hash, C.T., Shah, T., Upadhyaya, H.D., Riera-Lizarazu, O., Brown, P.J., Acharya, C.B., Mitchell, S.E., Harriman, J., Glaubitz, J.C., Buckler, E.S. and Kresovich, S. (2013). Population genomic and genome-wide association studies of agroclimatic traits in sorghum. Proc. Natl. Acad. Sci. U. S. A. **110**: 453-458.

Multani, D.S., Briggs, S.P., Chamberlin, M. a, Blakeslee, J.J., Murphy, A.S. and Johal, G.S. (2003). Loss of an MDR transporter in compact stalks of maize *br2* and sorghum *dw3* mutants. Science **302**: 81-84.

Murphy, R.L., Klein, R.R., Morishige, D.T., Brady, J. a, Rooney, W.L., Miller, F.R., Dugas, D. V, Klein, P.E. and Mullet, J.E. (2011). Coincident light and clock regulation of pseudoresponse regulator protein 37 (PRR37) controls photoperiodic flowering in sorghum. Proc. Natl. Acad. Sci. U. S. A. **108**: 16469–74.

Murphy, R.L., Morishige, D.T., Brady, J. a., Rooney, W.L., Yang, S., Klein, P.E. and Mullet, J.E. (2014). *Ghd7* (*Ma6*) Represses Sorghum Flowering in Long Days: Alleles Enhance Biomass Accumulation and Grain Production. Plant Genome **7**.-.

Nakamura, A., Fujioka, S., Sunohara, H., Kamiya, N., Hong, Z., Inukai, Y., Miura, K., Takatsuto, S., Yoshida, S., Ueguchi-Tanaka, M., Hasegawa, Y., Kitano, H. and Matsuoka, M. (2006). The role of *OsBRI1* and its homologous genes, *OsBRL1* and *OsBRL3*, in rice. Plant Physiol. **140**: 580–590.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, a, Yoshida, S., Li, J. and Chory, J. (1999). Arabidopsis *det2* is defective in the conversion of (24*R*)-24-methylcholest-4-En-3-one to (24*R*)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. Plant Physiol. **120**: 833–840.

Nole-Wilson, S., Rueschhoff, E.E., Bhatti, H. and Franks, R.G. (2010). Synergistic disruptions in *seuss cyp85A2* double mutants reveal a role for brassinolide synthesis during gynoecium and ovule development. BMC Plant Biol. **10**: 198.

Paterson, A.H., Bowers, J.E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A.K., Chapman, J., Feltus, F.A., Gowik, U., Grigoriev, I. V, Lyons, E., Maher, C. a, Martis, M., Narechania, A., Otillar, R.P., Penning, B.W., Salamov, A. a, Wang, Y., Zhang, L., Carpita, N.C., Freeling, M., Gingle, A.R., Hash, C.T., Keller, B., Klein, P., Kresovich, S., McCann, M.C., Ming, R., Peterson, D.G., Mehboob-ur-Rahman, Ware, D., Westhoff, P., Mayer, K.F.X., Messing, J. and Rokhsar, D.S. (2009). The *Sorghum bicolor* genome and the diversification of grasses. Nature **457**: 551-556.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P. and Harberd, N.P. (1997). The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev. **11**: 3194–3205.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, a J., Pelica, F., Sudhakar, D., Christou, P.,

Snape, J.W., Gale, M.D. and Harberd, N.P. (1999). 'Green revolution' genes encode mutant gibberellin response modulators. Nature **400**: 256-261.

Petruccelli, S., Dai, S., Carcamo, R., Yin, Y., Chen, S. and Beachy, R.N. (2001). Transcription factor RF2a alters expression of the rice tungro bacilliform virus promoter in transgenic tobacco plants. Proc. Natl. Acad. Sci. U. S. A. **98**: 7635–7640.

Quinby, J.R. *Sorghum Improvement and the Genetics of Growth.* (1974) Texas A&M University Press, College Station, TX.

Quinby, J.R. and Karper, R.E. (1954) Inheritance of height in sorghum. Agron. J. **46**: 211‑216.

Rao, P.S., Deshpande, S., Blümmel, M., Reddy, B.V.S. and Hash, T. (2012). Characterization of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). Eur. J. Plant Sci. Biotechnol. **6**: 71–75.

Roychoudhry, S., Del Bianco, M., Kieffer, M. and Kepinski, S. (2013). Auxin controls gravitropic setpoint angle in higher plant lateral branches. Curr. Biol. **23**: 1497–1504.

Saballos, A., Sattler, S.E., Sanchez, E., Foster, T.P., Xin, Z., Kang, C., Pedersen, J.F. and Vermerris, W. (2012). *Brown midrib2* (*Bmr2*) encodes the major 4-coumarate:coenzyme A ligase involved in lignin biosynthesis in sorghum (*Sorghum bicolor* (L.) Moench). Plant J. **70**: 818–830.

Sakamoto, T., Kitano, H. and Fujioka, S. (2013). An E3 ubiquitin ligase, ERECT LEAF1, functions in brassinosteroid signaling of rice. Plant Signal. Behav. **8**: e27117.

Sakamoto, T., Miura, K., Itoh, H., Tatsumi, T. and Ueguchi-Tanaka, M. (2004). An overview of gibberellin metabolism enzyme genes and their related mutants in rice. Plant Physiol. **134**: 1642–1653.

Sasaki, A., Ashikari, M., Ueguchi-Tanaka, M., Itoh, H., Nishimura, A., Swapan, D., Ishiyama, K., Saito, T., Kobayashi, M., Khush, G.S., Kitano, H. and Matsuoka, M. (2002). Green revolution: a mutant gibberellin-synthesis gene in rice. Nature **416**: 701–702.

Sato, Y., Sentoku, N., Miura, Y., Hirochika, H., Kitano, H. and Matsuoka, M. (1999). Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. EMBO J. **18**: 992–1002.

Sazuka, T., Kamiya, N., Nishimura, T., Ohmae, K., Sato, Y., Imamura, K., Nagato, Y., Koshiba, T., Nagamura, Y., Ashikari, M., Kitano, H. and Matsuoka, M.

(2009). A rice *tryptophan deficient dwarf* mutant, *tdd1*, contains a reduced level of indole acetic acid and develops abnormal flowers and organless embryos. Plant J. **60**: 227-241.

Smith, C.W. and Frederiksen, R.A. (2000) *Sorghum*: *Origin, history, technology, and production*, 191‑223 (John Wiley & Sons, New York).

Spielmeyer, W., Ellis, M.H. and Chandler, P.M. (2002). Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. Proc. Natl. Acad. Sci. U. S. A. **99**: 9043-9048.

Sun, T.P. and Kamiya, Y. (1994). The Arabidopsis *GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. Plant Cell **6**: 1509-1518.

Tanimoto, E. (2005). Regulation of root growth by plant hormones–Roles for auxin and gibberellin. Crit. Rev. Plant Sci. **24**: 249–265.

Thurber, C.S., Ma, J.M., Higgins, R.H. and Brown, P.J. (2013). Retrospective genomic analysis of sorghum adaptation to temperate-zone grain production. Genome Biol. **14**: R68.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T., Hsing, Y.C., Kitano, H., Yamaguchi, I. and Matsuoka, M. (2005). *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. Nature **437**: 693–698.

Ueguchi-Tanaka, M., Nakajima, M., Katoh, E., Ohmiya, H., Asano, K., Saji, S., Hongyu, X., Ashikari, M., Kitano, H., Yamaguchi, I. and Matsuoka, M. (2007). Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. Plant Cell **19**: 2140–2155.

Uriarte, V. T., Zambelli, D., Kaspar, M. and Pardo, A.P. (2013). Sorghum plants having mutant polynucleotide encoding the large subunit of mutated acetohydroxyacid synthase protein and increased resistance to herbicides. WO patent 2013/149674 A1, 2013.

Vogler, R.K., Tesso, T.T., Johnson, K.D. and Ejeta, G. (2009). The effect of allelic variation on forage quality of brown midrib sorghum mutants with reduced caffeic acid *O* - methyl transferase activity **3**: 70–76.

Wang, Y. and Li, J. (2005). The plant architecture of rice (*Oryza sativa*). Plant Mol. Biol. **59**: 75–84.

Wang, Y.H., Bible, P., Loganantharaj, R. and Upadhyaya, H.D. (2011). Identification of SSR markers associated with height using pool-based genome-wide association mapping in sorghum. Mol. Breed. **30**: 281–292.

Wolbang, C.M., Davies, N.W., Taylor, S.A. and Ross, J.J. (2007). Gravistimulation leads to asymmetry of both auxin and gibberellin levels in barley pulvini. Physiol. Plant. **131**: 140-148.

Wu, J., Kong, X., Wan, J., Liu, X., Zhang, X., Guo, X., Zhou, R., Zhao, G., Jing, R., Fu, X. and Jia, J. (2011). Dominant and pleiotropic effects of a *GAI* gene in wheat results from a lack of interaction between DELLA and GID1. Plant Physiol. **157**: 2120-2130.

Wu, Y., Yuan, L., Guo, X., Holding, D.R. and Messing, J. (2013). Mutation in the seed storage protein kafirin creates a high-value food trait in sorghum. Nat. Commun. **4**: 2217.

Xin, Z., Wang, M.L., Barkley, N.A., Burow, G., Franks, C., Pederson, G. and Burke, J. (2008). Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. BMC Plant Biol. **8**: 103.

Yabuta, T. and Sumiki, Y. (1938). On the crystal of gibberellin, a substance to promote plant growth. J. Agric. Chem. Soc. Japan **14**: 1526.

Yang, W., Ren, S., Zhang, X., Gao, M., Ye, S., Qi, Y., Zheng, Y., Wang, J., Zeng, L., Li, Q., Huang, S. and He, Z. (2011). *BENT UPPERMOST INTERNODE1* encodes the class II formin FH5 crucial for actin organization and rice development. Plant Cell **23**: 661–680.

Yonemaru, J. Ando, T.S., Mizubayashi, T.A., Kasuga, S.H., Matsumoto, T.A. and Yano, M.A. (2009). Development of genome-wide simple sequence repeat markers using whole-genome shotgun sequences of sorghum (*Sorghum bicolor* (L.) Moench). DNA Res. **16**: 187–193.

Zanta, C.A., Yang, X., Axtell, J.D. and Bennetzen, J.L. (1994). The *Candystrip* Locus, *y-cs*, Determines Mutable Pigmentation of the Sorghum Leaf, Flower, and Pericarp. J. Hered. **85**: 23–29.

Zhao, B. and Li, J. (2012). Regulation of brassinosteroid biosynthesis and inactivation. J. Integr. Plant Biol. **54**: 746-759.

Zhu, S., Gao, F., Cao, X., Chen, M., Ye, G., Wei, C. and Li, Y. (2005). The rice dwarf virus P2 protein interacts with *ent* -kaurene oxidases in vivo, leading to reduced biosynthesis of gibberellins and rice dwarf symptoms. Plant Physiol. **139**: 1935–1945.

Table and Figures

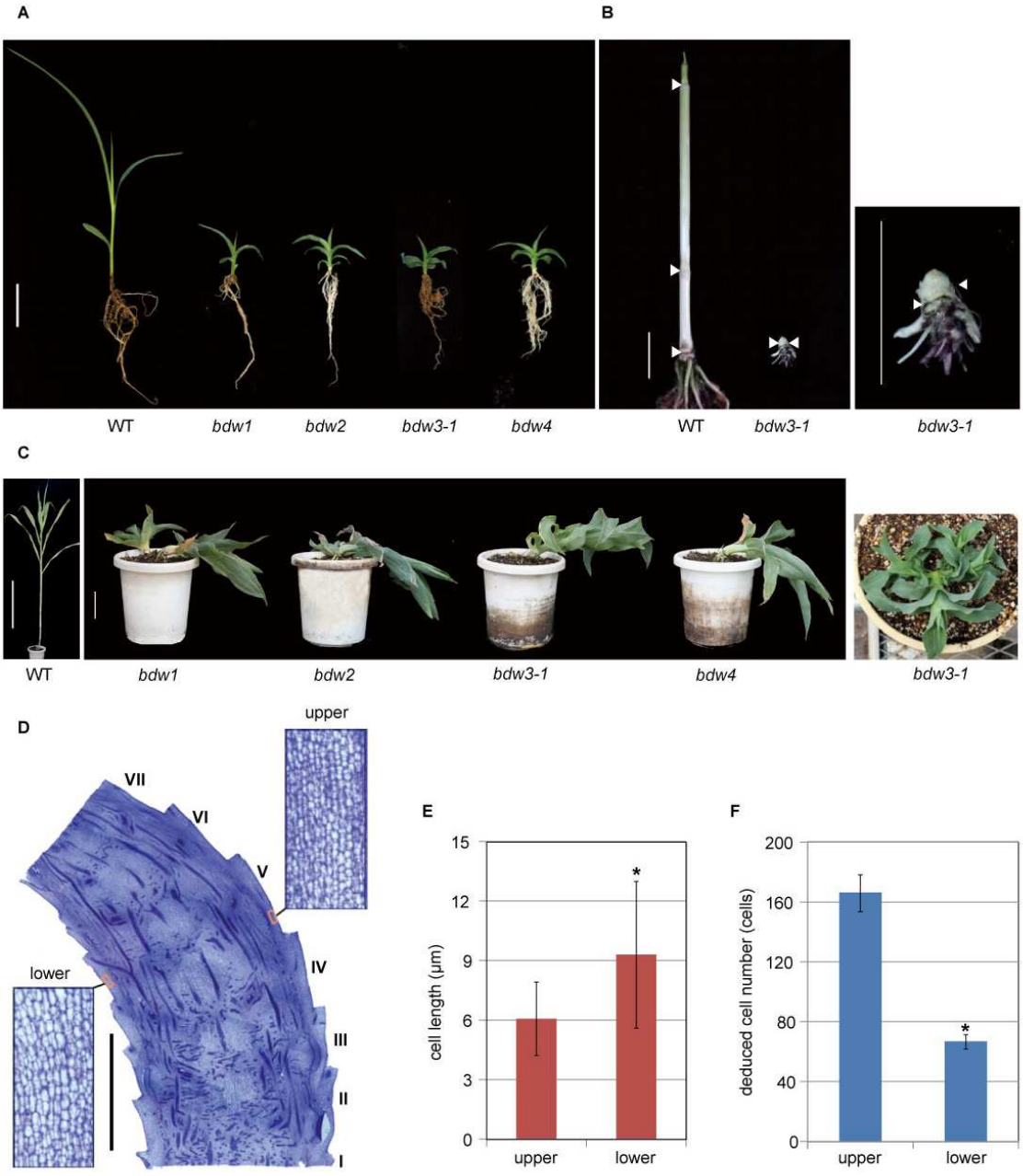

Figure 7. Phenotypic analysis of the *bdw* mutants. (A) The *bdw* and WT plants at the seedling stage (30 days after germination, DAS). Bar = 4 cm. (B) Culm structure of WT (left) and *bdw3* (center and right) at the vegetative stage. Bars = 2 cm. Nodes are indicated by arrowheads. (C) WT and the bent *bdw* mutants at the vegetative stage (110 DAS). Top view of two-month old *bdw3* is shown at the rightmost panel. Bars: 50cm, WT; 4 cm, mutants. (D) Longitudinal section of *bdw3* culm. Roman numerals at the

right side indicate internode position from bottom to top. Bar = 5 mm. (E-F) The length and number of cells at the upper and lower sides of the fifth internode in the bent region shown in panel D. Error bars for cell length represent the standard deviation calculated from approximately 100 cells. Error bars for cell number represent the standard deviation calculated from 5 cell files (see Methods). Asterisks indicate significant differences at 0.1% ($P<0.001$).

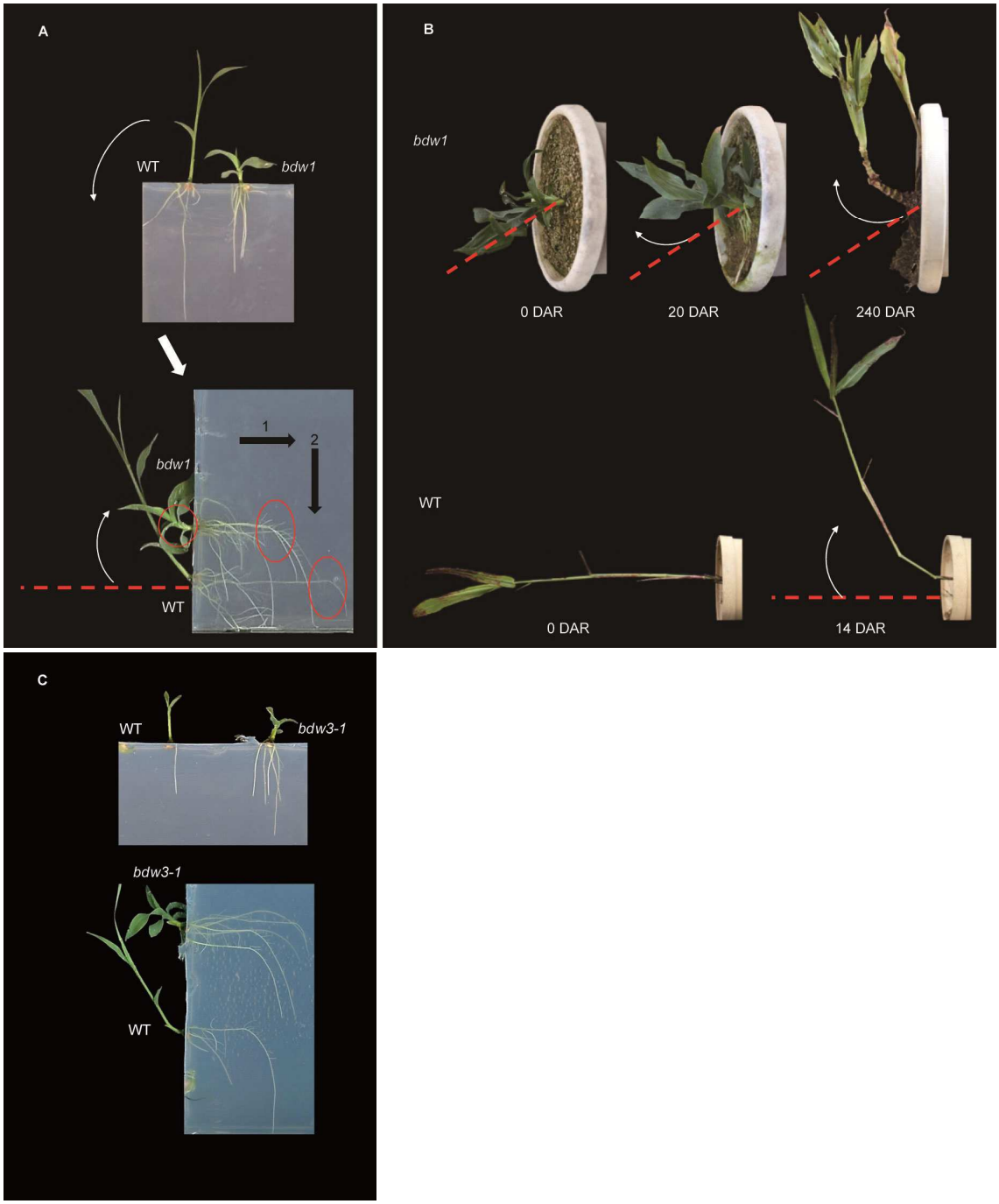

Figure 8. Gravitropism test of *bdw* mutants. (A) The gravitropic response of *bdw1* at the seedling stage. Top, plants at 4 days after germination (DAG) in upright position before gravistimulation; bottom, 4-DAG plants were rotated 90 degrees and further grown for 4 days after rotation (DAR). The horizontal "1" and vertical "2" arrows indicate the gravity directions before and after rotation, respectively. Red circles emphasize the

bending points. (B) The gravitropic response of *bdw1* (top) and WT (bottom) at the vegetative stage. Red dashed lines indicate the original direction of main culm before rotation. White arrows indicate the direction of culm movement. (C) The gravitropic response of *bdw3-1* at the seedling stage. The presentation is the same as in panel A.

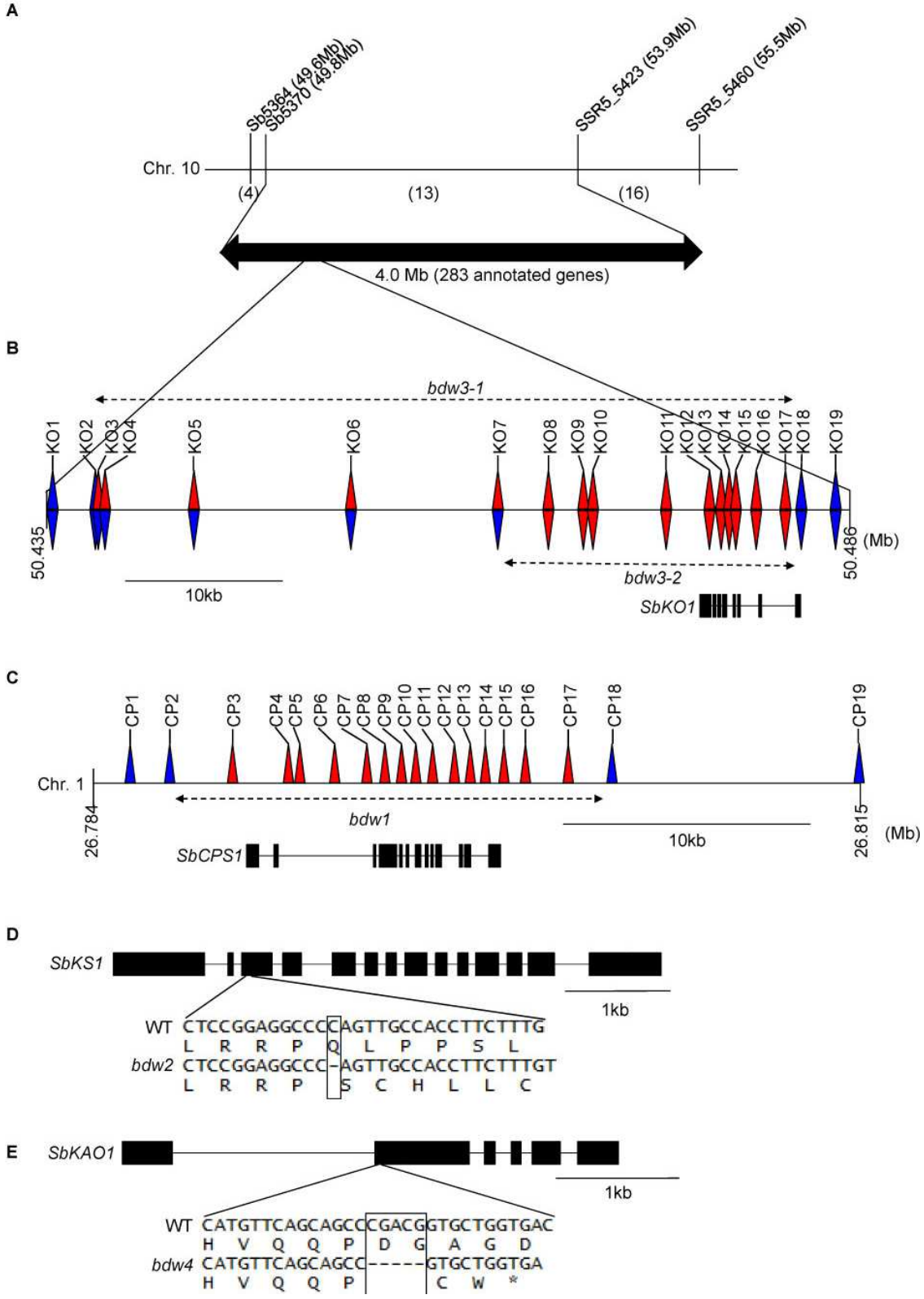

Figure 9. Identification of the causal genes of the *bdw* mutants. (A) A physical map of

the candidate region of *bdw3*. The top line shows the position of markers around the target region of chromosome 10. The numbers in parentheses indicate the number of recombinant plants between the markers. The *bdw3* mutation locates between Sb5370 and SSR5_5423. (B) Deletion in the *bdw3-1* and *bdw3-2* genome as detected by PCR analysis. Presence or absence of PCR products is indicated by blue and red triangles, respectively, whereas all PCR products were successfully obtained from the WT genome. Upright and inverted triangles correspond to the *bdw3-1* and *bdw3-2* genomes, respectively. Dashed arrows at the top and beneath the triangles show estimated deletions around the *SbKO1* gene. The *SbKO1* structure is shown at the bottom, where boxes and lines between them indicate exons and introns, respectively. (C) Deletion in the *bdw1* genome as detected by PCR analysis. The presentation is the same as in panel B. (D) One-nucleotide deletion in the *SbKS1* gene of *bdw2*. The presentation is the same as in panel B. (E) Five-nucleotide deletion in *SbKAO1* gene of *bdw4*. The presentation is the same as in panel B.

Table 3. Sorghum GA-related genes

Gene Name	Gene ID	Chromosome Location[a]	Function	Mutant line
CPS1	*Sobic.001G248600*	Chr01: 26790176 - 26800458	GA biosynthesis	*bdw1*[c]
CPS2	*Sobic.005G161200*	Chr05: 53944409 - 53957771	unknown	NI
KS1	*Sobic.006G211500*	Chr06: 57013802 - 57019007	GA biosynthesis	*bdw2*[c]
KS2	*Sobic.005G161600*	Chr05: 54053377 - 54062189	unknown	NI
KS3	*Sobic.006G211400*	Chr06: 57006141 - 5701073	unknown	NI
KO1	*Sobic.010G172700*	Chr10: 50476551 - 50482229	GA biosynthesis	*bdw3-1 & 2*[c]
KAO1	*Sobic.010G007700*	Chr10: 616990 - 621030	GA biosynthesis	*bdw4*[c]
GA20ox1	*Sobic.001G005300*	Chr01: 450531 - 452935	GA biosynthesis[b]	NI
GA20ox2	*Sobic.003G379500*	Chr03: 69354859 - 69357641	GA biosynthesis[b]	NI
GA20ox3	*Sobic.002G046500*	Chr02: 4373234 - 4374471	GA biosynthesis[b]	NI
GA20ox4	*Sobic.009G142400*	Chr09: 50000656 - 50005651	GA biosynthesis[b]	NI
GA3ox1	*Sobic.009G064700*	Chr09: 6922298 - 6923624	GA biosynthesis[b]	NI
GA3ox2	*Sobic.003G045900*	Chr03: 4213496 - 4215999	GA biosynthesis[b]	NI
GA2ox1	*Sobic.009G053700*	Chr09: 5482618 - 5487585	GA inactivation[b]	NI
GA2ox2	*Sobic.003G154100*	Chr03: 16728989 - 16735073	GA inactivation[b]	NI
GA2ox3	*Sobic.003G022700*	Chr03: 1958597 - 1961993	unknown	NI
GA2ox4	*Sobic.009G077500*	Chr09: 10344083 - 10349624	unknown	NI
GA2ox5	*Sobic.009G230800*	Chr09: 57097196 - 57099683	unknown	NI
GA2ox6	*Sobic.003G300800*	Chr03: 63204531 - 63207037	GA inactivation[b]	NI
GA2ox7	*Sobic.009G196300*	Chr09: 54711648 - 5471373	GA inactivation[b]	NI
GA2ox8	*Sobic.002G003100*	Chr02: 414500 - 419024	GA inactivation[b]	NI
GA2ox9	*Sobic.004G222500*	Chr04: 56529459 - 56534168	GA inactivation[b]	NI
GA2ox10	*Sobic.006G150800*	Chr06: 52074760 - 52079902	GA inactivation[b]	NI
GID1	*Sobic.009G134600*	Chr09: 48980261 - 48983253	GA signal transduction[b]	NI
GID2a	*Sobic.004G192400*	Chr04: 53720307 - 53722135	GA signal transduction[b]	NI
GID2b	*Sobic.001G470100*	Chr01: 67126469 - 67128158	unknown	NI
SLR1	*Sobic.001G120900*	Chr01: 9370541 - 9373961	GA signal transduction[b]	*slr1-1 & 2*[c]

[a] Number indicates the position of the gene; [b] putative function; [c] studied in this paper; NI, not isolated

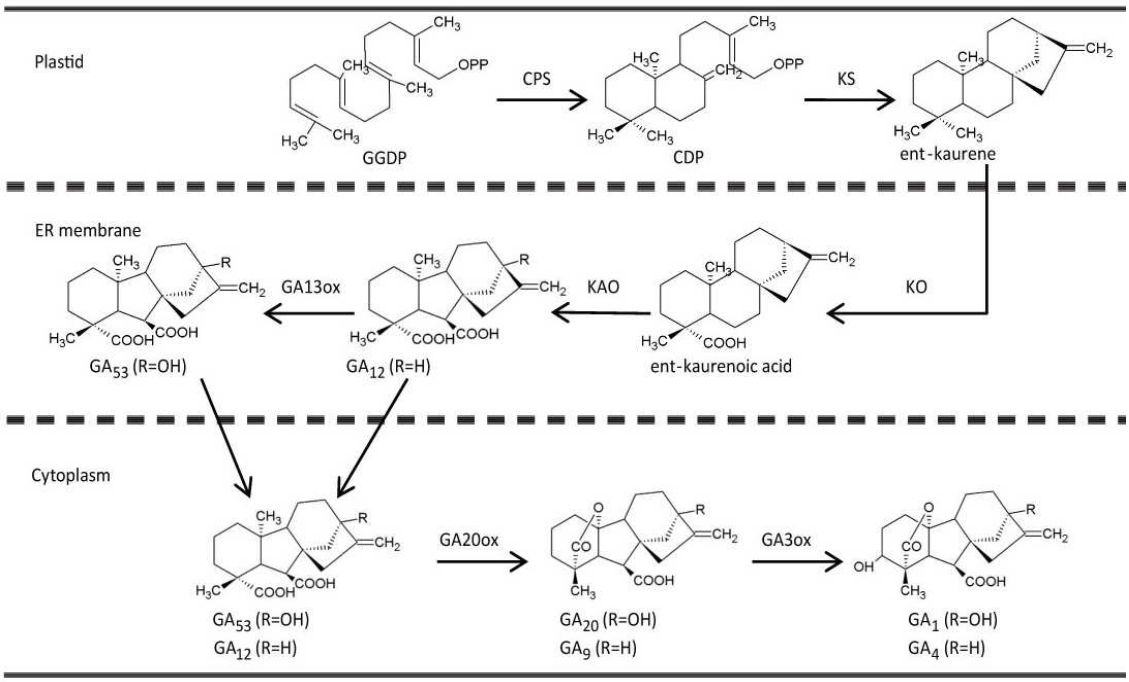

Figure 10. Estimated principal pathway of GA biosynthesis in sorghum. GA biosynthesis is carried out by several proteins (CPS, KS, KO, KAO, GA20ox and GA3ox) at different subcellular locations.

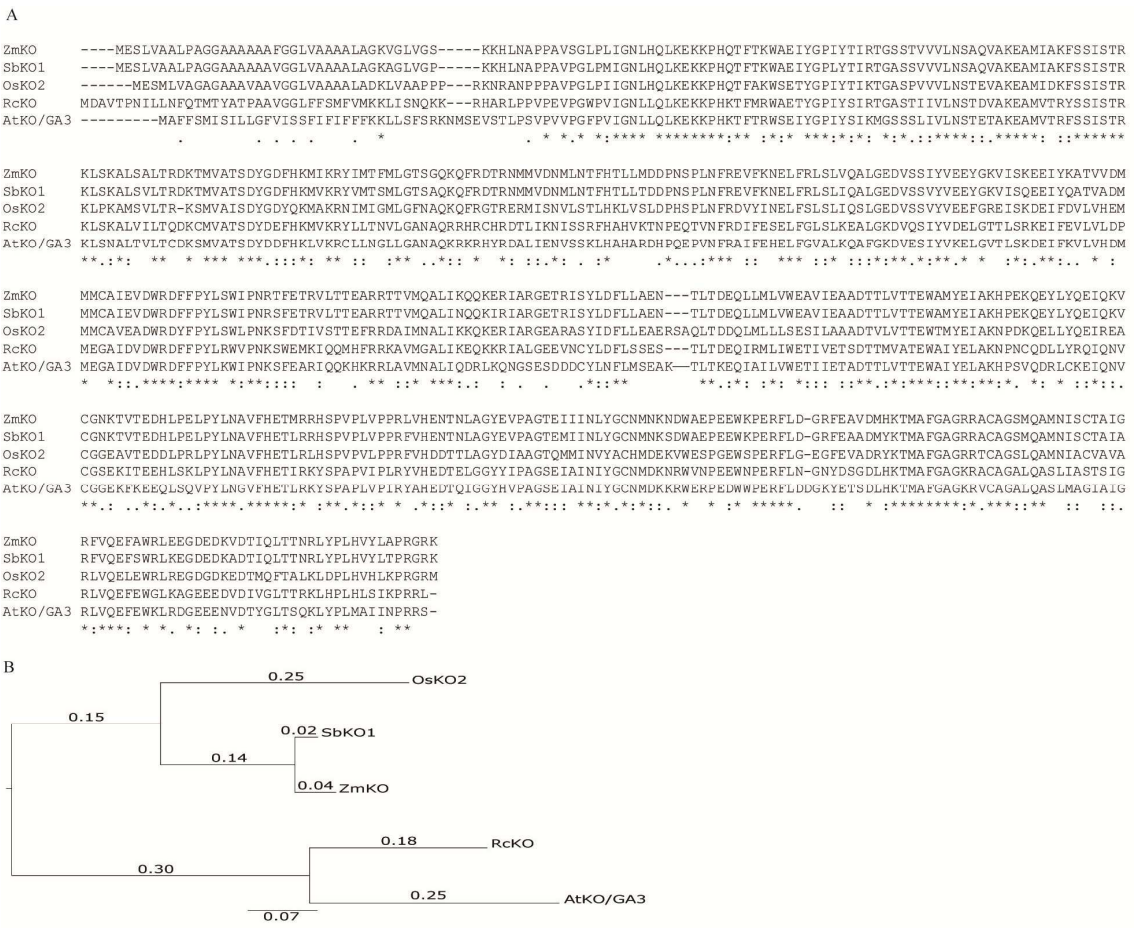

Figure 11. Amino acid alignment (A) and phylogenetic tree of KO (B). OsKO2, ZmKO, RcKO, and AtKO/GA3 indicate KO of rice, maize, *Ricinus communis*, and *Arabidopsis*, respectively. There is a single KO-like protein in sorghum and its loss of function induces bending and severe dwarfism.

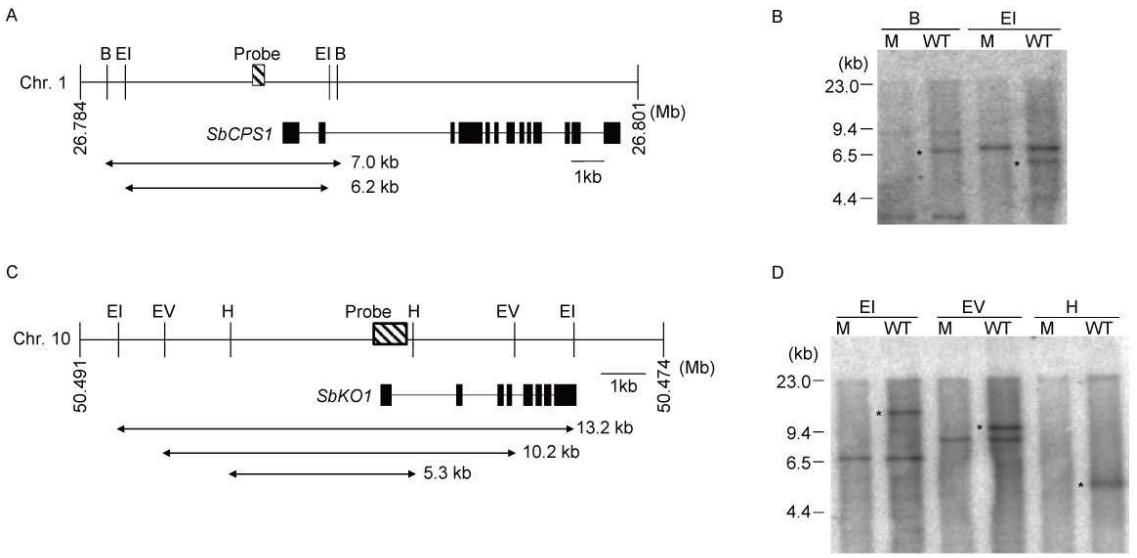

Figure 12. DNA blotting analysis of *SbCPS1* and *SbKO1* using the genome of *bdw1* and *bdw3-1*, respectively. (A) Genome structure around *SbCPS1*. The top line indicates the positions of the restriction enzymes such as *Bam*HI (B) and *Eco*RI (EI), and the probe. The second line represents the structure of *SbCPS1*. The presentation is the same as in Figure 9B. The third and fourth lines indicate the restriction fragments for *Bam*HI and *Eco*RI, respectively, which should be detected by the probe. (B) The result of the DNA blotting analysis of *SbCPS1* using the *bdw1* genome. The expected restriction fragments were detected in WT (shown by asterisks at the left) but not in *bdw1*. (C) Genome structure around *SbKO1*. The presentation is the essentially same as in panel A, except that *Hin*dIII (H) and *Eco*RV (EV) were used. (D) The result of DNA blotting analysis of *SbKO1* using the *bdw3-1* genome. The presentation is the same as in panel B.

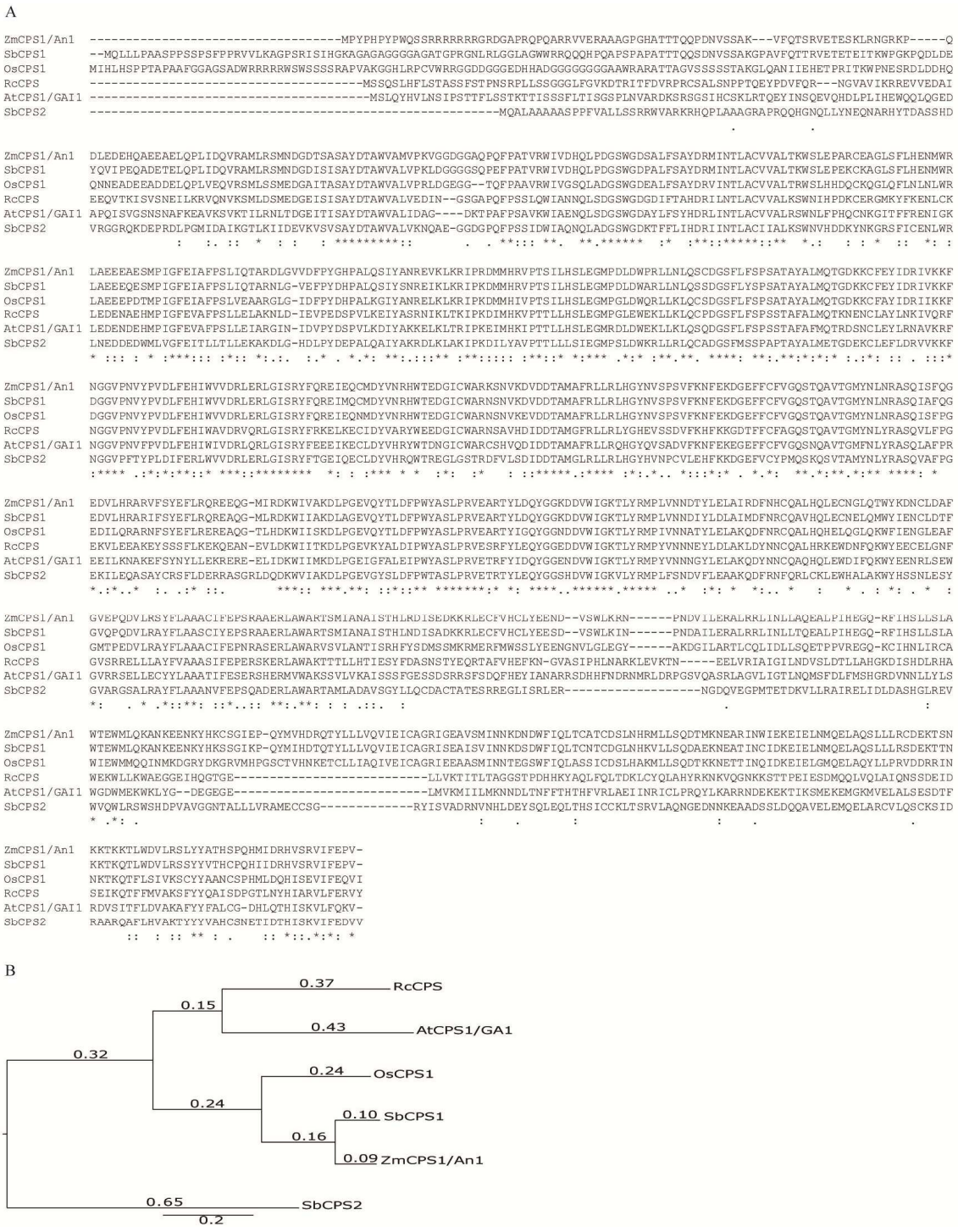

Figure 13. Amino acid alignment (A) and phylogenetic tree of CPS (B). RcCPS, AtCPS1/GA1, OsCPS1 and ZmCPS1/An1 indicate CPS of *Ricinus communis*, *Arabidopsis*, rice and maize, respectively. There are two CPS-like proteins (SbCPS1 and 2) in sorghum, but loss of function of SbCPS1 induced severe dwarfism, indicating that SbCPS1 should dominantly function for GA biosynthesis.

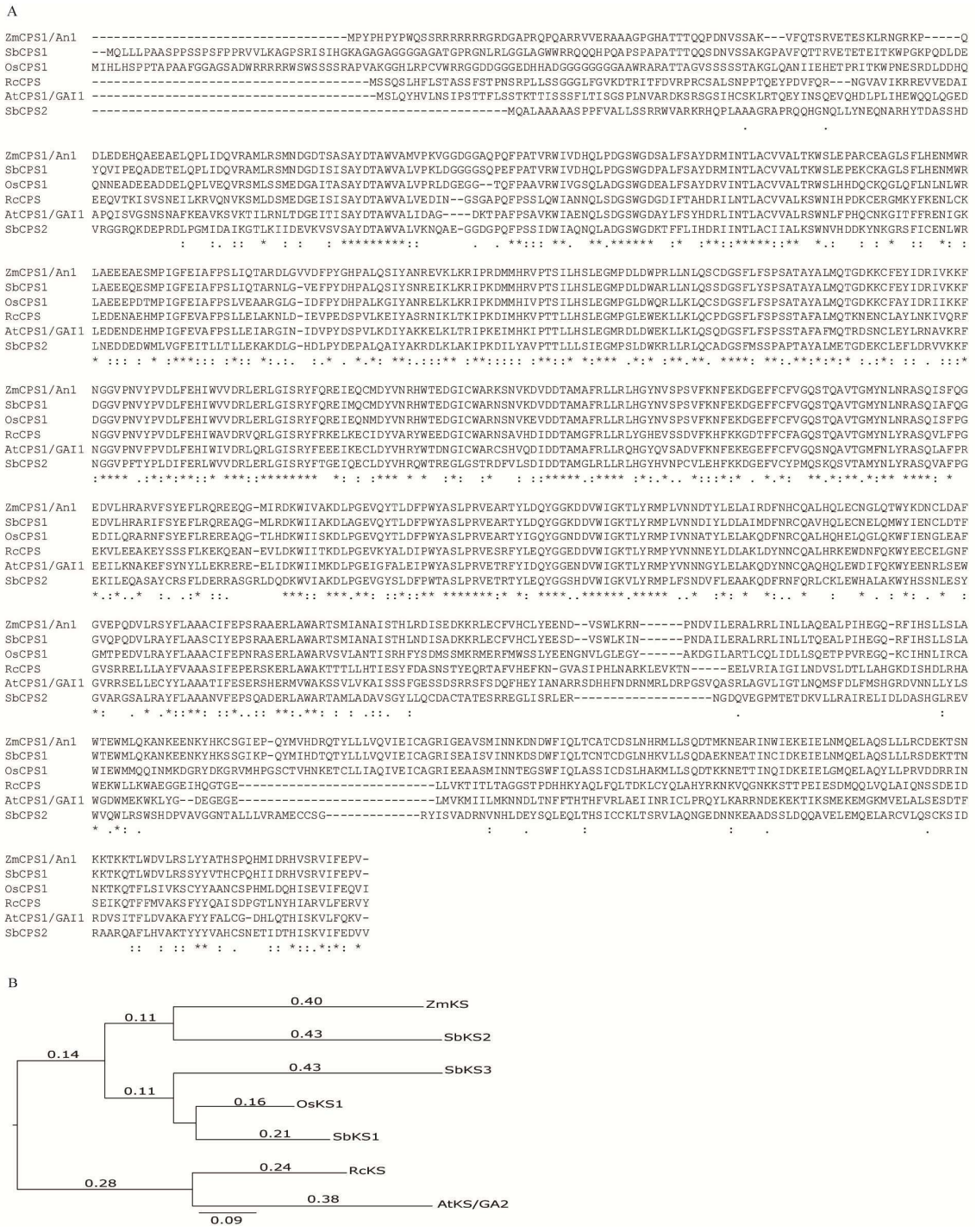

Figure 14. Amino acid alignment (A) and phylogenetic tree of KS (B). ZmKS, OsKS1, RcKS, and AtKS/GA2 indicate KS of maize, rice, *Ricinus communis*, and *Arabidopsis*, respectively. There are three KS-like proteins (SbKS1-3) in sorghum, and the loss of function of SbKS1 induced severe dwarfism, indicating that it should dominantly function for GA biosynthesis.

A

```
AtKAO1 --------------------------MAETTSWIPVWFPLMVLGCFGLNWLVRKVNVWLYESSLGE-NRHYLPPGDLGWPFIGNMLSFLRAFKTSDPDSFTRTLIKRYGPKGIYKAHMFG
AtKAO2 --------------------------MTETGLILMWFPLIILGLFVLKWVLKRVNVWIYVSKLGE-KKHYLPPGDLGWPVIGNMWSFLRAFKTSDPESFIQSYITRYGRTGIYKAHMFG
RcKAO  ---------------------------MEMGFVWVVLIWISGGFWCLKWILKRVNCWLYENQLGE-MQYSLPPGDLGWPFIGNMWSFLRAFKSNDPDSFMRNFTARYGSGGIYKAFMFG
ZmKAO  MLGVGMAAAVLLGAVALLLADAAARRAHWWYREAAEAVLVGAVALVVVDAAARRAHGWYREAALGAARRARLPPGEMGWPLVGGMWAFLRAFKSGKPDAFIASFVRRFGRTGVYRSFMFS
SbKAO1 ----------------------MLGEEAALLSWAAALLLGAAALVLVDAAARWAHGWYREAPLGAARRARLPPGEMGWPVVGGMWAFLRAFKSGKPDAFIASFVRRFGRTGVYRGFMFS
OsKAO1 ----------------MVMEGMGMAAAWAAGDLWVLAAAVVAGVVLVDAVVRRAHDWVRVAALGAERRSRLPPGEMGWPMVGSMWAFLRAFKSGNPDAFIASFIRRFGRTGVYRTFMFS
                                                .   :.  : .: *     **   :  ****::***.:*.* :******:..*::*  .   *:*  *:*: .**.

AtKAO1 NPSIIVTTSDTCRRVLTDDDAFKPGWPTSTMELIGRKSFVGISFEEHKRLRRLTAAPVNGHEALSTYIPYIEENVITVLDKWT--------KMGEFEFLTHLRKLTFRIIMYIFLSSESE
AtKAO2 YPCVLVTTPETCRRVLTDDDAFHIGWPKSTMKLIGRKSFVGISFEEHKRLRRLTSAPVNGPEALSVYIQFIEETVNTDLEKWS--------KMGEIEFLSHLRKLTFKVIMYIFLSSESE
RcKAO  KPSVLVTTSEACKRVLTDDDAFKPGWPSSTTELIGKKSFIGISYEEHKRLRRLTASPVNGHEALSVYMHYIEDKVKSALEKWS--------TMGEIQFLTQLRKLTFRIIMYIFLSSESH
ZmKAO  SPTVLVTTAEGCKQVLMDDDAFVTGWPKATVALVGPRSFVAMPYDEHRRIRKLTAAPINGFDALTGYLPFIDRTVTSSLRAWADHGG-------SVEFLTELRRMTFKIIVQIFLGGADQ
SbKAO1 SPTVLVTTPEACKQVLMDDDAFVTGWPKATVALIGPKSFVAMPYDEHRRLRKLTAAPINGFDALTAYLPFIDRTVTSSLRAWADESSSGAGAGTGVEFLTELRRMTFKIIVQIFLGGADE
OsKAO1 SPTILAVTPEACKQVLMDDEGFVTGWPKATVTLIGPKSFVNMSYDDHRRIRKLTAAPINGFDALTTYLSFIDQTVVASLRRWSSPESG------QVEFLTELRRMTFKIIVQIFMSGADD
        * ::..*.: *::** **:.*  ***.:*  *:* :**: :.:::*:*:*:**::*:** :**: *: :*: .* : *  *:           .:**:.**::**::*: **:.. ..

AtKAO1 NVMDALEREYTALNYGVRAMAVNIPGFAYHRALKARKTLVAAFQSIVTERRNQRK--QNILSNKKDMLDNLLNVKDEDGKTLDDEEIIDVLLMYLNAGHESSGHTIMWATVFLQEHPEVL
AtKAO2 HVMDSLEREYTNLNYGVRAMGINLPGFAYHRALKARKKLVAAFQSIVTNRRNQRK--QNISSNRKDMLDNLIDVKDENGRVLDDEEIIDLLLMYLNAGHESSGHLTMWATILMQEHPMIL
RcKAO  SVMEALEREYTTLNYGVRAMAINLPGFAYYKALKARKNLVAVLQFVVDARRNQKKGAEPNSSKKKDMMDALLDVEDEKGRKLSDEEIVDVLLMYLNAGHESSGHITMWATVFLQEHPEFL
ZmKAO  ATTRALERSYTELNYGMRAMAINLPGFAYRGALRARRRLVAVLQGVLDERRAARAK-GVS-GGGVDMMDRLIEAQDERGRHLDDDEIIDVLVMYLNAGHESSGHITMWATVFLQENPDMF
SbKAO1 PTTRALERSYTDLNYGMRAMAINLPGFAYHRALRARRRLVAVLQGVLDERRAAKAK-GVS-GAGVDMMDRLIEAEDERGRRLDDDEIIDVLIMYLNAGHESSGHITMWATVFLQENPDIF
OsKAO1 ATMEALERSYTDLNYGMRAMAINLPGFAYYRALRARRKLVSVLQGVLDGRRAAAAK-GFKRSGAMDMMDRLIEAEDERGRRLADDEIVDVLIMYLNAGHESSGHITMWATVFLQENPDIF
        .  :***.** ****:***.:*:*****  **:**: **:.:* ::  **          .   **:* *:::.:** *: * *:**:*:*:************  ****:::**:* .:

AtKAO1 QRAKAEQEMILKSRPEGQKGLSLKETRKMEFLSQVVDETLRVITFSLTAFREAKTDVEMNGYLIPKGWKVLTWFRDVHIDPEVFPDPRKFDPARWDNGFVPKAGAFLPFGAGSHLCPGND
AtKAO2 QKAKEEQERIVKKRAPGQK-LTLKETREMVYLSQVIDETLRVITFSLTAFREAKSDVQMDGYIIPKGWKVLTWFRNVHLDPEIYPDPKKFDPSRWEG-YTPKAGTFLPFGLGSHLCPGND
RcKAO  QKARQEQEEIIKRRPPTQKGLTLKEVRDMEYLSKVIDETLRLITFSLVVFREAKTNVNISGYVIPKGWKILVWFRSVHLDPEIYPNPREFNPSRWDN-HTAKAGTFLPFGAGSRMCPGND
ZmKAO  ARAKAEQEAIMRSIPSSQRGLTLRDFRKMEYLSQVIDETLRLVNISFVSFRQATRDVFVNGYLIPKGWKVQLWYRSVHMDPQVYPDPTKFDPSRWEG-HSPRAGTFLAFGLGARLCPGND
SbKAO1 AKAKAEQEAIMRSIPASQQGLTLRDFRKMEYLSQVIDETLRLVNISFVSFRQATKDVFVNGYLIPKGWKVQLWYRSVHMDPQVYPDPTKFNPSRWEG-HSPRAGTFLAFGLGARLCPGND
OsKAO1 ARAKAEQEEIMRSIPATQNGLTLRDFKKMHFLSQVVDETLRCVNISFVSFRQATRDIFVNGYLIPKGWKVQLWYRSVHMDDQVYPDPKMFNPSRWEG-PPPKAGTFLPFGLGARLCPGND
        :*: *** *::  .  *. *:*:: :.* :**:*:***** :.:*:. **:*. :: :.**:******:  *:*.**:* :::*:*  *:*:**:.   .:**:**.** *:::*****

AtKAO1 LAKLEISIFLHHFLLKYQVKRSNPECPVMYLPHTRPTDNCLARISYQ----
AtKAO2 LAKLEISIFLHHFLLKYRVERSNPGCPVMFLPHNRPKDNCLARITRTMP-
RcKAO  LAKLEIAIFLHHFLLNYELERLNPGSSMMYLPHSRPKDNCLARIKKIPSA-
ZmKAO  LAKLEISVFLHHFLLGYKLARTNPRCRVRYLPHPRPVDNCLAKITRVGS-
SbKAO1 LAKLEISVFLHHFLLGYRLARTNPRCRVRYLPHPRPVDNCLAKITRVSEEY
OsKAO1 LAKLEISVFLHHFLLGYKLKRANPKCRVRYLPHPRPVDNCLATITKVSDEH
       ******::******* *.: * ** . : :*** ** ***** *.
```

B

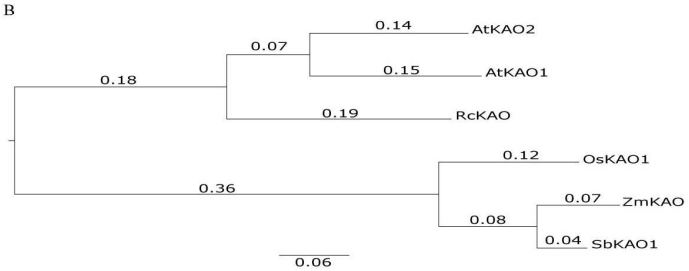

Figure 15. Amino acid alignment (A) and phylogenetic tree of KAO (B). AtKAO, RcKAO, OsKAO1 and ZmKAO indicate KAO of *Arabidopsis*, *Ricinus communis*, rice and maize, respectively. There is a single KAO-like protein in sorghum and its loss of function induces severe dwarfism.

Figure 16. Introduction of sorghum GA biosynthetic genes rescued the corresponding mutants of rice, *oscps1-1, osks1-1, osko2-1*, and *oskao-1*. Scale bar = 4 cm. Plants at the left and right in each set were transformed with the complementary WT genes from sorghum and the vector control, respectively.

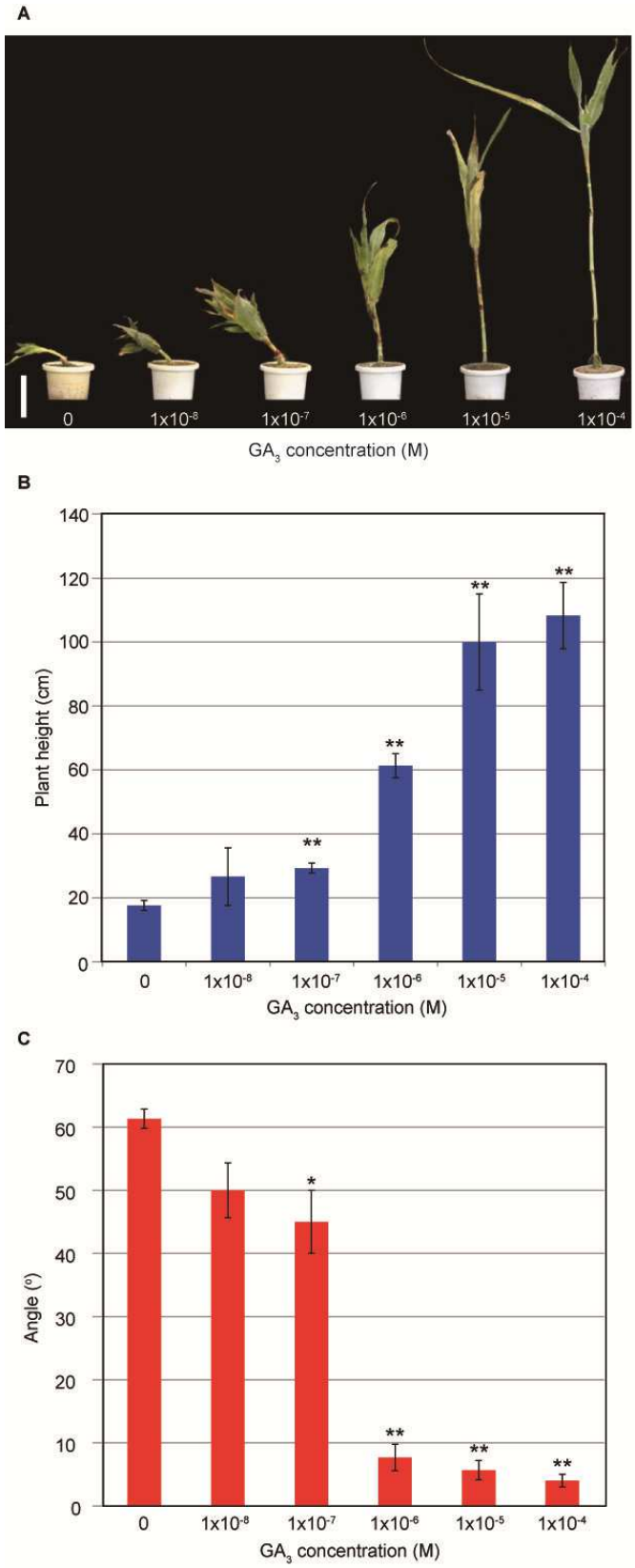

Figure 17. Dose-dependent recovery of the mutant phenotypes by exogenous GA

treatment. (A) The *bdw3-1* plants grown in different concentrations of GA_3 for four months. Bar = 10 cm. (B) Dose-dependent recovery of plant height. (C) Dose-dependent recovery of the bending culm phenotype, which was determined by measuring the angle between the main culm and the vertical axis. Error bars represent the standard deviation calculated from 3 replicates. Single (*) and double asterisks (**) indicate significant difference with the mock (0 M GA_3) at 0.5% ($P<0.005$) and 0.1% ($P<0.001$) levels of significance, respectively, as determined by t-test.

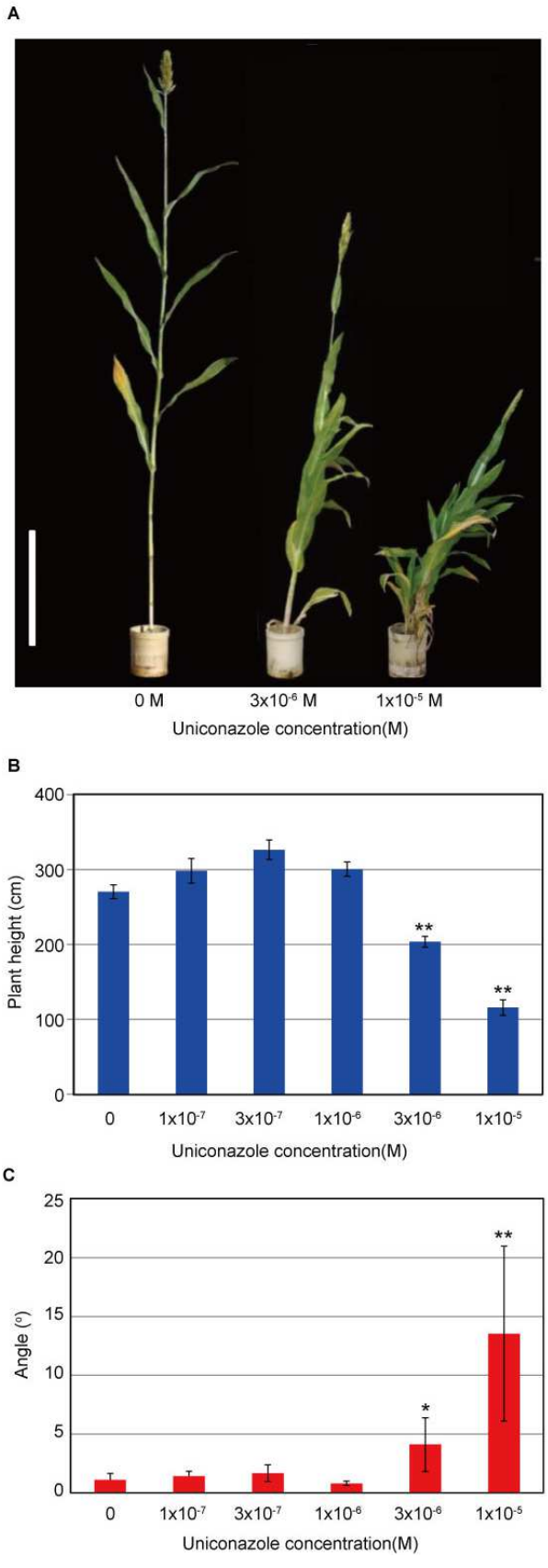

Figure 18. Effect of uniconazole on the plant height and culm bending of WT plants.

(A) Gross morphology of the uniconazole-treated plants. The plants at the left, center, and right were treated with 0, 3 x 10^{-6} and 1 x 10^{-5} M uniconazole, respectively. Bar = 50 cm. Dose-dependent inhibition of plant height (B) and induction of culm bending (C) in WT plants. Error bars represent the standard deviation calculated from 5 replicates. Single (*) and double asterisks (**) indicate significant difference between the mock and 3 x 10^{-6} and 1 x 10^{-5} M uniconazole at 5% ($P<0.05$) and 1% ($P<0.01$) levels of significance, respectively, as determined by t-test.

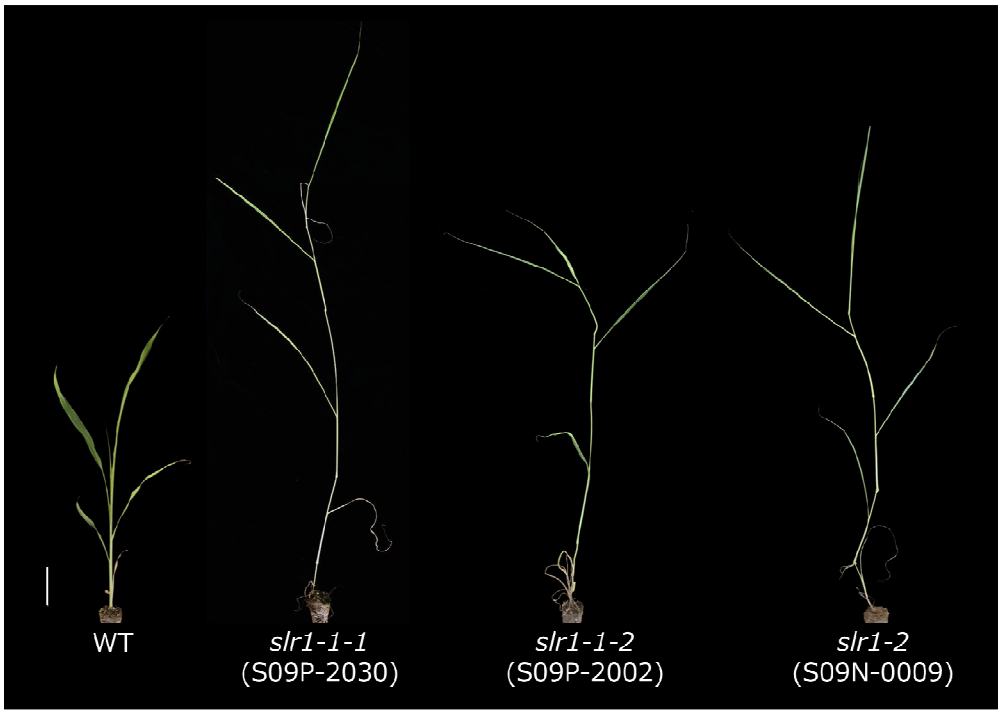

Figure 19. The gross morphology of the *slr1* mutants at the seedling stage. Bar = 5 cm.

Figure 20. Some abnormal culm twisting anomalies observed in *slr1-1* (S09P-2030). (A) The mutant at the seedling stage. (B) The mutant at the heading stage. The white arrowhead points at the twisted panicle (C) The enlarged picture of the panicle in B. Red arrowheads point at the twisted culms. Scale bars = 5 cm.

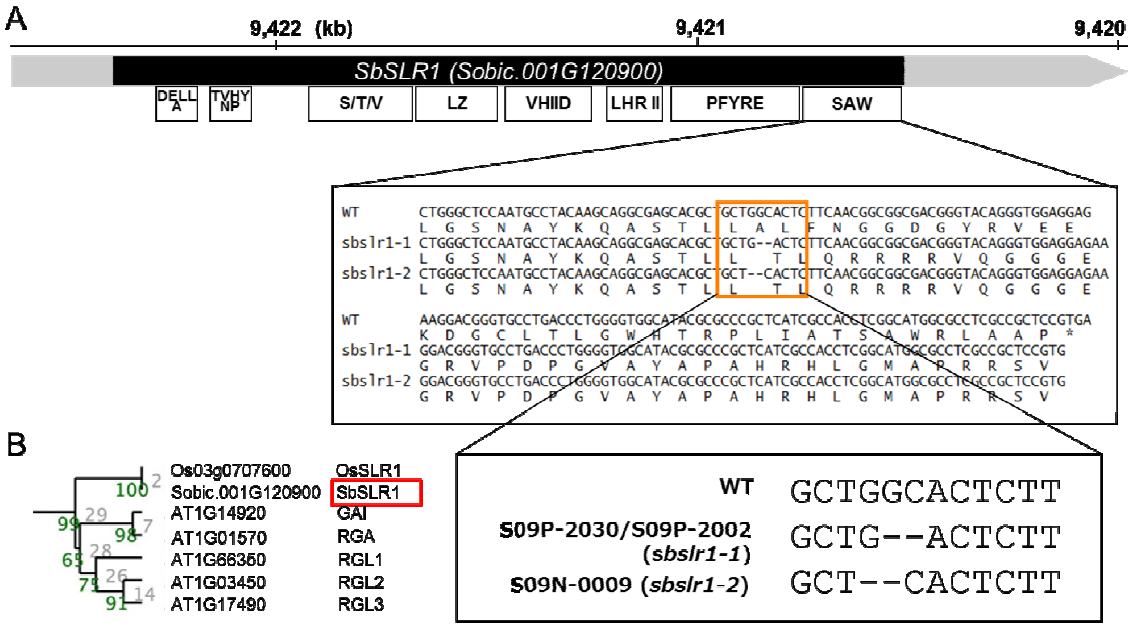

Figure 21. Mutation of *SbSLR1* in the slender mutants. (A) The gene structure of *SbSLR1*, where gray and black shades indicate UTRs and exon, respectively. White boxes underneath the exon indicate the protein domain that they code for. The loss of function of the gene is due to a 2-base pair deletion at the SAW domain-coding region of the gene. (B) Phylogenetic analysis of SbSLR1. A single copy of the protein exists in sorghum.

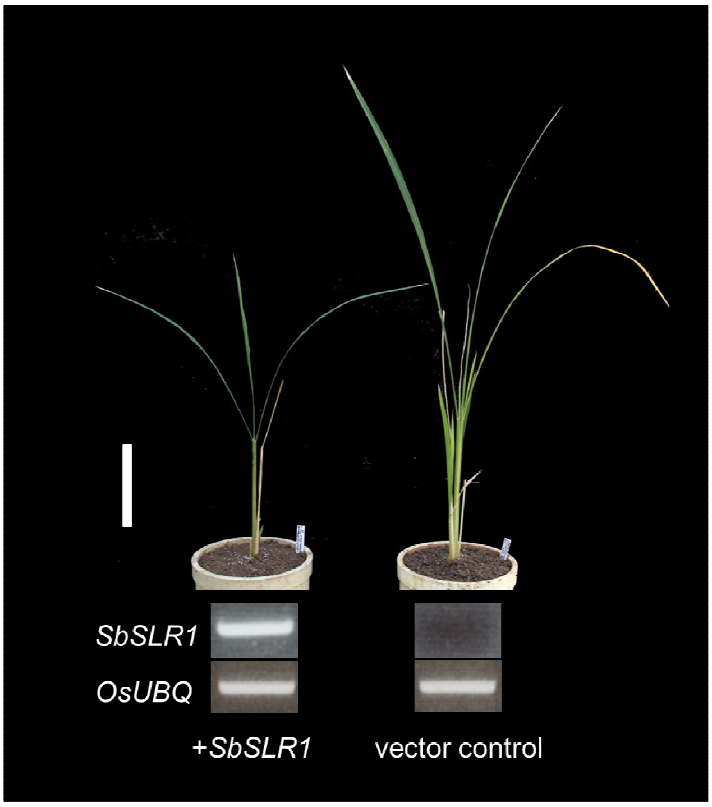

Figure 22. Height reduction in T65 transformed with the WT *SLR1* gene from sorghum. Scale bar = 15 cm. Expression of the introduced *SbSLR1* was verified by RT-PCR using rice *Ubiquitin* gene (*OsUBQ*) as control.

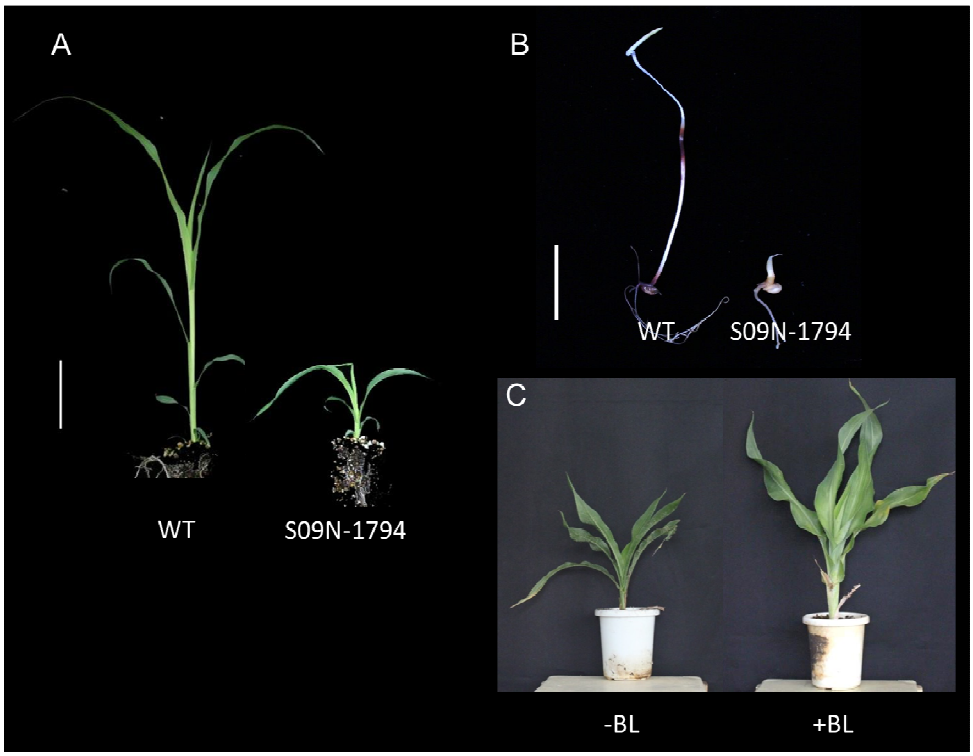

Figure 23. Morphological characteristics of S09N-1794 (*sbbr6ox*). (A) The mutant at the seedling stage. Scale bar = 2 cm. (B) Abnormal skotomorphogenesis in the mutant after being germinated in the dark. Scale bar = 2 cm. (C) BL treatment for three weeks considerably increased the plant height of the mutant.

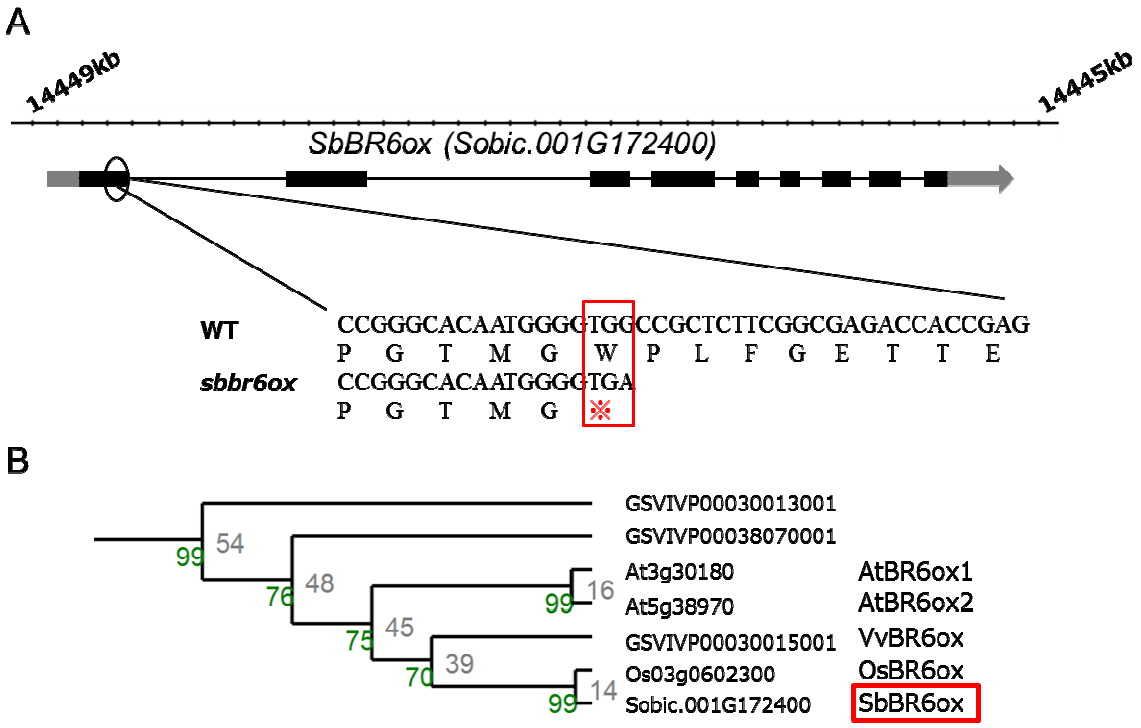

Figure 24. Mutation of *SbBR6ox* in the mutant S09N-1794. (A) Gene structure of the *SbBR6ox* gene. The presentation is the same as in Figure 9B except for the UTRs shown in gray. A base substitution in the exon 1 of the gene resulted in a premature stop codon. (B) Phylogenetic tree of SbBR6ox. A single copy of the protein exists in sorghum.

Figure 25. Introduction of sorghum *BR6ox* rescued the severe dwarf phenotype of rice T2-177. Plant on the left was transformed with the WT *BR6ox* gene from sorghum while the other one was transformed with the empty vector. Scale bar = 4 cm.

Figure 26. Gross morphology of the semi-dwarf *dw3-sd3* mutant line (S09P-2101). Black bar = 1 m.

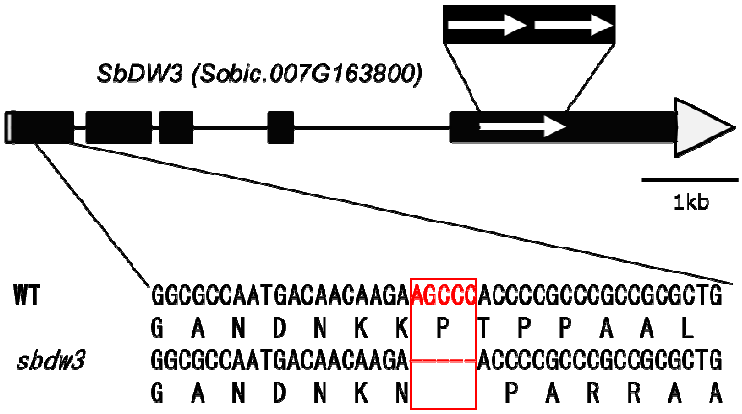

Figure 27. Mutation of *SbDw3* in the mutant S09P-2101. The presentation is the same as in Figure 24. Red box and letters indicate the 5-bp deletion in the exon 1 of the gene. Two abutting arrows above the gene indicate the duplication in the classical *dw3* mutation used in many sorghum semi-dwarf varieties.

Table 4. Previous researches regarding sorghum dwarfing genes

Author	QTL Name	Chromosome Location	Candidate Gene	Description
Morris *et al*. (2012)	*Dw1/Sb-HT9.1*	Chr 9 (~57.2 Mb)*	*GA2ox5*	Top association peak is 29 kb from a *GA2ox5*
	Dw2	Chr 6 (39.7-42.6 Mb)	*histone deacetylase* (*Sobic.006G067600*)	
	Dw3	Chr 7 (~58.6 Mb)	*SbPGP1*	Top association peak is 114 kb from *dw3* locus
	Dw4	Chr 6 (~6.6 Mb)	unknown	
Thurber *et al*. (2012)	*Dw1*	Chr 9 (57.4-57.6 Mb)*	unknown	
	Dw2	Chr 6 (~40.3 Mb)	unknown	*Dw2* locus is several Mb away from tightly linked *Ma1* at 40.3 Mb
	Dw3	Chr 7 (58.2-58.7 Mb)	*SbPGP1*	
Wang *et al*. (2012)	23-1062	Chr 2 (~65.5 Mb)	*Ugt (UDP-glycosyltransferase/indole-3-acetate beta-glucosyltransferase*)	The marker 23-1062 was 385 kb from *Ugt*
	37-1740	Chr 6 (~9.8Mb)	unknown	
	40-1897	Chr 6 (~61.1Mb)	*Floricaula/leafy-like 2* or *FtsZ*	The marker 40-1897 was 0.44 cM from *Ftz* and 4.89 cM from *Floricaula/leafy-like 2*
	44-2080	Chr 9 (~57.2Mb)*	*GA2ox5*	The marker 44-2080 was 47 kb from *GA2ox5*
Brown *et al*. (2008)	*Sb-HT9.1*	Chr 9 (57.14-57.21 Mb)*	unknown	

* Analyzed in this study

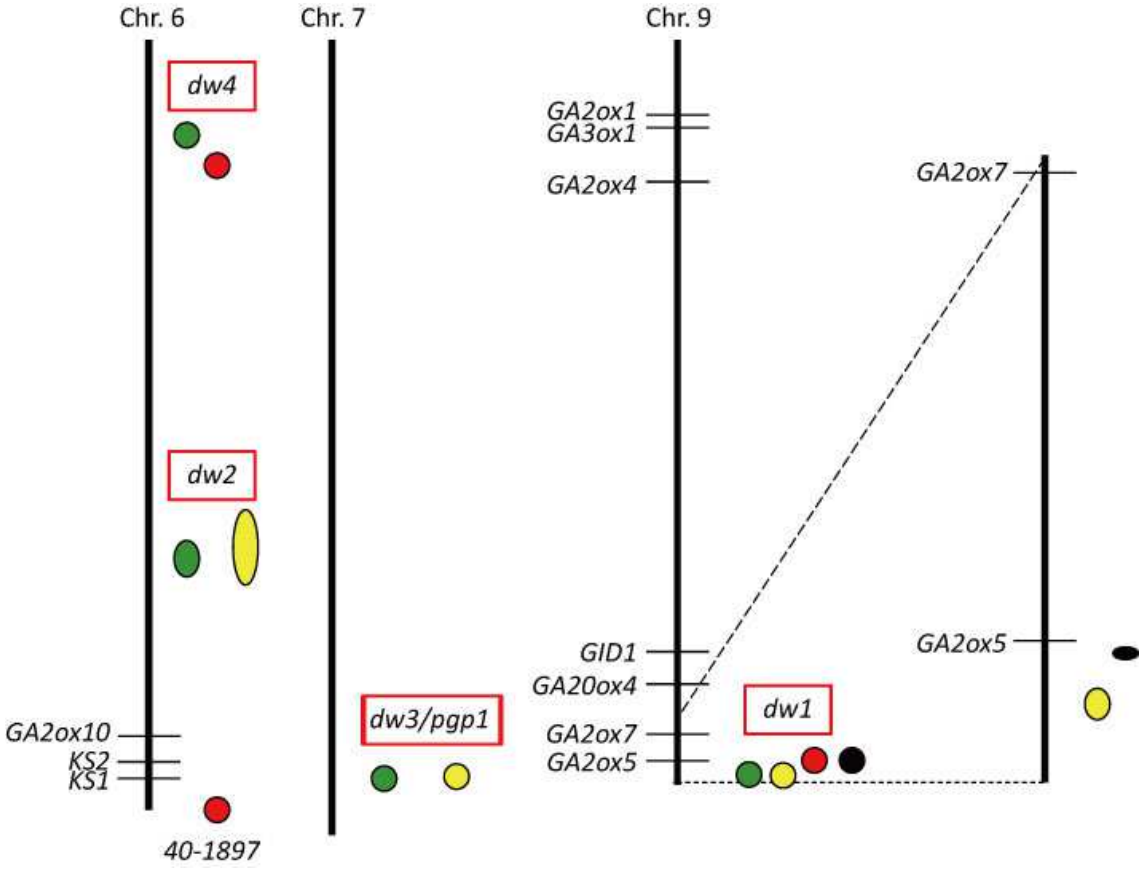

Figure 28. Comparison between the map positions of previously reported dwarfing QTL genes and the GA-related genes of sorghum. The chromosomes carrying the dwarfing QTL genes previously reported are presented. Ovals and circles at the right side of each chromosome indicate the positions of respective previously reported dwarfing QTLs (red rectangles), where green, yellow, red and black shades represent the mapping work of Morris *et al*. (2013), Thurber *et al*. (2013), Wang *et al*. (2012) and Brown *et al*. (2008), respectively. The positions of GA-related genes are presented at the left side of each chromosome with their corresponding names as presented in Table 3.

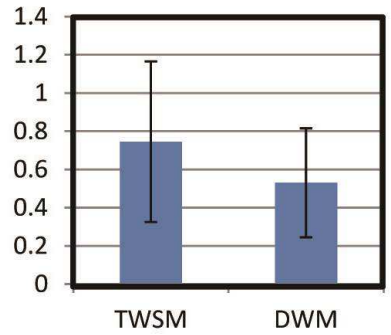

Figure 29. Comparison of the DNA sequence and expression levels of *SbGA2ox5* (*Sobic.009G230800*) between two sorghum isogenic varieties carrying gain- and loss-of-function alleles of Dw1. (A) Alignment of the DNA sequences of the two varieties, tall white sooner milo (TWSM) and dwarf white milo (DWM). No difference in sequence was found at the 2-kb upstream sequence, ORF (black shade), introns (gray shade), and 886-bp downstream sequence of *SbGA2ox5*. (B) Reads Per Kilobase of exon model per Million mapped reads (RPKM) of *SbGA2ox5* in TWSM and DWM as determined by RNA-seq experiment. There was no significant difference between their RPKMs ($P<0.29$), although the taller TWSM expressed slightly higher level of *SbGA2ox5* than the shorter DWM. Error bars represent the standard deviation calculated from 3 replicates.

Table 5. Primers used to analyze the identified genes in this study

Name	Sequence	Hormone relation	Remarks*
CP1F	TGCCTAATGGACAGTGGCACCACT	GA	
CP1R	CTCATCATCCTATGCCAGGGTGACCAAG	GA	
CP2F	ACCCTGATGCGACCATCTCGGGA	GA	
CP2R	GGTTCCAGTCCGTGCGCGTTT	GA	
CP3F	GGATCAAGGGCGGCCAACAGA	GA	DNA blotting probe
CP3R	TGGAGTCGGACATCCCCCCACA	GA	DNA blotting probe
CP4F	CTGTAGTGCTAGGAGATGCTTTGTGC	GA	Used for sequencing
CP4R	GATGGACACGGCCAACAA	GA	Used for sequencing
CP5F	ACACTCAGAGATGCTGTGCT	GA	Used for sequencing
CP5R	AACCGTAAGCAACCCCCTCAT	GA	Used for sequencing
CP6F	GCTTACGGTTTTATGCAGGTCG	GA	Used for sequencing
CP6R	CGAATCGGTGTTCGGTTCGG	GA	Used for sequencing
CP7F	GTTCGGATCGAGCGAAGTC	GA	Used for sequencing
CP7R	GTCTGGAACACTGCAGGTC	GA	Used for sequencing
CP8F	CAGTTTCAGGTCAGCCCAAT	GA	Used for sequencing
CP8R	GATCATACGGAAACTCAACACCCA	GA	Used for sequencing
CP9F	AGCAGGAGTCGATGCCCAT	GA	Used for sequencing
CP9R	CATCATAAGGTTCGGTGCTCACT	GA	Used for sequencing
CP10F	AGACTCGGGATCTCCCGCTA	GA	Used for sequencing
CP10R	TCGCCAGCTAGATCCTTCGC	GA	Used for sequencing
CP11F	ACTCAAGCCGTCACTGGG	GA	Used for sequencing
CP11R	GTTCTAGCCCATGCAAGCCG	GA	Used for sequencing
CP12F	GCATTTATGAACCTAGCCGTGCTG	GA	Used for sequencing
CP12R	TGGCAATGCTTCTTGCGTCA	GA	Used for sequencing
CP13F	CTCGAGAGGGCACTTCGA	GA	Used for sequencing
CP13R	TGGGACAGTAACACCTTGTGGT	GA	Used for sequencing
CP14F	GTGATGGTCTTAACCACAAGGT	GA	Used for sequencing
CP14R	CTCATCAGACCTCAGGAGCA	GA	Used for sequencing
CP15F	CCATGCGCAGGATGCTGAG	GA	Used for sequencing
CP15R	CATCAAGTGCATCATGGTGGT	GA	Used for sequencing
CP16F	AGCAAGGTCATCTAAGCCCTAGCGCACT	GA	
CP16R	ACACGCCCATGGCACAAAGGTTGGCTCT	GA	
CP17F	CCAACTCAGACATCAGGTTAGGCAGCTT	GA	
CP17R	CGCTCGATGTGCTTGGTCGCTCA	GA	
CP18F	CGCCATGGATGGATTGCAGTGGTT	GA	
CP18R	AGCACCTGGACCCACCAAGGT	GA	
CP19F	GAGGAGGAGGTGTACTCTCTGGCG	GA	
CP19R	TGCCTCTACCTTGGCACAGGCAT	GA	
CP20F	CGAGCTCGGATTCTCTTACGGAAGATT	GA	Used for cloning
CP20R	CGAGCTCATTGCATGCTTCCACTACCG	GA	Used for cloning
KS1F	CAAAGCCACATACATCGGCA	GA	Used for sequencing
KS1R	CGGCCATAACGTTGGGCAT	GA	Used for sequencing
KS2F	TCTTCAGGGCGCCAAGTC	GA	Used for sequencing
KS2R	CTTCAACGCAAGAACACATGCCAAC	GA	Used for sequencing
KS3F	AGCCTGGGTTTCTATGGTGCC	GA	Used for sequencing
KS3R	ACAAATCAGGAGCACCCCTTT	GA	Used for sequencing
KS4F	AAGACAAACTGATGTCTGTGGG	GA	Used for sequencing
KS4R	CCATTCATCCTCAAAAGGCGA	GA	Used for sequencing
KS5F	TTCAGGTGGGTTGCCGTA	GA	Used for sequencing
KS5R	GCATCAGAGGACCCCAAGCA	GA	Used for sequencing

Table 5. (continuation)

Name	Sequence	Hormone relation	Remarks*
KS6F	AGGATTCTGGTCTGGCAACT	GA	Used for sequencing
KS6R	GCCCATGAAATGCGAGCG	GA	Used for sequencing
KS7F	GTTTACCAGGATGAACTTCGGCAT	GA	Used for sequencing
KS7R	CGAAATACAATGCTGGGAGCACAA	GA	Used for sequencing
KS8F	CTTGGAACACATTTGCAGTGGCTG	GA	Used for sequencing
KS8R	GGACTGTGAGTGGCTCGTT	GA	Used for sequencing
KS9F	GAAAGCGTTGTTCCTAGGCCAT	GA	Used for sequencing
KS9R	GAAGGAGAACTGGAGCATCTG	GA	Used for sequencing
KS10F	CGCGGATCCCTCGAGTGAGACACAGATTTAGCGTT	GA	Used for cloning
KS10R	CGGGGTACCGGGACCAGATTTTTGCAGGATGTGGCA	GA	Used for cloning
KO1F	ACGTCCAATGGGCTTCAGCTGTTGGA	GA	
KO1R	TGGTAAGTGGATTCGTGAGCACTCGCT	GA	
KO2F	TGTGCACATAAGTTAGCTCGC	GA	
KO2R	ATTTCTCGGGTGGATCGTGA	GA	
KO3F	CATGACCTTCACGATCCACCCGAGAAA	GA	
KO3R	CTCCGTCACATCGAATCTTGCGGCA	GA	
KO4F	CCTAAGCACGTCAAAGGCTCAATCG	GA	
KO4R	ACTGGTGGAAGTTCAAGTGCGA	GA	
KO5F	AGCAACCACGAACCCGACGTCCT	GA	
KO5R	GCCAGAACTGTTAGCAAGAGCATTCCGG	GA	
KO6F	TGGCCATCAGGTATGCGGCCGATTA	GA	
KO6R	GCAGAGCTCACCATGCATGTGGTG	GA	
KO7F	GTTTTCCTATTATGGCCCTGCTGGTTGC	GA	
KO7R	GTGGTAGTGGAGGTCGGTGGTTCG	GA	
KO8F	CCATAGTTGGACTCGTCACCGCCAA	GA	
KO8R	AGGCCTTTAGAATTAGTAGGAGGCAGCACAC	GA	
KO9F	CATTGTTCCAGCCACGTTCG	GA	
KO9R	CGTCCGAGGCAAGTTTCAGA	GA	
KO10F	CACGTGCACATGTTGGTTTAC	GA	
KO10R	GCGTCCGGTGATTCTCAGT	GA	
KO11F	GTGGGTGGAAGCTCCACCACTCCT	GA	
KO11R	GGCATCGAAACCAGGTACGCATCACT	GA	
KO12F	TATGAGATTGCGAAGCACCC	GA	Used for sequencing
KO12R	CAAGTTCGTGTCCAAGTCCT	GA	Used for sequencing
KO13F	GATATCCTAGAGCTCGTGGT	GA	Used for sequencing
KO13R	ACTGTCTTATTGCCGCACAC	GA	Used for sequencing
KO14F	GTGACTATGGTGACTTCCAC	GA	Used for sequencing
KO14R	CACCTAAAGCCTGGAATTGC	GA	Used for sequencing
KO15F	TAGGCTATCTGGACTCACCA	GA	Used for sequencing
KO15R	CGTGGTTGACACTGAAGAAC	GA	Used for sequencing
KO16F	CCTATGAGTACCTCCGAAGA	GA	Used for sequencing
KO16R	GTCATCTGCTACCCGAACTT	GA	Used for sequencing
KO17F	GCCATCTGTAAGTGGGACAA	GA	Used for sequencing
KO17R	TCGGTAGTGGTCCAGTATAC	GA	Used for sequencing
KO18F	GGGAGTCATCTTCTTGGGCACGACCTC	GA	
KO18R	AGCAGGAGCTAGGGCAGCCAACAG	GA	
KO19F	CAACACTCCTATCCACCCACAGCACAT	GA	
KO19R	CTGCTGTGCTTGGGATGGTGGGCT	GA	
KO20F	GCCAATCCAAGCTAACATGCTGAG	GA	DNA blotting probe
KO20R	ACGTTGGGCAGCATGCGT	GA	DNA blotting probe
KO21F	GCTCTAGATTGTGCAGATTCAGATCAAC	GA	Used for cloning
KO21R	GCTCTAGAAATGCTTGAGTAGAATTCAG	GA	Used for cloning
KAO1F	CGCTTGCGTGTGCATAATAC	GA	Used for sequencing

Table 5. (continuation)

Name	Sequence	Hormone relation	Remarks*
KAO1R	CACACTTGACAGTAACAGGG	GA	Used for sequencing
KAO2F	GCCTCTGATCTTGATCAGTC	GA	Used for sequencing
KAO2R	TTCTCCTGCAGGAACACGGT	GA	Used for sequencing
KAO3F	ATGATGGACCGGCTGATCGA	GA	Used for sequencing
KAO3R	TTGAACTTGGTGGGGTCAGG	GA	Used for sequencing
KAO4F	CAACATCTCCTTCGTCTCCT	GA	Used for sequencing
KAO4R	CAGAGCATGCCATGTTGGAG	GA	Used for sequencing
KAO5F	CGGGATCCTGATTGCCCGCAACACCCGA	GA	Used for cloning
KAO5R	GGGAAGCTTTCAGGCCAAACGCTACATGT	GA	Used for cloning
SbSLR1F1	TAGCAACCGCAGTACACATCGGC	GA	Used for sequencing
SbSLR1R1	TGGGCTCCAATGCCTACAAGC	GA	Used for sequencing
SbSLR1F2	CCATGCCGAGGTGGCGATGA	GA	Used for sequencing
SbSLR1R2	TCACCGCGAACCAGGCAATC	GA	Used for sequencing
SbSLR1F3b	CGAGGATTGCCTGGTTCGCGGT	GA	Used for sequencing
SbSLR1R3b	CAGCAGCACCTACGCCCTGAAG	GA	Used for sequencing
SbSLR1F4	GGCTTCAGGGCGTAGGTGCT	GA	Used for sequencing
SbSLR1R4	CCGTCCATCGATCCAACGCTGTGCG	GA	Used for sequencing
SLR1_KpnI_F	GGGGTACCTCATGTTCAATGGTTCAACG	GA	Used for cloning
SLR1_KpnI_R	GGGGTACCTCCAATCCGAGGAGTACATG	GA	Used for cloning
SOL6OX1_5	CCTCCTCCTGCTCCAAGCAC	BR	Used for sequencing
SOL6OX1_3	GGGACGGCGAGCGGAATGCGG	BR	Used for sequencing
SOL6OX2_5	GAAGCTGATGGATGGTACC	BR	Used for sequencing
SOL6OX2_3	CAACTGCTGTGCATCTG	BR	Used for sequencing
SOL6OX3_5	CTTCCATAGCGTAGCATGGC	BR	Used for sequencing
SOL6OX3b_5	CACTTGGCTGGGTGCTG	BR	Used for sequencing
SOL6OX3_3	GGGCTCAGTTCAGCTCGAC	BR	Used for sequencing
SOL6OX4b_5	GAGCCCTCTAACAGCAGCAG	BR	Used for sequencing
SOL6OX4_3	GGGATTGAACTTGGTAGCG	BR	Used for sequencing
SOL6OX5_5	CGCTACCAAGTTCAATCCC	BR	Used for sequencing
SOL6OX5_3	CTGGGTGTATCATCTCCAAC	BR	Used for sequencing
SOL6OX6_5	GTTGGAGATGATACACCCAG	BR	Used for sequencing
SOL6OX6_3	GGATCCTGTAGGATTCTC	BR	Used for sequencing
SOL6OX7_5	GAGAATCCTACAGGATCC	BR	Used for sequencing
SOL6OX7_3	CTGAGATGTTTGCTTGTTC	BR	Used for sequencing
SOL6OX8b_5	GACAATAGGAGACGAACCTC	BR	Used for sequencing
SOL6OX8_3	GAGGCCAGCAGCATCAGCATC	BR	Used for sequencing
SB6OX_F8c	CGGAACCGATGGTTTTCAAC	BR	Used for sequencing
6OX_BGLII_Fb	GAAGATCTGCCTATGAACACAAAGTATC	BR	Used for cloning
6OX_BGLII_Rb	GAAGATCTATTCAACGGGCTCATGAAGC	BR	Used for cloning
SBDW3_F4c	GAAAGTGGCCTTACACCGGTCCTCAGCGA	Auxin	Used for sequencing
SBDW3_R4d	CGTACCAGAGCAGGAGGCCGTAGCA	Auxin	Used for sequencing
DW3F10c	ATCACGGGGCTCTTCGAG	Auxin	Used for sequencing
DW3R10c	GAGTGCGACCCTTGCTCCA	Auxin	Used for sequencing
DW3F11b	CGGTGGCTCTTCGAGGCCAA	Auxin	Used for sequencing
DW3R11c	CTTGTTGCGATCATGGAGTACCA	Auxin	Used for sequencing

* Primers successfully used for DNA sequencing, DNA blotting analysis and gene cloning are indicated.

Acknowledgements

I would like to express my sincerest gratitude to the following people who, in one way or another, have contributed toward the completion of this thesis:

Dr. Takashi Sazuka, for his supervision and for the support he has extended for my research. I am truly grateful for the new things I learned from him.

Dr. Makoto Matsuoka, for his utmost concern and for assisting and training me in publishing scientific papers.

Dr. Yusuke Ito, for his patience, kindness, infectious laughter and most of all, for his brilliant ideas.

Drs. Hidemi Kitano, Miyako Ueguchi-Tanaka, and Motoyuki Ashikari for their kindness and for their invaluable comments and suggestions in connection to my thesis.

To the members of the sorghum team, namely, Tsuyoshi Tanaka, Etsuko Ichikawa, Tsubasa Hosaki, Asako Hatakeyama, Satoko Araki-Nakamura, Kozue Ohmae-Shinohara, Kenshiro Nakamura, Keijiro Kurami, Kei-ichi Nakagawa, Haruka Fujimoto, Miki Yamaguchi, Kazuyuki Hamada and Ayako Kondo-Watanabe for their camaraderie and support in good and in trying times.

To the people of the Laboratory of Plant Molecular Breeding, Plant Bioresource, and Molecular Biosystem, particularly to Rico Gamuyao, Xian-Jun Song, Huan Phung, Rosalyn Angeles-Shim, Keisuke Nagai, Kanako Uehara, Yusuke Kurokawa, Tomoyuki Furuta, Ko Hirano, Mayuko Ikeda, Mayuko Kawamura, Eriko Kouketsu, Kenji Yano, Wakana Takase, Sayaka Takehara, Kumiko Furukawa and Kotoe Otani for their help and concern.

To the Monbukagakusho Scholarship for sponsoring my PhD studies in Japan, and to Ms. Nobuko Abe and Ms. Naoko Kamiya for their assistance and friendliness.

To the Panasonic Scholarship Foundation, Japan for their continued support and guidance since 2007, most especially to Mr. Yoshiro Koezuka.

To the NC-CARP members and staff, especially to Prof. Satoshi Naramoto, Prof. Wataru Sakamoto, Prof., Chieko Saito, Koji Nobuta and Yusuke Mizokami, for the valuable knowledge I acquired and the unforgettable experiences in Kenya.

To all my friends in Japan, namely Roel, Peter, Rency, Dr. Oogawa, Randy, Jhunie, Jane, Mam Joyce and to those not mentioned here.

To the staff and students of ICAN, especially to Ms. Aya and Mika and Mr. Keiichi Kasuga and Jacky for our lively weekly interaction.

To my PhilRice family, and to friends and relatives whom I interact with via Facebook.

To my beloved parents, Mr. Reynaldo G. Ordonio and Mrs. Cresinta L. Ordonio, for their utmost love and concern, and for their ardent prayers; and my brothers Reynaldo, Jeremias, and Ruferto for their support and assistance in the preparation of this manuscript.

Above all, my supreme praises to the Almighty GOD, who has always been very kind to me. He has always been my foremost source of strength, wisdom, spiritual guidance and salvation.

REYNANTE L. ORDONIO
September 2014

List of Publications

(1) Ordonio, R.L., Ito, Y., Hatakeyama, A., Ohmae-Shinohara, K., Kasuga, S., Tokunaga, T., Mizuno, H., Kitano, H., Matsuoka, M. and Sazuka, T. (2014). Gibberellin deficiency pleiotropically induces culm bending in sorghum: an insight into sorghum semi-dwarf breeding. Sci. Rep. 4: 5287.

(2) Ordonio, R.L., Ito, Y., Matsuoka, M. and Sazuka, T. Gamma-ray mutagenesis of sorghum: Characterization of morphological mutants reveals a bent culm phenotype unseen in other species carrying similar mutations. (in preparation)

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
PLANT BREEDING
PLANT MOLECULAR BIOLOGY
GIBBERELLINS

Received
17 April 2014

Accepted
23 May 2014

Published
13 June 2014

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Gibberellin deficiency pleiotropically induces culm bending in sorghum: an insight into sorghum semi-dwarf breeding

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Regulation of symmetrical cell growth in the culm is important for proper culm development. So far, the involvement of gibberellin (GA) in this process has not yet been demonstrated in sorghum. Here, we show that GA deficiency resulting from any loss-of-function mutation in four genes (*SbCPS1*, *SbKS1*, *SbKO1*, *SbKAO1*) involved in the early steps of GA biosynthesis, not only results in severe dwarfism but also in abnormal culm bending. Histological analysis of the bent culm revealed that the intrinsic bending was due to an uneven cell proliferation between the lower and upper sides of culm internodes. GA treatment alleviated the bending and dwarfism in mutants, whereas the GA biosynthesis inhibitor, uniconazole, induced such phenotypes in wild-type plants— both in a concentration-dependent manner, indicating an important role of GA in controlling erectness of the sorghum culm. Finally, we propose that because of the tight relationship between GA deficiency-induced dwarfism and culm bending in sorghum, GA-related mutations have unlikely been selected in the history of sorghum breeding, as could be inferred from previous QTL and association studies on sorghum plant height that did not pinpoint GA-related genes.

Dwarfism in plants is brought about by an irregularity in one or more of the various growth-related mechanisms, and may involve physical defects in some cellular growth processes, or problems in the production and action of phytohormones. So far, aberrant cellular division or elongation has already been reported to cause dwarfism in some mutants such as the rice *d6*[1], *bent uppermost internode 1* (*bui1*)[2], *jmj703*[3] and *dwarf and gladius leaf* (*dgl-1*)[4], and the *Arabidopsis lue1*[5]. However, the relationship between dwarfism and phytohormones has been more widely studied using various kinds of plant species. In the case of auxin, some of the reported dwarf mutants include the maize *brachytic 2* (*br2*)[6,7] and the sorghum *dwarf 3* (*dw3*)[6] which both have semi-dwarfism due to a defect in auxin transport; the rice *small organ 1* (*smos1*), which has a defective transcription factor that disrupts auxin signaling[8], and the auxin-deficient *tdd1*[9]; and the *Arabidopsis sax1* which shows high sensitivity to auxin[10], and *bud1* which has a defect in auxin metabolism[11]. Another growth-promoting hormone is brassinosteroid (BR). Mutants that have a defect in BR biosynthesis and signalling show severe dwarf phenotypes as in the case of the *Arabidopsis* BR-deficient *de-etiolated 2* (*det2*)[12] and *seuss-1* (*seu-1*)[13], and the brassinosteroid-insensitive *bri1*[14]; the rice BR signalling mutants, *d61-4*[15] and *erect leaf 1* (*elf1-1*)[16], and the BR-deficient *ebisu dwarf* (*d2*)[17]; and the tomato *br6ox* (BR biosynthesis) mutant "Micro-Tom", a commercial cultivar[18]. BR-related mutations can also give rise to semi-dwarfism, and among them, a barley mutant, *uzu*, was used for producing a lodging-resistant variety[19]. Recently, strigolactones (SLs), a group of newly identified plant hormones that control plant shoot branching, have also been implicated for dwarfism in high-tillering dwarf mutants as in the case of the SL-deficient *d10* and the SL-insensitive *d53* and *d14* mutants of rice[20,21].

Among the phytohormones, gibberellin (GA) is the most well known to be involved in controlling stem elongation, and a deficiency or insensitivity to GA could easily result in severe dwarfism as reported in many different kinds of plant species such as the rice mutants independently mutated in any of the six GA biosynthetic enzymes, ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO), ent-kaurenoic acid oxidase (KAO), GA 20 oxidase (GA20ox), and GA 3-oxidase (GA3ox)[22]; the pea GA-deficient *na-1*[23]; the *Arabidopsis* GA-insensitive *short internodes* (*shi*)[24] and *ga-insensitive* (*gai*)[25], and the GA-deficient *ga1 – 3* mutants[26]; the barley GA-deficient *grd2c* mutant[27], and the potato *andigena* (*adg*) mutant[28]. Across different

species, GA-related mutants with severe dwarfism show several consensus phenotypes such as small dark-green leaves, delayed germination, defective flowering, reduced seed production and male sterility[22,28–34], whereas, semi-dwarf mutants only show mild height reduction with no adverse effects on overall morphology or agronomical traits. This is the reason why GA-related mutants with semi-dwarfism were widely used in the history of crop production. Actually, the rice *semi-dwarf1* (*sd1*) and wheat *Reduced height-1* (*Rht-1*) mutants became the highlights of the Green Revolution, which avoided the imminent food shortage of the mid-20th century[35]. Later on, the *SD1* gene was characterized as a GA 20-oxidase[36,37], which catalyses the penultimate step of GA biosynthesis, while *Rht-1* gene was found to encode a constitutively active repressor of GA signaling[38]. Further, another GA-deficient mutant defective in KO, *Tan-ginbozu*/*d35*, also contributed to rice crop productivity due to its lodging resistance[39].

In contrast to the other plant species above, there are still no other reports of dwarfing mechanisms (including those relating to GA) in sorghum aside from the auxin-related *dw3* mutant mentioned earlier. Until now, even the underlying mechanisms behind the major dwarfing QTL genes (*dw1*, *dw2* and *dw4*) used in the history of sorghum breeding have remained unknown[40–42]. Aside from these classical dwarfing genes, increasing the number of available dwarfing mechanisms for sorghum should be important not only for breeding but also for basic research. Thus, in this study, we mutagenized sweet sorghum to create a mutant library, and searched it for plants with varying degrees of dwarfism. In the process, we found five severe dwarf mutants showing identical unusually bent culms which have never been reported in sorghum. We hereby attempted to isolate and analyse these mutants namely, *bending dwarf 1* (*bdw1*), *bdw2*, *bdw3-1*, *bdw3-2* and *bdw4*. We found that the mutants were mutated in four GA biosynthetic genes encoding enzymes catalysing the earlier steps of GA biosynthesis (Supplementary Fig. S1), and that their loss of function directly promotes culm bending. Thus, we reveal for the first time that GA-deficiency can pleiotropically induce culm bending or prostrate growth in sorghum. We also hypothesize that this could possibly be related to why GA-related mutations have not been utilized for sorghum breeding, unlike the case of rice and wheat.

Results

The *bdw* mutants show an abnormal bent culm. In order to increase the diversity of sorghum dwarf mutants and to further our knowledge of sorghum dwarfing mechanisms, we produced a mutant library through gamma-ray irradiation. During the process of screening for dwarf phenotypes, we isolated several unusual dwarf mutants, which developed bent culms. Succeeding analyses revealed that they were mutated in four different loci, thus we named them as *bending dwarf* (*bdw*)*1 - bdw4*, with *bdw3* having two different alleles (*bdw3-1* and *-2*). At the seedling stage, the bending culm phenotype was not evident in the mutants (Fig. 1a) but severe inhibition of internode elongation was observed (Fig. 1b). At about three and a half months after sowing, the bent culm phenotype was clearly observed (Fig. 1c). The mutants bent in a prostrate manner with leaves extending from the sides of the culm (rightmost in Fig. 1c).

In order to study the underlying reason for the bending, we carried out a histological analysis of the *bdw3-1* culm (Fig. 1d). The cell length at the upper side of the bent region was significantly shorter than that of the lower side (Fig. 1d, e), suggesting that there is a faster rate of cell proliferation at the upper side. Actually, the cell number at the upper side was more than two-folds higher than that at the lower side (Fig. 1f). These observations suggest that the bending was due to a difference in cell proliferation rates between the upper and lower sides of the bent culm.

The *bdw* mutants have reduced gravitropic response. Since we suspected that the gravitropic perception of the mutants might be defective, we directly examined the response of the *bdw1* culm to gravity at two stages, seedling and vegetative (Fig. 2). The aerial part of *bdw1* at the seedling stage responded positively to gravistimulation, but with a much weaker response compared to the WT (Fig. 2a), possibly due to its naturally inhibited culm elongation. Such weak gravitropic response was also observed at the late vegetative stage (Fig. 2b). Aside from the culm, the roots of *bdw1* also responded positively but much more slowly to gravistimulation at the seedling stage (Fig. 2a), as observed in another *bdw* mutant, *bdw3-1* (Supplementary Fig. S2), strongly suggesting that the *bdw* mutants have diminished ability to respond to gravity.

A loss of function of GA biosynthetic genes causes the bending dwarf phenotype. To further characterize the *bdw* mutants, we carried out positional cloning of the *BDW3* gene since there was a pair of allelic mutants for this gene among the five *bdw* mutants. We used F_2 plants derived from the cross between *bdw3-1* and the cultivar bmr-6. These plants segregated into two phenotypic groups, tall and dwarf, with a segregation ratio of 3 : 1 ($P<0.05$), respectively. By genotyping 249 F_2 plants, we narrowed down the locus of *BDW3* to approximately 4.0-Mb region between the markers Sb5370 and SSR5_5423 (Fig. 3a). This region contains 283 genes annotated in the sorghum genomic DNA sequence database (http://www.phytozome.net/), including *Sobic.010G172700* (Supplementary Table S1), a gene which is homologous to the rice *ent-kaurene oxidase* (*KO*) that encodes a GA biosynthetic enzyme[22]. In rice and *Arabidopsis*, the loss of function of KO causes a severe dwarf phenotype with small dark-green leaves[22,43], which was also evident in *bdw3-1* except that it had an added bent culm trait (Fig. 1c). The deduced amino acid sequence of *Sobic.010G172700* showed a high similarity to the entire sequence of rice KO (67%) and also to that of *Arabidopsis* KO (56%) (Supplementary Fig. S3c)[22,43]. We searched for mutations in the genomic DNA sequence encompassing the *SbKO* gene by PCR, and obtained no PCR products from the genome of *bdw3-1* (upright red triangles, Fig. 3b), whereas the same primers produced PCR products from the WT genome at the same condition. By using primers covering the flanking sequences of the *SbKO1* gene, we successfully located sequences that yielded PCR products (upright blue triangles KO1, KO2, KO18 and KO19, Fig. 3b) and predicted the deletion around the *SbKO1* gene to be at about 45 kb from KO3 to KO17. We also performed the same experiment on another *bdw3* mutant, *bdw3-2*, and found that it contained a shorter deletion (~17 kb) involving all portions of its *SbKO1* gene (inverted red or blue triangles in Fig. 3b). A genomic DNA blotting analysis using the promoter sequence of the *SbKO1* gene as probe also confirmed the large deletion in the corresponding region of the *bdw3-1* genome (Supplementary Fig. S4c, d).

The above results suggest that mutation in the *SbKO1* gene could be the reason for the severe dwarfism in the *bdw3* mutants. To determine whether such loss of function of GA biosynthetic genes is behind the phenotype of all the *bdw* mutants, we examined the sequences of the other GA biosynthetic genes (*CPS*, *KS*, *KAO*, *GA20ox*, and *GA3ox*) in the remaining mutants. As expected, the genome of *bdw1* contained an approximately 16-kb deletion involving the entire genomic region of *SbCPS1* (*Sobic.001G248600*) (Fig. 3c; Supplementary Fig. S4a, b). That of *bdw2* had a one-nucleotide deletion in the exon 3 of *SbKS1* (*Sobic.006G211500*) (Fig. 3d), while *bdw4* contained a five-nucleotide deletion in the exon 2 of *SbKAO1* (*Sobic.010G007700*) (Fig. 3e). These demonstrate that all of the five *bdw* mutants have defects in GA biosynthetic genes.

To confirm that the concerned GA biosynthetic genes in sorghum really function *in vivo*, we used them to transform corresponding GA-deficient mutants of rice, namely, *oscps1-1, osks1-1, osko2-1* and *oskao-1*[22]. We introduced a 7,545-bp sorghum genomic DNA frag-

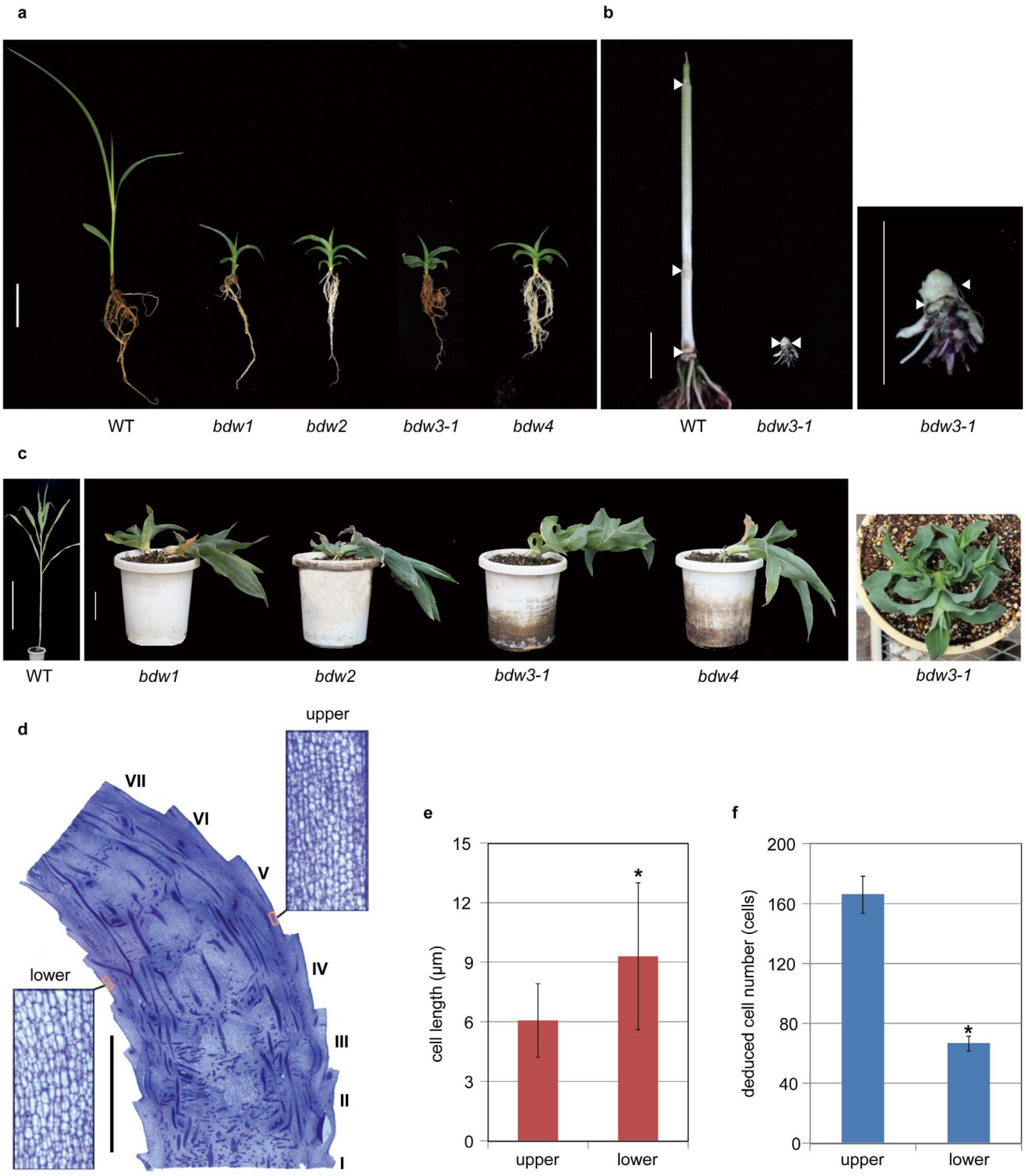

Figure 1 | Phenotypic analysis of the *bdw* mutants. (a) The *bdw* and WT plants at the seedling stage (30 days after germination, DAS). Bar = 4 cm. (b) Culm structure of WT (left) and *bdw3* (center and right) at the vegetative stage. Bars = 2 cm. Nodes are indicated by arrowheads. (c) WT and the bent *bdw* mutants at the vegetative stage (110 DAS). Top view of two-month old *bdw3* is shown at the rightmost panel. Bars: 50 cm, WT; 4 cm, mutants. (d) Longitudinal section of *bdw3* culm. Roman numerals at the right side indicate internode position from bottom to top. Bar = 5 mm. (e–f) The length and number of cells at the upper and lower sides of the fifth internode in the bent region shown in panel (d). Error bars for cell length represent the standard deviation calculated from approximately 100 cells. Error bars for cell number represent the standard deviation calculated from 5 cell files (see Methods). Asterisks indicate significant differences at 0.1% ($P<0.001$).

ment containing the entire *SbKO1* sequence into rice *osko2-1* via *Agrobacterium tumefaciens*-mediated transformation and found that it completely rescued the severe dwarfism of the mutant (Fig. 4). Similarly, introduction of *SbCPS1*, *SbKS1*, and *SbKAO1* genes rescued the dwarfism of corresponding rice mutants (Fig. 4). These observations confirmed that the isolated sorghum genes have biological activity in rice, and therefore the dwarf phenotype of the *bdw* mutants should be caused by the loss of function of these genes.

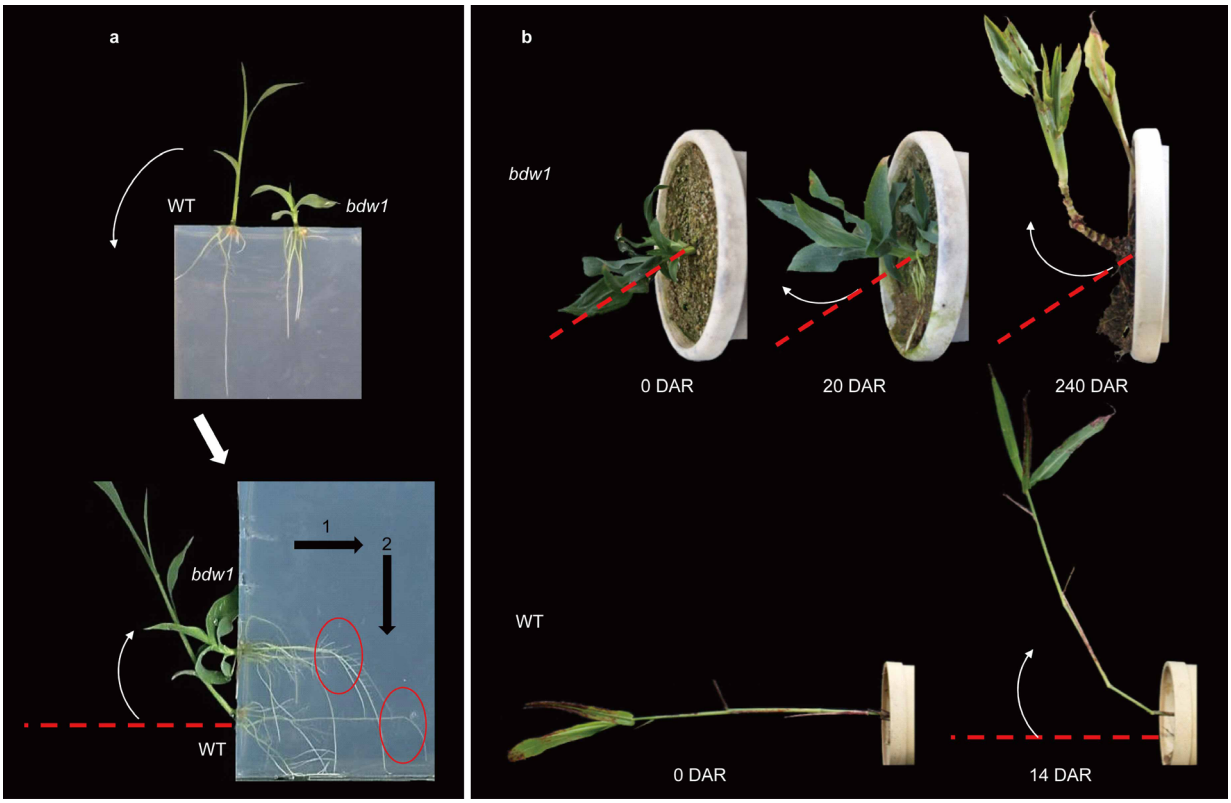

Figure 2 | Gravitropism test of *bdw1*. (a) The gravitropic response at the seedling stage. Top, plants at 4 days after germination (DAG) in upright position before gravistimulation; bottom, 4-DAG plants were rotated 90 degrees and further grown for 4 days after rotation (DAR). The horizontal "1" and vertical "2" arrows indicate the gravity directions before and after rotation, respectively. (b) The gravitropic response of *bdw1* (top) and WT (bottom) at the vegetative stage. Red dashed lines indicate the original direction of main culm before rotation. White arrows indicate the direction of culm movement.

GA rescues the bending culm phenotype, whereas, uniconazole induces bending in WT plants. The results described above indicate that loss of function of GA biosynthetic genes causes dwarfism accompanied by unusual culm bending in the mutants. We further confirmed that GA deficiency causes the culm bending by using two approaches. First, we treated the *bdw3-1* mutant with different concentrations of GA_3 for 4 months. Both the dwarf phenotype and the bending culm phenotype were rescued in a dose-dependent manner by GA_3, and were almost normal at 10^{-4} M GA_3 (Fig. 5). At 10^{-6} M GA_3, the dwarfism was partially rescued to about 60% but the culm remained at a significantly bent state (Fig. 5). Secondly, we treated WT with various concentrations of uniconazole (Fig. 6), an inhibitor of GA biosynthesis. As a result, treatment with 10^{-5} M uniconazole induced WT to bend in a similar manner observed in the mutants (Fig. 6a). At 3×10^{-6} M uniconazole, the plant height was reduced to about 25% while the bending was significantly induced from 0 to about 5 degrees against the vertical axis. These results show that the alleviation of the dwarf and bent phenotype of the mutants by GA was exactly opposite to the effect of uniconazole on WT plants, and that dwarfism and bending are tightly correlated (Fig. 5a, 6a).

Discussion

In this study, we were able to isolate five dwarf mutants (*bdw1*, *2*, *3-1*, *3-2*, and *4*) with abnormal culm bending from a mutant population of sweet sorghum (Fig. 1). Each of the *bdw* mutants had a defect in a specific GA biosynthetic gene, namely, *SbCPS1*, *SbKS1*, *SbKO1* and *SbKAO1* (Fig. 3; Supplementary Fig. S1)— the genes involved in the early part of GA biosynthesis. Further analyses of these mutants revealed that both dwarfism and culm bending were caused by GA deficiency (Fig. 5, 6).

Although previous studies in maize, rice, and *Arabidopsis* have shown that loss-of-function mutations in GA biosynthetic genes result in the suppression of internode elongation and dwarfism[22,43,44], there was no mention of culm bending or GA being involved in such a phenomenon. In barley, however, the loss of function of GA3ox1, which is involved in the last step of GA biosynthesis, resulted not only in dwarfism but also prostrate growth in the *grd2c* mutant[27]. Also in wheat, a strong allele of *Rht-B1, Rht-B1c*, induced severe dwarfism that correlated with an increase in tiller angle[45]. These indicate that culm bending (prostrate growth) is one of the pleiotropic effects of GA-related gene mutations and such event appears to be species-dependent. Our conclusion that GA deficiency causes culm bending in sorghum was supported by three experimental evidences. First, the independent loss-of-function mutants defective in four different GA biosynthetic genes were found to have bent culms (Fig. 1, 3). Second, the bent culm phenotype was reverted by exogenous GA treatment in a dose-dependent manner (Fig. 5). Lastly, uniconazole treatment of WT mimicked the bending of the mutants also in a dose-dependent manner (Fig. 6).

Although the exact molecular mechanism controlling bending in sorghum under GA deficiency is still unclear, there was a strong indication that the bending in the *bdw3* mutants was due to the asymmetric growth of culm internodes (Fig. 1d, f), which could be partially due to the reduced gravitropic response of the mutants as compared to the WT (Fig. 2; Supplementary Fig. S2). A comparable situation was observed in the GA-deficient barley *grd2c* mutant[27], which showed a slower pulvinar gravitropic response than the WT, and to a greater extent, to *sln1c*, a GA-hypersensitive mutant. The evidence indicated that gravity-induced auxin asymmetry leads to an asymmetry of GA distribution, and that GAs play a role in facilitating faster gravitropic response by inducing rapid cell elongation on the

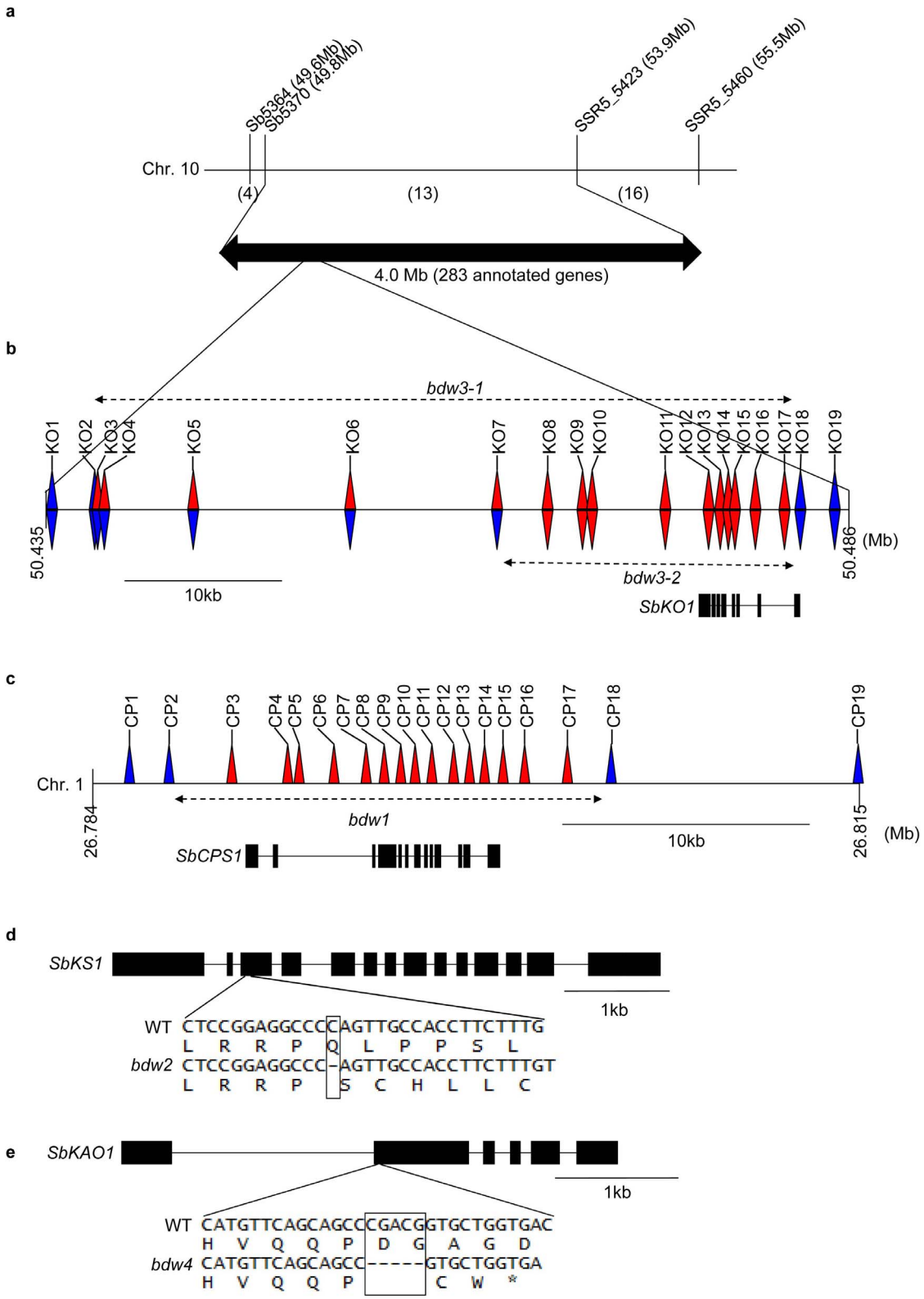

Figure 3 | Identification of the causal genes of the *bdw* mutants. (a) A physical map of the candidate region of *bdw3*. The top line shows the position of markers around the target region of chromosome 10. The numbers in parentheses indicate the number of recombinant plants between the markers. The *bdw3* mutation locates between Sb5370 and SSR5_5423. (b) Deletion in the *bdw3-1* and *bdw3-2* genome as detected by PCR analysis. Presence or absence of PCR products is indicated by blue and red triangles, respectively, whereas all PCR products were successfully obtained from the WT genome. Upright and inverted triangles correspond to the *bdw3-1* and *bdw3-2* genomes, respectively. Dashed arrows at the top and beneath the triangles show estimated deletions around the *SbKO1* gene. The *SbKO1* structure is shown at the bottom, where boxes and lines between them indicate exons and introns, respectively. (c) Deletion in the *bdw1* genome as detected by PCR analysis. The presentation is the same as in panel (b). (d) One-nucleotide deletion in the *SbKS1* gene of *bdw2*. The presentation is the same as in panel (b). (e) Five-nucleotide deletion in *SbKAO1* gene of *bdw4*. The presentation is the same as in panel (b).

lower side of the pulvini, following an asymmetric localization of auxin which serves as an initial trigger for bending. Further, just like the *bdw* mutants, the barley *grd2c* mutant was also reported to have a prostrate growth habit and GA appears to be affecting the gravitropic set-point angle (GSA) of its lateral shoots (tillers)[27]. Recently, using pea and *Arabidopsis*, GSA values were found to be dynamically specified by auxin throughout development by regulating the magnitude of the anti-gravitropic offset component via TIR1/AFB-Aux/IAA-ARF-dependent auxin signalling within the gravity-sensing cells of the root and shoot[46], suggesting that the bending in the *bdw* mutants could also be auxin-related. Our results show that GA deficiency triggers prostrate growth habit in sorghum in the presence of auxin, which is consistent with the case of barley *grd2c*[27], strongly indicating that GA also has a crucial role in controlling the GSA of the culm, at least in these species.

Mutations involving partial GA-deficiency and semi-dominant GA-insensitivity have long been harnessed in breeding programs to induce the favourable semi-dwarf trait made famous by the Green Revolution rice (*sd1*) and wheat (*Rht-B1b* and *Rht-D1b*), respectively[36,38]. In such instances, semi-dwarfism led to increased lodging resistance and high yield. However, the present study strongly suggests that the use of GA-related genes for sorghum breeding remains to be a big challenge because of the bending side-effect linked with GA deficiency. Actually, results presented in Fig. 5 and 6 demonstrated that even semi-dwarf sorghum plants show a potentially destabilizing (e.g. lodging-prone) culm curvature under reduced GA levels. Our assumptions are also being upheld by the fact that there are still no reports of GA-related genes used for sorghum semi-dwarf breeding to date.

So far, it is widely known that four major classical dwarfing genes (*dw1* - *dw4*) had been introgressed into elite varieties in the early history of sorghum breeding[47–51]. Among these dwarf genes, only the causal gene of *dw3* had been identified, namely, a gene encoding a phosphoglycoprotein involved in auxin transport[6]. Because the location of *dw1*, *dw2* and *dw4* have also been identified[40–42,52] (Supplementary Table S2), we compared them against the loci of GA-related genes (Fig. 7 and Supplementary Table S1) and found that the loci of *dw2*, *dw3* and *dw4* do not coincide with mapped positions of known GA-related genes. On the other hand, *dw1* is in close proximity to a *GA 2-oxidase*-like gene (Fig. 7), *Sobic.009G230800* (referred to as *GA2ox5* in Supplementary Table S1), which was previously discussed by two independent groups to be the possible causal gene of *dw1*[40,41]. However, based on our experiments, it is unlikely that a *GA2ox* is behind the phenotype of *dw1* because in that case, bending would become inevitable. Since GA2ox is involved in GA catabolism, its effect on sorghum should be similar to that of a GA synthesis inhibitor, uniconazole. To investigate whether *dw1* is the same as *GA2ox5*, we directly compared the DNA sequence and expression level of the gene in a tall (*Dw1Dw1*) and dwarf (*dw1dw1*) isogenic sorghum cultivar carrying gain-of-function and loss-of-function alleles of *Dw1*, respectively. As expected, there was no difference in the genome sequence and expression level of *GA2ox5* between the two cultivars (Supplementary Fig. S5). This result supports the above idea that a *GA2ox* is unlikely to be the causal gene of *dw1*.

As mentioned earlier, in wheat, the use of a strong allele of *Rht-B1* caused severe dwarfism and bending[45], and such traits were further aggravated by overexpression of a GA-inactivating enzyme, GA2ox[29,53]. In contrast, the weak *Rht-B1* mutation used in the Green Revolution only resulted in semi-dwarfism, with no obvious bending[38]. On the other hand, the Green Revolution rice and even the severe dwarf rice mutants carrying null alleles for GA biosynthetic or signalling genes do not show culm bending at all[22,36,54]. These observations suggest that, because of the exceptionally tight relationship between GA deficiency-induced dwarfism and bending in sorghum, the use of GA-related mutations to induce semi-dwarfism, without compromising the straightness and lodging resistance of the culm, may not be possible. Taken altogether, our results strongly suggest

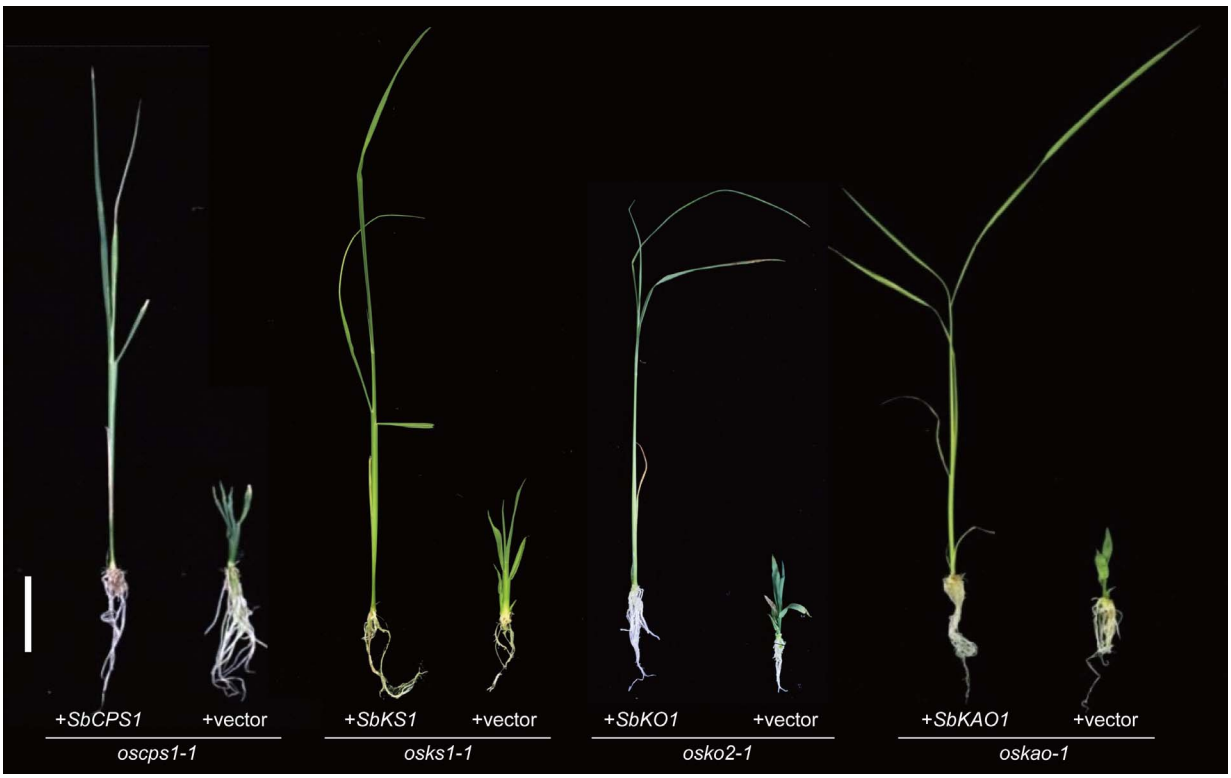

Figure 4 | Introduction of sorghum GA biosynthetic genes rescued the corresponding mutants of rice, *oscps1-1, osks1-1, osko2-1*, and *oskao-1*. Scale bar = 4 cm. Plants at the left and right in each set were transformed with the complementary WT genes from sorghum and the vector control, respectively.

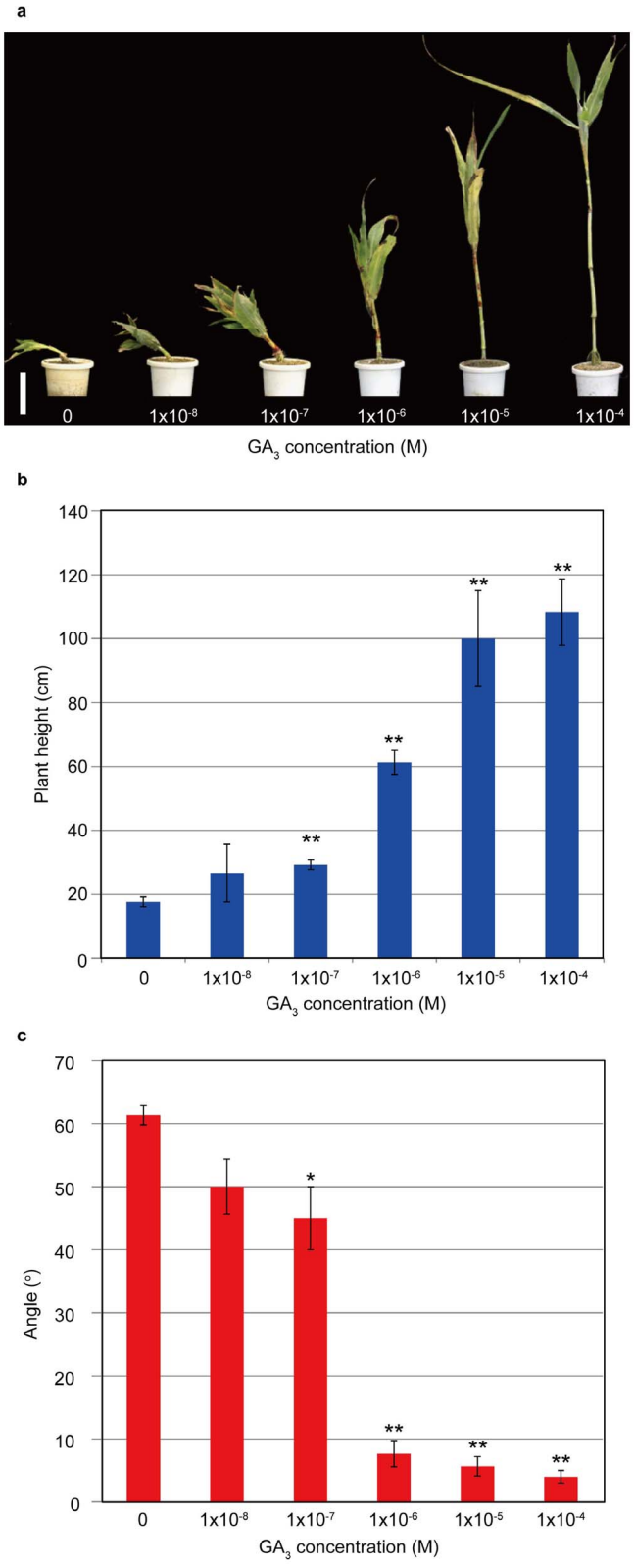

Figure 5 | Dose-dependent recovery of the mutant phenotypes by exogenous GA treatment. (a) The *bdw3-1* plants grown in different concentrations of GA_3 for four months. Bar = 10 cm. (b) Dose-dependent recovery of plant height. (c) Dose-dependent recovery of the bending culm phenotype, which was determined by measuring the angle between the main culm and the vertical axis. Error bars represent the standard deviation calculated from 3 replicates. Single (*) and double asterisks (**) indicate significant difference with the mock (0 M GA_3) at 0.5% ($P<0.005$) and 0.1% ($P<0.001$) levels of significance, respectively, as determined by t-test.

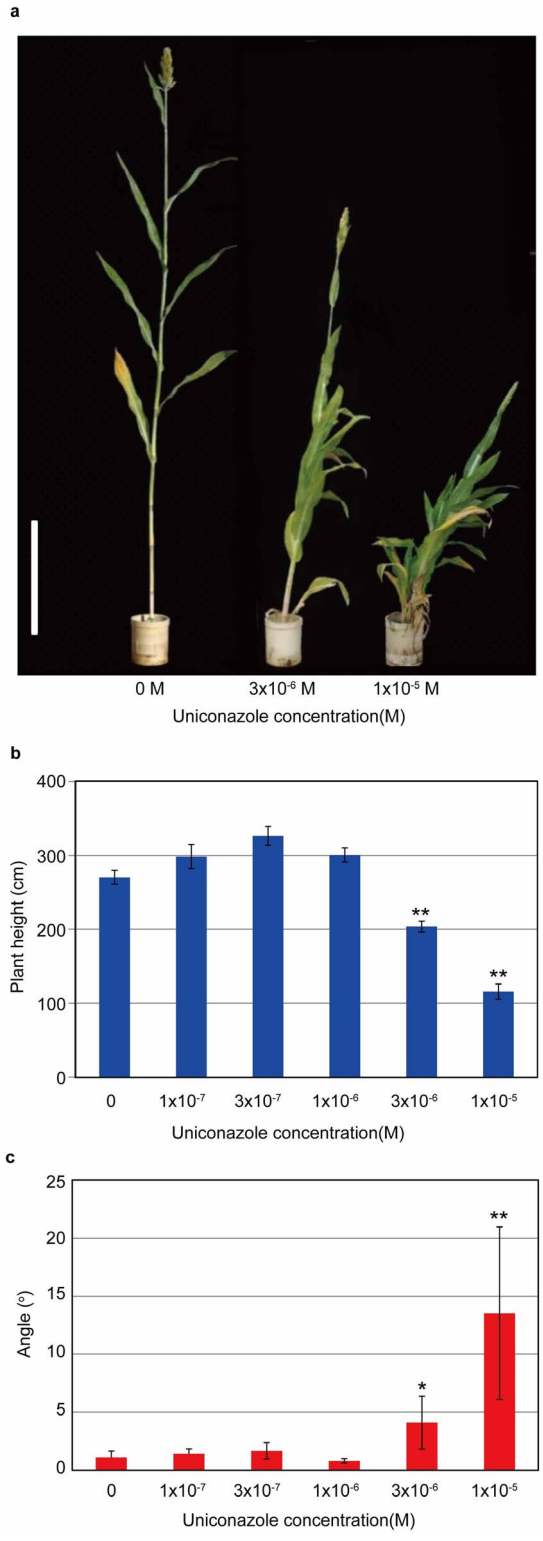

Figure 6 | Effect of uniconazole on the plant height and culm bending of WT plants. (a) Gross morphology of the uniconazole-treated plants. The plants at the left, center, and right were treated with 0, 3×10^{-6} and 1×10^{-5} M uniconazole, respectively. Bar = 50 cm. Dose-dependent inhibition of plant height (b) and induction of culm bending (c) in WT plants. Error bars represent the standard deviation calculated from 5 replicates. Single (*) and double asterisks (**) indicate significant difference between the mock and 3×10^{-6} and 1×10^{-5} M uniconazole at 5% ($P<0.05$) and 1% ($P<0.01$) levels of significance, respectively, as determined by t-test.

that the classical dwarfing genes that have been utilized for sorghum breeding in the past may not be related to GA. As in the case of *dw3*[6], the isolation and characterization of *dw1*, along with those of *dw2* and *dw4* will reveal novel mechanism(s) for semi-dwarfism in sorghum. Once identified, these genes can also be exploited in rice and other crops as additional or alternative options for lodging resistance breeding.

Methods

Plant materials and mutant library construction. We used *Sorghum bicolor* cv. SIL-05 (sweet sorghum) for constructing a mutant library. Mutagenesis was carried out by irradiating seeds with gamma-ray (175–250 Gray (Gy)). The resulting M_1 seeds were sown in seedling trays and were grown inside the greenhouse at 25°C for more than 3 weeks before planting in the field. About 5,000 of such plants were self-pollinated to obtain M_2 lines which were screened for dwarfism at the seedling (greenhouse) and vegetative (field) stages. Identified mutants and all plants for analysis were grown in pots inside the greenhouse under ambient lighting conditions.

Microscopic analysis. A culm longitudinal section of the *bdw3-1* mutant was made with a cryotome. For staining, hematoxylin and eosin stains were used. Photographs were taken using an Olympus photomicroscope connected to a CCD camera. Resulting photographs were stitched to create a panoramic image (Photostitch, Canon). In order to understand the underlying cause of the bending in the *bdw3-1* mutant, the length and number of the cells at both the upper side and lower side of the fifth internode, where the greatest degree of bending took place, was quantified. This was done by measuring the length and number of cells in 5 cell files within a 500-μm long region (orange rectangles, Fig. 1d) at both the upper and lower sides of the fifth internode and then estimating the total number of cells in the internode.

Gravitropism test. Seeds of the *bdw1* and *bdw3-1* mutants (as representatives of the phenotypically identical *bdw* mutants) and WT were disinfected with 0.5% benlate overnight at 4°C and sowed on MS medium with 0.9% agar in plastic rectangular culture plates. Plants were grown for 4 days after germination (DAG) in an upright position and then rotated 90 degrees and examined 4 days after rotation (DAR). The culture plates were imaged by using a flatbed digital scanner. Also, bent *bdw1* plants at the vegetative stage were rotated 90 degrees following the direction of culm bending and compared their response with the WT.

Genome mapping of the *bdw3* locus and sequence analysis. To carry out gene mapping, we crossed heterozygous siblings of the *bdw3-1* mutant (sweet sorghum (SIL-05) background) with bmr-6 (grain sorghum cultivar) to produce F_1 plants that were progeny-tested to obtain the desired F_2 population for analysis. Segregation ratio of the tall and dwarf phenotypes was observed and analysed by chi-square test.

We screened about 1,500 Simple Sequence Repeats (SSR) markers[55] and selected 162 polymorphic markers. For rough mapping of the candidate region, we used the SSR markers on 16 F_2 seedlings with tall phenotype. For the fine mapping, we expanded the analysis to 249 F_2 tall plants.

We used the amino acid sequences of known GA biosynthetic genes of rice as queries for the TBLASTN program (http://www.phytozome.net/search.php?show=blast&org=Org_Sbicolor_v2.1) to identify homologous sequences in the sorghum genome. PCR analysis of the identified genes, namely, *SbCPS1* (*Sobic.001G248600*), *SbKS1* (*Sobic.006G211500*), *SbKO1* (*Sobic.010G172700*), *SbKAO1* (*Sobic.010G007700*), *SbGA20ox2* (*Sobic.003G379500*) and *SbGA3ox2* (*Sobic.003G045900*) was done on all the *bdw* mutants and the WT. PCR primers used are listed in Supplementary Table S3. DNA sequencing was done with the ABI Prism 310-10 sequencer following the manufacturer's protocol.

Amino acid sequences of sorghum CPS1, KS1, KO1 and KAO1 were compared with those of other species by aligning with ClustalW (http://www.genome.jp/tools/clustalw/) using default settings (slow/accurate) and then manually adjusted to optimize alignments. The unrooted phylogenetic tree with branch length (N-J) tree file output was used to create a phylogenetic tree with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) using default settings for the rectangular tree layout.

For DNA gel blot analysis of *SbCPS1* and *SbKO1*, 1 μg genomic DNAs from *bdw1*, *bdw3-1* and WT were analysed as previously described[56]. Primers used to amplify the probes are listed in Supplementary Table S3.

Complementation of rice GA-deficient mutants with WT GA biosynthetic genes from sorghum. To examine the bioactivities of the identified sorghum GA biosynthetic genes, we first cloned 11,287, 6,938, 7,628, and 5,909 bp of sorghum genomic DNA fragments containing entire *SbCPS1, SbKS1, SbKO1* and *SbKAO1*

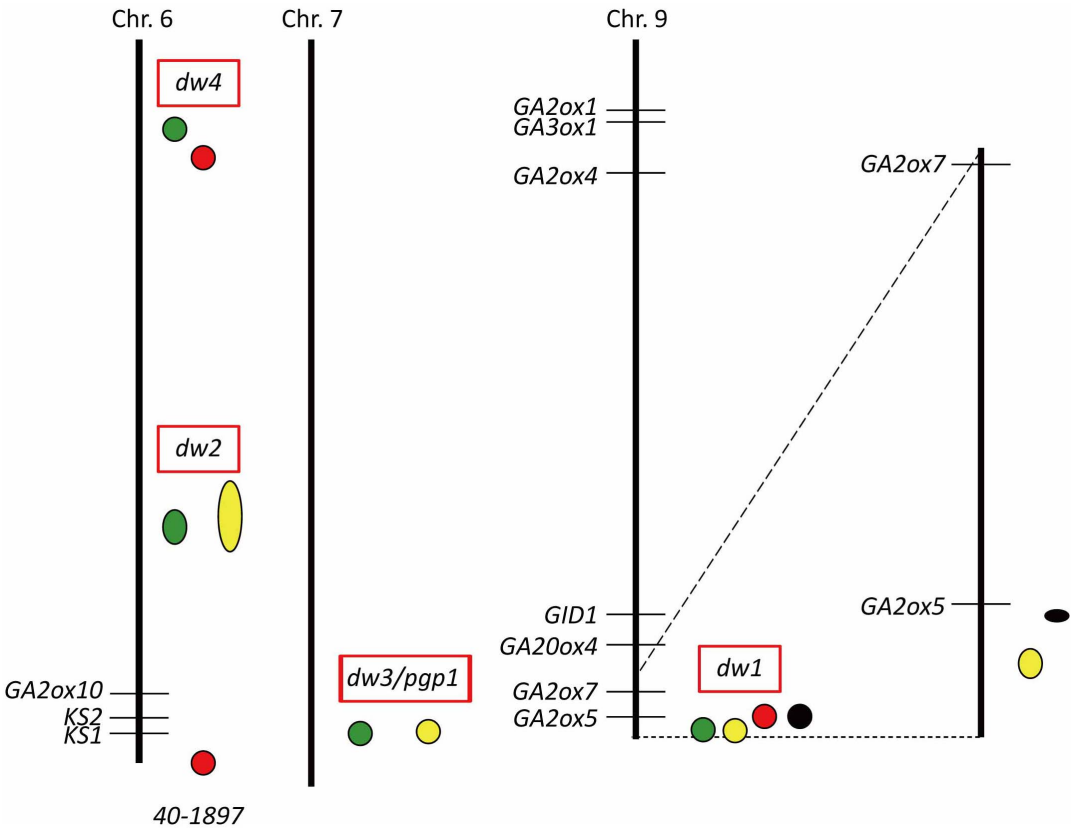

Figure 7 | Comparison between the map positions of previously reported dwarfing QTL genes and the GA-related genes of sorghum. The chromosomes carrying the dwarfing QTL genes previously reported are presented. Ovals and circles at the right side of each chromosome indicate the positions of respective previously reported dwarfing QTLs (red rectangles), where green, yellow, red and black shades represent the mapping work of Morris *et al.* (2013), Thurber *et al.* (2013), Wang *et al.* (2012) and Brown *et al.* (2008), respectively. The positions of GA-related genes are presented at the left side of each chromosome with their corresponding names as presented in Supplementary Table S1.

genes along with their native promoters into a pCAMBIA1300 binary vector, respectively. These chimeric plasmids were introduced into corresponding mutants of rice, *oscps1-1, osks1-1, osko2-1* and *oskao-1*, through *Agrobacterium*-mediated transformation[22,57]. The transformants were screened on selection medium with hygromycin and the regenerated plants were documented.

Exogenous gibberellin treatment and uniconazole treatment. For GA treatment, two-week old *bdw3* seedlings were transplanted into pots. A set of three pots (with holes in the bottom) were placed in a square plastic vat containing a designated concentration of GA_3 (0, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} and 1×10^{-4} M) and were grown continuously in the solution for four months inside the greenhouse. For uniconazole treatment, a set of five 4-week-old WT plants (SIL-05) were grown continuously for 2 months in a vat containing specified concentrations of uniconazole (0, 1×10^{-7}, 3×10^{-7}, 1×10^{-6}, 3×10^{-6} and 1×10^{-5} M). In both GA and uniconazole experiments, the vats were regularly checked and augmented with water whenever necessary to guard against evaporation and fluctuation in the concentration of the original solution until the completion of the experiment. Measurement of plant height was done with a rope by tracing the contour of the main culm from the base to the longest tip. Culm angle was taken by measuring the angle formed by the culm against the vertical axis.

DNA and RNA-seq analysis by Next Generation Sequencer (NGS). Two isogenic varieties, tall white sooner milo (*Dw1Dw1*) and dwarf white milo (*dw1dw1*) carrying gain-of-function and loss-of-function alleles of *Dw1*, respectively, were utilized for genomic DNA sequence analysis using NGS. Sequencing was performed at Hokkaido System Science Co. Ltd. (Sapporo, Japan). Library for sequencing was prepared using TruSeq DNA/RNA Sample Prep Kit (Illumina, Inc.) according to the manufacturer's protocol. The resultant library was sequenced (2×101 cycles, paired-end) on HiSeq 2500/2000 instrument using TruSeq PE Cluster Kit and TruSeq SBS Kit (Illumina, Inc.) to obtain approximately 40 million raw sequence reads for analysis. Sequences were mapped to the BTx623 sorghum reference genome[58] by using BWA version 0.6.1-r104 (http://bio-bwa.sourceforge.net/), and SNPs were called with the SAMtools version 0.1.18 (http://samtools.sourceforge.net/). The two varieties were also subjected to RNA-seq analysis by using elongating internode total RNAs isolated by a modified Trizol method[59]. Total RNAs were utilized for RNA-seq experiment as described previously[60].

1. Sato, Y. *et al.* Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. *EMBO J.* **18**, 992–1002 (1999).
2. Yang, W. *et al. BENT UPPERMOST INTERNODE1* encodes the class II formin FH5 crucial for actin organization and rice development. *Plant Cell* **23**, 661–680 (2011).
3. Cui, X. *et al.* Control of transposon activity by a histone H3K4 demethylase in rice. *Proc. Natl. Acad. Sci. USA.* **110**, 1953–1958 (2013).
4. Komorisono, M. *et al.* Analysis of the rice mutant *dwarf and gladius leaf 1*. Aberrant katanin-mediated microtubule organization causes up-regulation of gibberellin biosynthetic genes independently of gibberellin signaling. *Plant Physiol.* **138**, 1982–1993 (2005).
5. Bouquin, T., Mattsson, O., Naested, H., Foster, R. & Mundy, J. The *Arabidopsis lue1* mutant defines a katanin p60 ortholog involved in hormonal control of microtubule orientation during cell growth. *J. Cell Sci.* **116**, 791–801 (2003).
6. Multani, D. S. *et al.* Loss of an MDR transporter in compact stalks of maize *br2* and sorghum *dw3* mutants. *Science* **302**, 81–84 (2003).
7. Knoller, A. S., Blakeslee, J. J., Richards, E. L., Peer, W. A. & Murphy, A. S. Brachytic2/ZmABCB1 functions in IAA export from intercalary meristems. *J. Exp. Bot.* **61**, 3689–3696 (2010).
8. Aya, K. *et al.* A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. *Plant Cell Physiol.* **55**, 897–912 (2014).
9. Sazuka, T. *et al.* A rice *tryptophan deficient dwarf* mutant, *tdd1*, contains a reduced level of indole acetic acid and develops abnormal flowers and organless embryos. *Plant J.* **60**, 227–241 (2009).
10. Ephritikhine, G., Fellner, M., Vannini, C., Lapous, D. & Barbier-Brygoo, H. The *sax1* dwarf mutant of *Arabidopsis* thaliana shows altered sensitivity of growth responses to abscisic acid, auxin, gibberellins and ethylene and is partially rescued by exogenous brassinosteroid. *Plant J.* **18**, 303–314 (1999).
11. Dai, Y., Fu, Z. & Li, J. Isolation and characterization of an *Arabidopsis* bush and dwarf mutant. *Acta Bot. Sin.* **45**, 621–625 (2003).
12. Noguchi, T. *et al.* Arabidopsis *det2* is defective in the conversion of (24*R*)-24-methylcholest-4-En-3-one to (24*R*)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiol.* **120**, 833–839 (1999).
13. Nole-Wilson, S., Rueschhoff, E. E., Bhatti, H. & Franks, R. G. Synergistic disruptions in *seuss cyp85A2* double mutants reveal a role for brassinolide synthesis during gynoecium and ovule development. *BMC Plant Biol.* **10**, 198 (2010).
14. Clouse, S. D., Langford, M. & McMorris, T. C. A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. *Plant Physiol.* **111**, 671–678 (1996).
15. Nakamura, A. *et al.* The role of *OsBRI1* and its homologous genes, *OsBRL1* and *OsBRL3*, in rice. *Plant Physiol.* **140**, 580–590 (2006).
16. Sakamoto, T., Kitano, H. & Fujioka, S. An E3 ubiquitin ligase, ERECT LEAF1, functions in brassinosteroid signaling of rice. *Plant Signal. Behav.* **8**, e27117 (2013).
17. Hong, Z. *et al.* A rice brassinosteroid-deficient mutant, *ebisu dwarf* (*d2*), is caused by a loss of function of a new member of cytochrome P450. *Plant Cell* **15**, 2900–2910 (2003).
18. Marti, E., Gisbert, C., Bishop, G. J., Dixon, M. S. & Garcia-Martinez, J. L. Genetic and physiological characterization of tomato cv. Micro-Tom. *J. Exp. Bot.* **57**, 2037–2047 (2006).
19. Chono, M. *et al.* A semidwarf phenotype of barley uzu results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. *Plant Physiol.* **133**, 1209–1219 (2003).
20. Arite, T. *et al. d14*, a strigolactone-insensitive mutant of rice, shows an accelerated outgrowth of tillers. *Plant Cell Physiol.* **50**, 1416–1424 (2009).
21. Jiang, L. *et al.* DWARF 53 acts as a repressor of strigolactone signalling in rice. *Nature* **504**, 401–405 (2013).
22. Sakamoto, T. *et al.* An overview of gibberellin metabolism enzyme genes and their related mutants in rice. *Plant Physiol.* **134**, 1642–1653 (2004).
23. Davidson, S. E., Elliott, R. C., Helliwell, C. A., Poole, A. T. & Reid, J. B. The pea gene *NA* encodes *ent*-kaurenoic acid oxidase. *Plant Physiol.* **131**, 335–344 (2003).
24. Fridborg, I., Kuusk, S., Moritz, T. & Sundberg, E. The Arabidopsis dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. *Plant Cell* **11**, 1019–1031 (1999).
25. Peng, J. *et al.* The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes Dev.* **11**, 3194–3205 (1997).
26. Sun, T. P. & Kamiya, Y. The Arabidopsis *GA1* locus encodes the cyclase *ent*-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* **6**, 1509–1518 (1994).
27. Wolbang, C. M., Davies, N. W., Taylor, S. A. & Ross, J. J. Gravistimulation leads to asymmetry of both auxin and gibberellin levels in barley pulvini. *Physiol. Plant.* **131**,140–148 (2007).
28. Bamberg, J. B. & Hanneman, R. E. Characterization of a new gibberellin related dwarfing locus in potato (*Solanum tuberosum* L.). *Am. Potato J.* **68**, 45–52 (1991).
29. Hedden, P. & Phillips, A. L. Gibberellin metabolism: new insights revealed by the genes. *Trends Plant Sci.* **5**, 523–530 (2000).
30. Fleet, C. M. & Sun, T. P. A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Curr. Opin. Plant Biol.* **8**, 77–85 (2005).
31. Tanimoto, E. Regulation of root growth by plant hormones–Roles for auxin and gibberellin. *Crit. Rev. Plant Sci.* **24**, 249–265 (2005).
32. Wang, Y. & Li, J. The plant architecture of rice (*Oryza sativa*). *Plant Mol. Biol.* **59**, 75–84 (2005).
33. Chhun, T. *et al.* Gibberellin regulates pollen viability and pollen tube growth in rice. *Plant Cell* **19**, 3876–3888 (2007).
34. Lo, S. F. *et al.* A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. *Plant Cell* **20**, 2603–2618 (2008).
35. Hedden, P. The genes of the Green Revolution. *Trends in Genet.* **19**, 5–9 (2003).
36. Sasaki, A. *et al.* Green revolution: a mutant gibberellin-synthesis gene in rice. *Nature* **416**, 701–702 (2002).
37. Spielmeyer, W., Ellis, M. H. & Chandler, P. M. Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. *Proc. Natl. Acad. Sci. USA* **99**, 9043–9048 (2002).
38. Peng, J. *et al.* 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**, 256–261 (1999).
39. Itoh, H. *et al.* A rice semi-dwarf gene, *Tan-Ginbozu (D35)*, encodes the gibberellin biosynthesis enzyme, *ent*-kaurene oxidase. *Plant Mol. Biol.* **54**, 533–547 (2004).
40. Wang, Y. H., Bible, P., Loganantharaj, R. & Upadhyaya, H. D. Identification of SSR markers associated with height using pool-based genome-wide association mapping in sorghum. *Mol. Breed.* **30**, 281–292 (2012).
41. Morris, G. P. *et al.* Population genomic and genome-wide association studies of agroclimatic traits in sorghum. *Proc. Natl. Acad. Sci. USA* **110**, 453–458 (2013).
42. Thurber, C. S., Ma, J. M., Higgins, R. H. & Brown, P. J. Retrospective genomic analysis of sorghum adaptation to temperate-zone grain production. *Genome Biol.* **14**, R68 (2013).
43. Helliwell, C. A. *et al.* Cloning of the *Arabidopsis ent*-kaurene oxidase gene *GA3*. *Proc. Natl. Acad. Sci. USA* **95**, 9019–9024 (1998).
44. Fujioka, S. *et al.* Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1, dwarf-2, dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. *Plant Physiol.* **88**, 1367–1372 (1988).
45. Wu, J. *et al.* Dominant and pleiotropic effects of a *GAI* gene in wheat results from a lack of interaction between DELLA and GID1. *Plant Physiol.* **157**, 2120–2130 (2011).
46. Roychoudhry, S., Del Bianco, M., Kieffer, M. & Kepinski, S. Auxin controls gravitropic setpoint angle in higher plant lateral branches. *Curr. Biol.* **23**, 1497–1504 (2013).
47. Quinby, J. R. & Karper, R. E. Inheritance of height in sorghum. *Agron. J.* **46**, 211–216 (1954).
48. Hadley, H. H. An analysis of variation in height in sorghum. *Agron. J.* **49**, 144–147 (1957).
49. Quinby, J. R. *Sorghum Improvement and the Genetics of Growth* (Texas A&M University Press, College Station, TX, 1974).
50. Smith, C. W. & Frederiksen, R. A. *Sorghum: Origin, history, technology, and production,* 191–223 (John Wiley & Sons, New York, 2000).

51. Klein, R. R. *et al*. Effect of tropical sorghum conversion and inbred development on genome diversity as revealed by high-resolution genotyping. *Crop Sci.* **48**, S12–S26 (2008).
52. Brown, P. J., Rooney, W. L., Franks, C. & Kresovich, S. Efficient mapping of plant height quantitative trait loci in a sorghum association population with introgressed dwarfing genes. *Genetics* **180**, 629–637 (2008).
53. Appleford, N. E. *et al*. Decreased shoot stature and grain α-amylase activity following ectopic expression of a gibberellin 2-oxidase gene in transgenic wheat. *J. Exp. Bot.* **58**, 3213–3226 (2007).
54. Ueguchi-Tanaka, M. *et al*. *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. *Nature* **437**, 693–698 (2005).
55. Yonemaru, J. *et al*. Development of genome-wide simple sequence repeat markers using whole-genome shotgun sequences of sorghum (*Sorghum bicolor* (L.) Moench). *DNA Res.* **16**, 187–193 (2009).
56. Miura, K. *et al*. A metastable *DWARF1* epigenetic mutant affecting plant stature in rice. *Proc. Natl. Acad. Sci. USA* **106**, 11218–11223 (2009).
57. Hiei, Y. & Komari, T. *Agrobacterium*-mediated transformation of rice using immature embryos or calli induced from mature seed. *Nat. Protoc.* **3**, 824–834 (2008).
58. Paterson, A. H. *et al*. The *Sorghum bicolor* genome and the diversification of grasses. *Nature* **457**, 551–556 (2009).
59. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
60. Mizuno, H. *et al*. Global transcriptome analysis reveals distinct expression among duplicated genes during sorghum-*Bipolaris sorghicola* interaction. *BMC Plant Biol.* **12**, 121 (2012).

Acknowledgments

This work was partially supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation), QTL5501, 5505, Genomics-based Technology for Agricultural Improvement, IVG-1004; Core Research for Evolutional Science and Technology (CREST) Program "Novel techniques of tailor-made breeding for energy crop improvement using high-throughput genotyping" of Japan Science and Technology Agency (JST); and Grants-in-Aid from the NC-CARP project, the Ministry of Education, Science, Sports and Culture of Japan. We thank Prof. Y. Inukai for the gamma-ray treatment of sorghum seeds, A. Kondo-Watanabe and S. Araki-Nakamura for their technical assistance, and K. Aya, K. Yano and T. Hosaki for handling DNA- and RNA-seq results.

Author contributions

R.L.O., Y.I., S.K., H.K., M.M. and T.S. designed the experiments and wrote the manuscript. R.L.O. conducted almost all of the molecular analyses and plant assays. T.S. and T.T. grew the M1 and M2 populations. A.H. and Y.I. conducted the uniconazole test and mutant phenotyping in the field. K.O.S. made the construct for *SbKO1* and assisted in genetic transformation, while H.M. did the RNA-seq analysis. And H.M. did the RNA-seq analysis.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ordonio, R.L. *et al*. Gibberellin deficiency pleiotropically induces culm bending in sorghum: an insight into sorghum semi-dwarf breeding. *Sci. Rep.* **4**, 5287; DOI:10.1038/srep05287 (2014).

List of Publications (Appendix)

(1) Hirano, K., Okuno, A., Hobo, T., Ordonio, R., Shinozaki, Y., Asano, K., Kitano, H. and Matsuoka, M. (2014). Utilization of stiff culm trait of rice *smos1* mutant for increased lodging resistance. PLoS One 9: e96009.

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Utilization of Stiff Culm Trait of Rice *smos1* Mutant for Increased Lodging Resistance

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Abstract

Although the introduction of semi-dwarf trait into rice has led to improved lodging resistance making it capable of supporting high grain yield, lodging still remains a concern when attempting to further increase the grain yield of rice. However, improving the lodging resistance in rice by depending on the semi-dwarf trait alone is possible only up to a certain limit, beyond which other traits may be needed for reinforcement. To search for alternative traits relating to high lodging resistance, we identified 9 rice mutant lines possessing improved culm strength. To evaluate whether such lines can be useful for breeding lodging resistant rice, *small organ size1 (smos1)* mutant having increased lodging resistance but low tiller number and low grain yield, was chosen as a representative for a breeding trial. *smos1* was crossed with ST-4 (from the Stock rice collection of Nagoya University Togo field #4), a cultivar with high tiller number and high grain yield, and from their progeny, LRC1 (lodging resistance candidate-1) was selected. Although the low tiller number trait of *smos1* was not fully reversed in LRC1, this was compensated by an increase in grain weight per panicle, thereby resulting in high grain yield per plant. This important attribute of LRC1 was further enhanced by the improved lodging resistance trait inherited from *smos1*. Such improved lodging resistance in LRC1 and *smos1* was revealed to be mainly due to increased culm diameter and culm thickness, which led to a high section modulus (SM) value, a parameter defining the physical strength of the culm. Since *smos1* possesses high breaking-type lodging resistance which is different from semi-dwarf plants with high bending-type lodging resistance, an alternative approach of using thick culm lines for the creation of rice with increased lodging resistance is hereby proposed.

Citation: Hirano K, Okuno A, Hobo T, Ordonio R, Shinozaki Y, et al. (2014) Utilization of Stiff Culm Trait of Rice *smos1* Mutant for Increased Lodging Resistance. PLOS ONE 9(7): e96009. doi:10.1371/journal.pone.0096009

Editor: Fan Chen, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China

Received February 12, 2014; **Accepted** April 1, 2014; **Published** July 2, 2014

Funding: This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics-based for Agricultural Improvement, RBS2003) and Grants-in-Aid from the NC-CARP project, the Ministry of Education, Science, Sports and Culture of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In the 1960's, introduction of semi-dwarf trait into rice varieties combined with high nitrogen fertilizer input doubled rice grain yield, a success referred to as the "Green Revolution" [1–3]. Use of high amount of fertilizer allowed yield increase, while reducing plant height through the introduction of semi-dwarf gene increased lodging resistance, making rice capable of supporting grain-laden panicles. To avoid the future food crisis that may arise from human population explosion and climate change, it is urgent to increase rice grain yield per land area. In this context, lodging still remains a concern.

Lodging of cereal crops can be classified into three types [4]. Culm bending-type lodging is exhibited by plants when they cannot withstand the bending pressure, and is often observed in the upper internodes of rice during strong winds and rain. Culm breaking is another type of lodging that usually occurs at lower internodes (below the third internode counted from the top) as a result of excessive bending pressure at the higher internodes, and is determined primarily by the morphology and quality of the culm [5,6]. The third type is root lodging, which results from the inability of the roots to support the above-ground part [7]. Root lodging is infrequently observed in transplanted rice, as they possess well-established root system.

The widespread use of gibberellin (GA)-related semi-dwarf plants during the "Green Revolution" was due to their improved bending-type lodging resistance. Short-statured plants have lower "center of gravity" than normal plants, which improves their resistance against bending pressure [8]. However, attaining lodging resistance through the use of GA-related semi-dwarf trait has some limitations. First of all, further improving the bending resistance does not necessarily result in increased lodging resistance. This is because although semi-dwarf plants have increased bending resistance in the culm, their breaking resistance is significantly reduced [9]. In addition, GA dependent semi-dwarf plants have lower grain yield than their original cultivars [9].

From the reasons mentioned above, a rather different approach is required to further improve lodging resistance in rice. One possible way is to increase the physical strength of the culm to improve breaking-type lodging resistance. This should satisfy the requirement for supporting higher grain yield without depending on semi-dwarfism.

In this study, we searched for rice mutants with stiff culm and improved breaking resistance, and 9 such lines were identified

from more than 3000 rice mutant accessions. To investigate whether these traits could be utilized in the breeding program for improved lodging resistance, *small organ size1* (*smos1*) was chosen from the mutant lines and used for a breeding trial. Just recently, molecular characterization of *smos1* mutant revealed that it contains a mutation in a gene coding for APETALA2 (AP2)-type transcription factor which acts as an auxin-dependent regulator of cell expansion [10]. Thus, the mutation results in decreased ability of the cells to expand, hence, reducing the cell size in various organs of *smos1*. Surprisingly, the mutant also exhibits an increase in cell numbers in several organs which could account for *smos1*'s high lodging resistance. Although the molecular mechanism leading to increased cell numbers is unknown, expression of DNA replication-related genes is altered in *smos1* mutant. From such observation, Aya et. al. suggested one possibility that SMOS1 directly suppress cell proliferation through regulating these genes [10]. Unfortunately, the advantage of increased cell numbers of *smos1* is severely undermined by its poor number of tillers which translates into reduced grain yield per plant. Thus, as a crossing partner for *smos1*, we chose ST-4 (from the Stock rice collection of Nagoya University Togo field #4), a cultivar with high yield and large number of tillers. Through such a crossing combination, we succeeded to identify one progeny line possessing increased lodging resistance and grain yield. We hereby present that the stiff culm trait is yet another available option for breeding.

Materials and Methods

Growth conditions

Experiments were conducted at the Nagoya University Togo Field. Each rice line was planted in a 1×1 m plot with a planting density of 18 plants per square meter (15 cm horizontal and 30 cm vertical spacing between plants).

Plant materials

smos1 is a mutant derived from T65. ST-4 originated from inbred *japonica-indica* multiple crossing lines, but the parents were undeterminable. Crossing *smos1* with ST-4 was conducted using the latter as pollen donor. Among the self-fertilized F5 population of *smos1*×ST4 lines, a line tentatively named LRC1 (lodging resistance candidate-1), was selected for investigation. In the F5 lines, we did not observe any obvious differences within individual lines, suggesting that most of their genome regions were already fixed.

Analyses of morphological and agronomical traits

For the measurement of each trait, plant samples were taken 40 days after heading, air-dried for 2 weeks, and analyzed.

Analyses of lodging resistance

Lodging resistance parameters were measured from rice plants 40 days after heading. Culm bending resistance was investigated by measuring the cLr value (lodging resistance factor), following the method of Grafius and Brown [11]. Bending moment at breaking was measured at a distance of 4 cm between two supporting points as described previously [12]. Physical parameters were calculated using the formula: M = section modulus × bending stress [13]. M is the bending moment of the basal internode at breaking (g · cm). Section modulus (SM) = $\Pi/32\times(a_1{}^3b_1-a_2{}^3b_2)/a_1$, where, a_1 is the outer diameter of the minor axis in an oval cross-section, b_1 is the outer diameter of the major axis in an oval cross-section, a_2 is the inner diameter of the minor axis in an oval cross-section and b_2 is the inner diameter of the major axis in an oval cross-section.

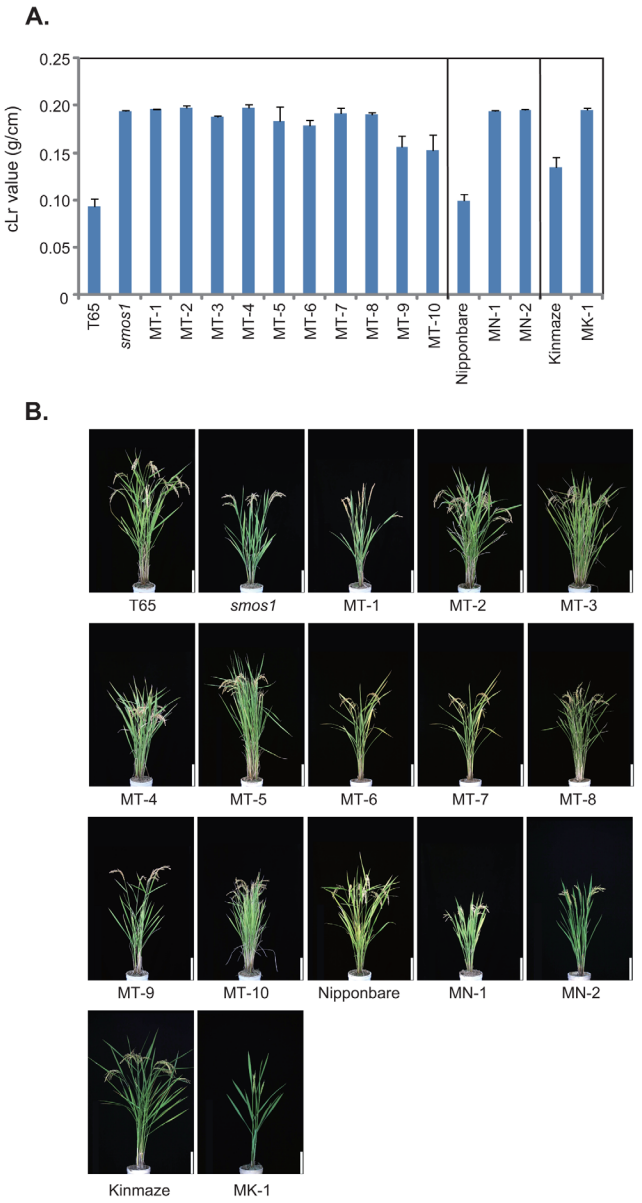

Figure 1. Rice lines with improved lodging resistance. (A) Bending-type lodging resistance of selected lines evaluated in terms of cLr value. Data are means ± SD (n>=3). (B) Gross morphology of the selected lines. First up to the second panel of the third row show T65 (original cultivar) and T65 mutant lines. MN-1 and MN-2 at the third row are Nipponbare mutants and MK-1 at the fourth row is a Kinmaze mutant, respectively. Bar = 20 cm.
doi:10.1371/journal.pone.0096009.g001

Observation of the cross-section of the internode

The uppermost down to the fourth internode of rice plants at the matured seed stage were sampled. The center of each internode was hand-sectioned and stained with toluidine blue.

Confirmation of *smos1* mutation by PCR

Genomic DNA of the F5 generation plants crossed with *smos1* and ST-4 were PCR amplified using the primers designed to amplify the *SMOS1* gene. Mutation was confirmed by sequencing the PCR amplified DNA.

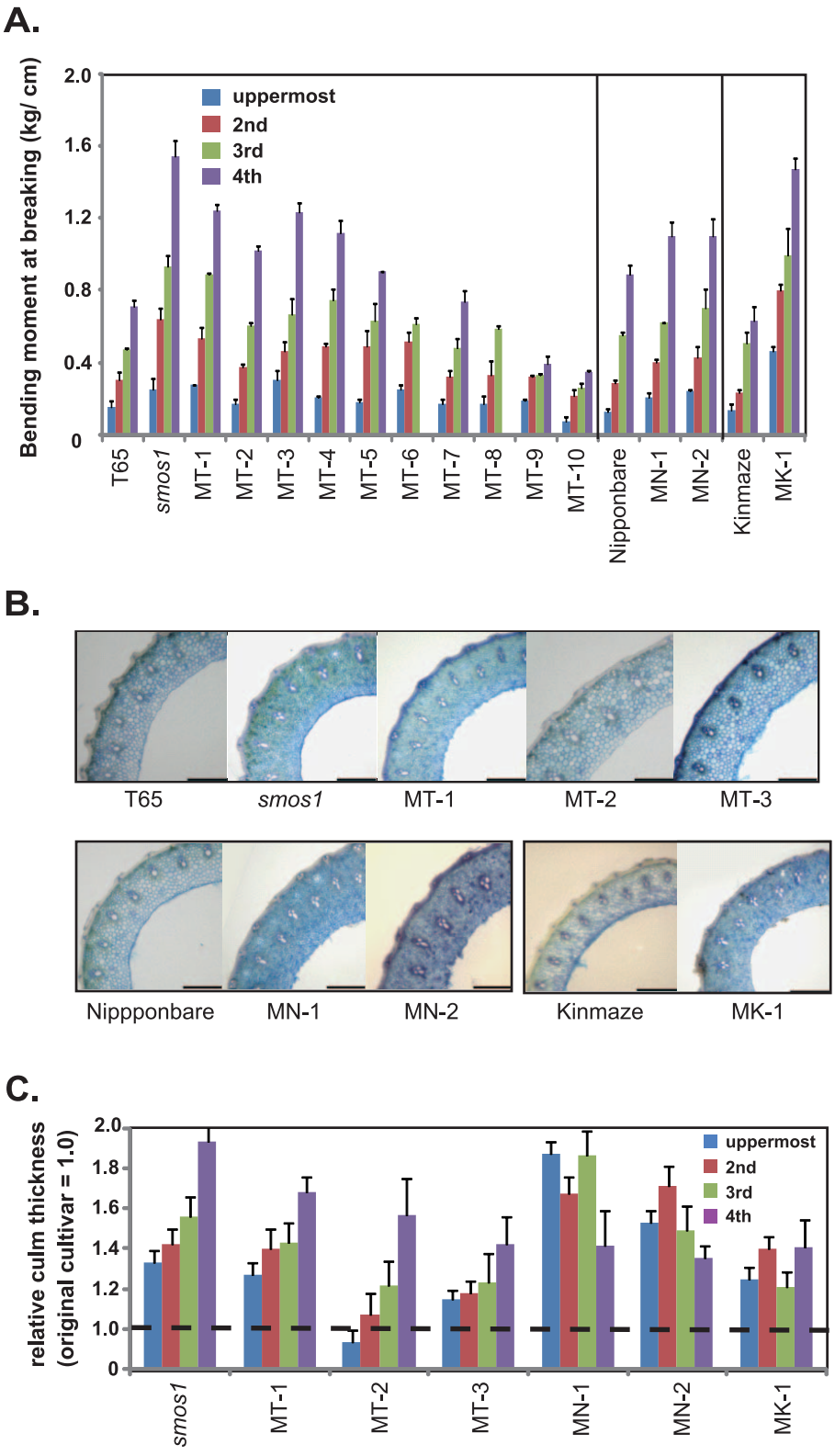

Figure 2. Breaking-type lodging resistance and culm morphologies of selected lines. (A) Breaking-type lodging resistance evaluated in terms of bending moment at breaking. Data are means ± SD (n> = 3). The fourth internodes of MT-6 and MT-8 could not be evaluated due to short internode lengths. (B) Magnified view of the third internode cross-section of 7 lines showing high bending-moment-at-breaking value in comparison with original cultivars (T65, Nipponbare, and Kinmaze). Bar = 500 μm. (C) Relative culm thickness of each line. Thickness of the original cultivars is set as 1. Data are means ± SD (n> = 3). The uppermost to fourth internodes were measured.
doi:10.1371/journal.pone.0096009.g002

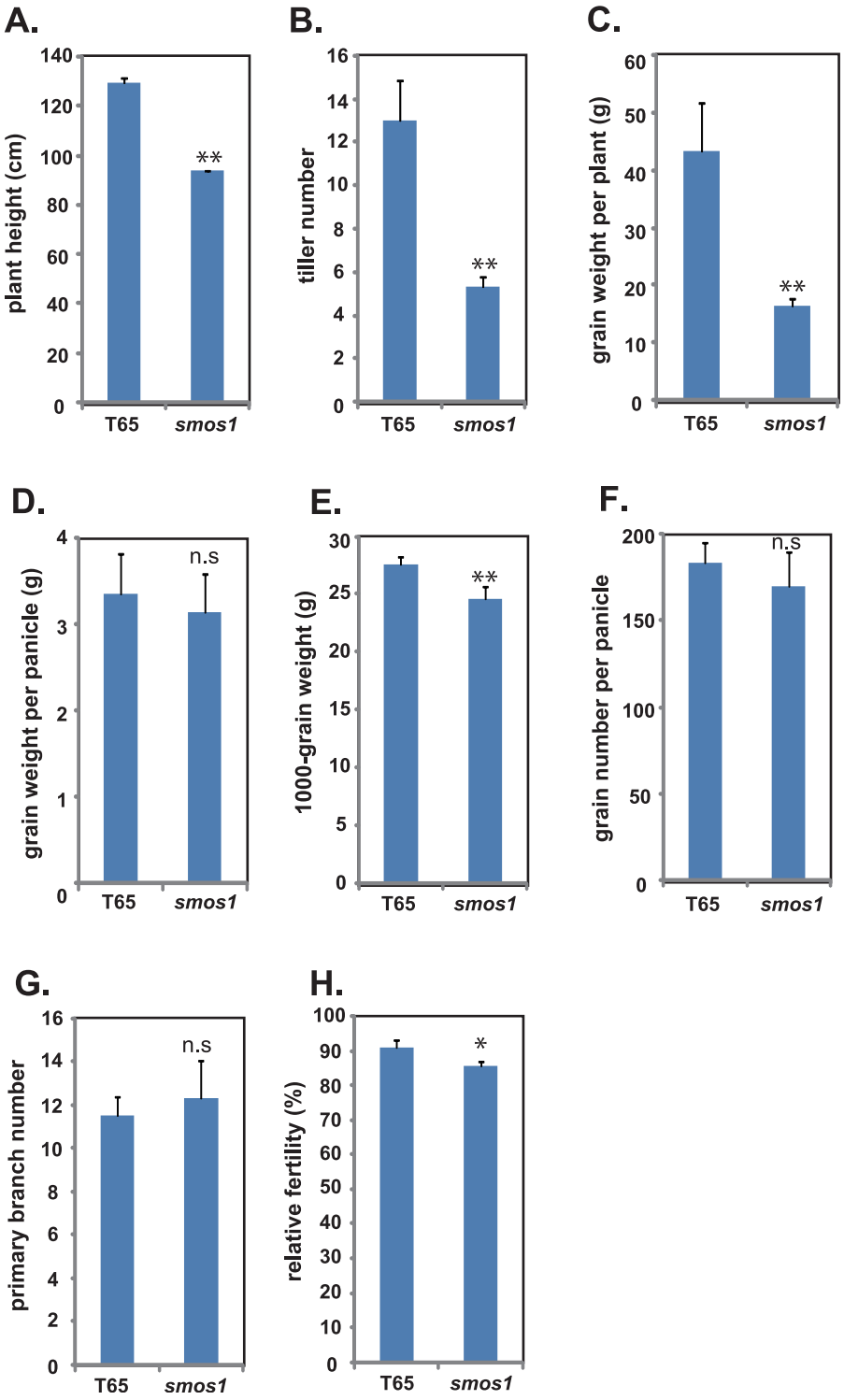

Figure 3. Plant height and agronomical traits of T65 and *smos1*. (A to H) Each trait of *smos1* was compared to that of its original cultivar T65. Data are means ± SD (n> = 3). **, $^{n.s}$, significantly different at P<0.01, and not significant, respectively (two-tailed Student's *t*-test).
doi:10.1371/journal.pone.0096009.g003

Statistical analysis

Data on morphological and agronomical traits, and lodging resistance were examined using three or more individual plants for replication. Statistical analyses were carried out using R version 2.12.1 software. Significance test was conducted using the two-tailed Student's *t*-test or the Tukey's significant difference test.

Results

Screening for rice mutants with improved lodging resistance parameters

From among the >3000 rice mutant accessions of the Nagoya University Togo Field that we initially screened, we selected 14

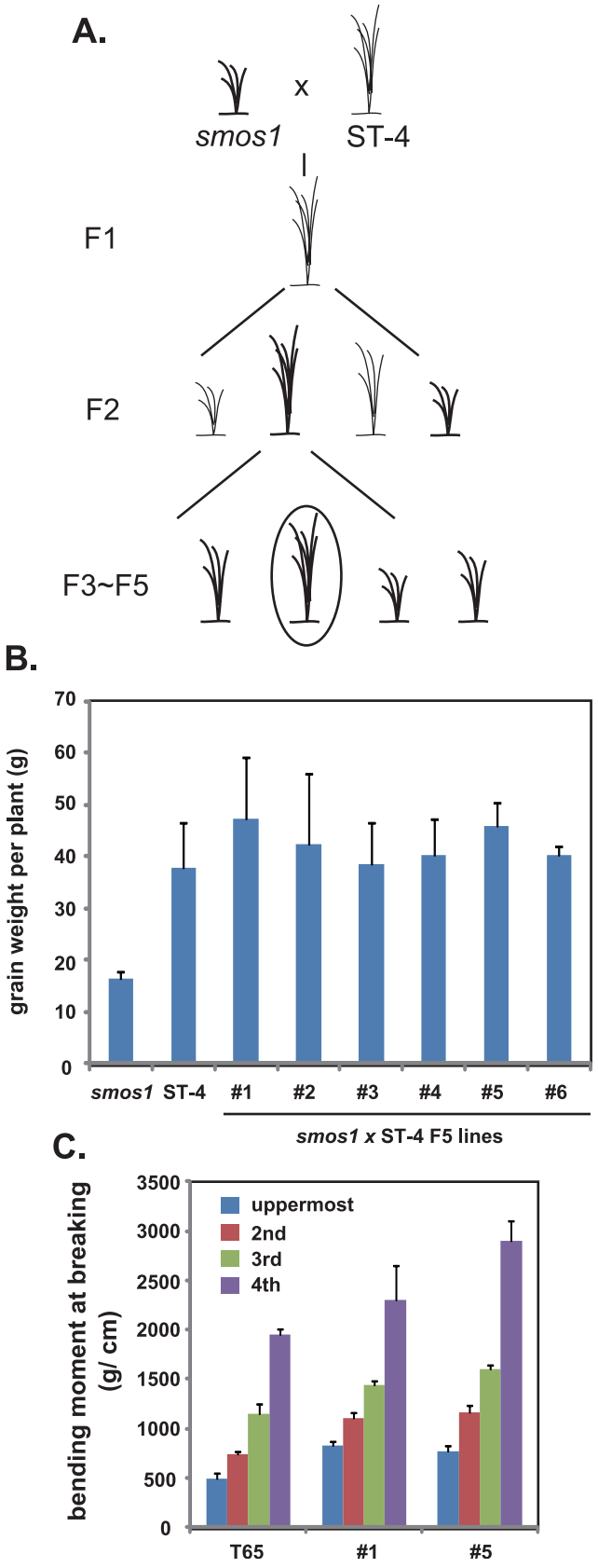

Figure 4. Selection of lines with high grain yield and improved lodging resistance from the F5 population of the *smos1* and ST-4 cross. (A) Procedure to select for high grain yield and improved lodging resistance. (B) Grain weight per plant of lines from the F5 population of the *smos1* and ST-4 cross. Data are means ± SD (n> = 3). (C) Bending-moment-at-breaking values of line #1 and line #5 (n> = 3).
doi:10.1371/journal.pone.0096009.g004

lines showing improved cLr values (a measure for bending-type lodging resistance) for two seasons as compared to their respective original lines (T65, Nipponbare, and Kinmaze) (Figure 1A). Their gross morphologies are shown in Figure 1B. Next, we evaluated the bending moment at breaking (BMB, a measure for breaking-type lodging resistance) of the mutants (Figure 2A) using a high throughput handmade load-testing apparatus in the paddy field. Through these steps, we were able to classify the lines into two groups: 9 lines showed both increased cLr and BMB values, and 5 lines that only showed increased cLr values (MT-6 to -10).

Among the 9 mutants showing increased BMB, we further analyzed 7 lines, namely, *smos1*, MT-1, MT-2, MT-3, MN-1, MN-2, and MK-1 in terms of their culm morphology. The magnified view of the fourth internode of each culm is shown in Figure 2B. Almost all lines showed increased culm thickness in all the internodes tested as compared to original cultivars (Figure 2C).

Since our aim in this study is to find out whether there are other available options for breeding rice with improved lodging resistance other than the traditional use of semi-dwarf trait (which improves cLr), we decided to do a breeding trial using a rice line with improved BMB, while maintaining a good cLr value. For this purpose, we selected *smos1* from among the candidate mutant lines since it showed the highest BMB (Figure 2A). Previously, we identified the causal gene for *smos1* and found that it codes for an APETALA2 (AP2)-type transcription factor which controls organ size downstream of auxin signaling pathway [10]. Mutation in *smos1* results in reduced cell elongation but is accompanied by an increased cell number in several organs.

We initially evaluated the global agronomical attributes of *smos1* such as plant height, tiller number, grain weight per plant, grain weight per panicle, 1000-grain weight, grain number per panicle, primary branch number, and relative fertility in comparison to those of the original cultivar T65 (Figure 3). As a result, the plant height (Figure 3A), tiller number (Figure 3B), and total grain weight per plant (Figure 3C) of *smos1* were reduced to 72.0%, 40.8% and 38.1% relative to that of T65, respectively. As for the remaining grain-related traits, those of *smos1* did not vary significantly from that of T65, except for a slight 1000-grain weight and relative fertility reduction in *smos1* (Figure 3E and 3H; 11% and 5.6% reduction, respectively). Overall, this shows that the low tiller number of *smos1* is the main reason for the reduced grain weight per plant of this otherwise high yielding and lodging resistant line.

Morphological traits of *smos1* x ST-4

For the breeding trial, we chose ST-4 as an ideal crossing partner to complement *smos1*'s unfavorable trait because of its high tiller number (14.3) and high grain yield (37.9 g of grain weight per plant). After crossing *smos1* with ST-4 (Figure 4A), the resulting F1 plant was self-fertilized to proceed to the next generation (F2). Such procedure was conducted until the F5 generation, each time selecting for lines with high grain yield and thick culm through visual and manual checking in the field. In the F5 generation, there were six independent lines (#1 to #6) that possess *smos1* mutant allele as confirmed by PCR, and showing similar or higher grain weight per plant relative to ST-4 (Figure 4B). Among them, we evaluated the BMB of the two highest yielding lines, #1 and #5, by using a precision load-testing machine (Tensilon RTM-25) for accurate measurement of BMB. Although both lines were

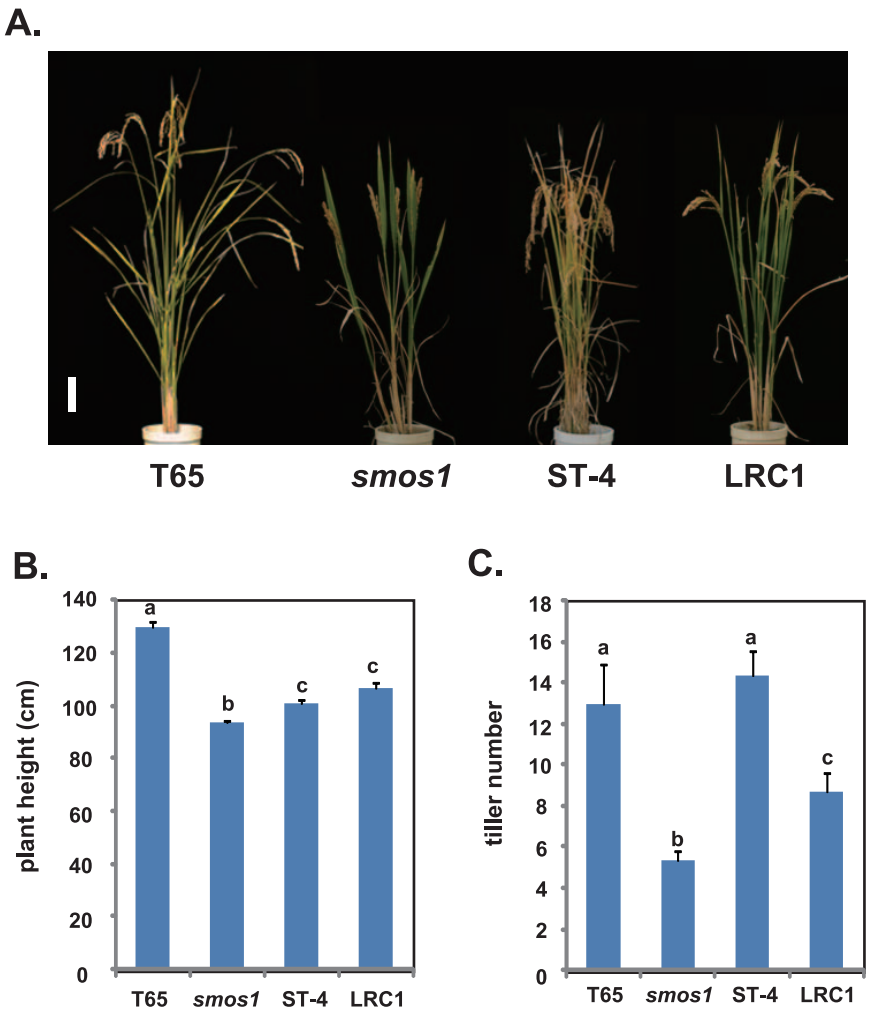

Figure 5. Morphology, plant height, and tiller numbers of T65, *smos1*, ST-4, and LRC1. (A) Gross morphology of plants at 30 days after heading. Bar = 10 cm. (B) Diagram for plant height. (C) Diagram for tiller numbers. Plant height and tiller numbers (n > = 3) were measured at 40 days after heading. Tukey's test was conducted for panels (B) and (C).
doi:10.1371/journal.pone.0096009.g005

superior to T65, #5 showed a larger BMB than #1 line (Figure 4C). Thus, we renamed #5 line as LRC1 (lodging resistance candidate-1) and studied it in more detail.

Agronomical traits of LRC1

Figure 5A shows the gross morphology of LRC1 and its parentals *smos1* and ST-4, together with T65. The plant height of LRC1 was slightly higher than that of *smos1* and similar to that of ST-4, whereas T65 was significantly higher than the three plants (Figure 5A and 5B). As mentioned, the tiller number of *smos1* was reduced compared to that of its original strain, T65 (Figure 5C). Compared to *smos1*, although LRC1 showed increased tillering with an average of 8.7, it was not fully restored to that of ST-4 (14.3) or T65 (13.0).

Next, we evaluated the panicle structure of LRC1 (Figure 6). The 1000-grain weight of LRC1 (26.2 g) was similar to that of *smos1* (24.6 g) and T65 (27.6 g), whereas that of ST-4 was inferior (20.3 g; Figure 6B). On the other hand, the primary branch number (16.3) and grain number per panicle (264.7) of LRC1 were more similar to that of ST-4 (14.0 and 236.3, respectively) than to that of *smos1* (12.3 and 169.7, respectively; Figure 6C and 6D). Relative fertility was similar between the four plants, although *smos1* showed a slight decrease (Figure 6E). These data imply that LRC1 inherited the favorable 1000-grain weight trait from *smos1* and the high grain number and primary branch number traits from ST-4, which led to a marked increase in grain weight per panicle (5.3 g; Figure 6F) compared to the two parentals and T65 (2.6 to 3.3 g). Thus, despite the relatively low tiller number of LRC1, it achieved a grain weight per plant (shown as grain yield per hectare in Figure 6G) that was 20.1% and 5.7% higher than that obtained from ST-4 and T65, respectively, though these values are not statistically significant since the standard deviation for each plant was relatively high.

Lodging resistance of LRC1

Next, we evaluated the lodging resistance of LRC1 in terms of cLr and BMB values. The cLr value of LRC1 was similar to that of *smos1* and ST-4, but 1.9 times higher than that of T65 (Figure 7A). As for the BMB (Figure 7B), LRC1 had a value significantly higher than that of ST-4 and T65 in all the internodes analyzed, and *smos1* showed a similar trend. Thus, the improved lodging resistance of *smos1* was maintained in LRC1.

BMB can be further subdivided into two parameters. That is, the section modulus (SM), which is determined by the morphology

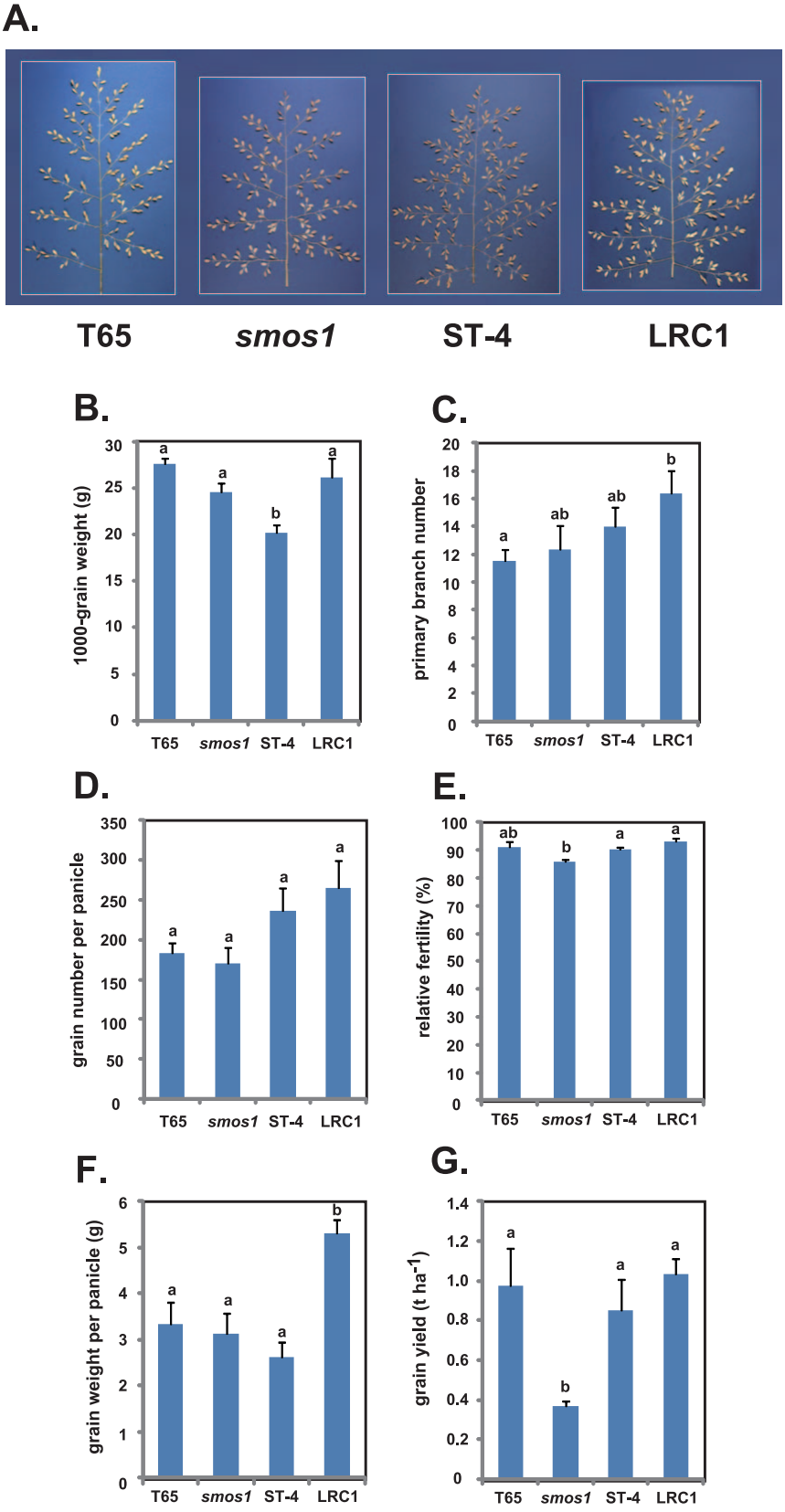

Figure 6. Panicle structure and yield-related traits of T65, *smos1*, ST-4, and LRC1. (A) The gross panicle morphology of each line at 40 days after heading. Diagrams for (B) 1000-grain weight, (C) primary branch number, (D) grain number per panicle, (E) relative fertility, (F) grain weight per panicle, and (G) grain yield per hectare are also shown. Data are means ± SD (n>=3). Tukey's test was conducted for panels (B) to (G).
doi:10.1371/journal.pone.0096009.g006

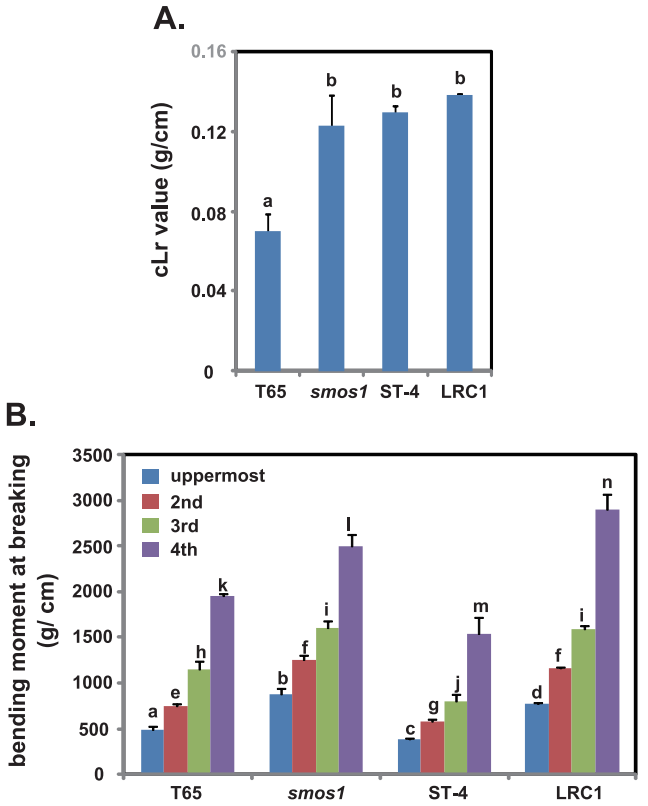

Figure 7. Comparison of lodging resistance between T65, *smos1*, ST-4, and LRC1. (A) Bending-type lodging resistance was analyzed in terms of cLr value. (B) Bending-moment-at-breaking lodging resistance. The uppermost to the fourth internode of each plant was used for analysis. Data are means ± SD (n> = 3). Tukey's test was conducted for each panel.
doi:10.1371/journal.pone.0096009.g007

of the culm (thickness and diameter), and the bending stress (BS) coefficient, which is influenced by the quality of the culm such as the amount of cellulose and lignin within the cell wall [12]. To investigate the mechanism behind the improved culm stiffness of *smos1* and LRC1, culm thickness, culm diameter, SM, and BS were measured at the fourth internodes of each line (Figure 8). Initially, we observed the transverse sections of the uppermost to the fourth internode of *smos1* and LRC1 and found that they are apparently different from those of T65 and ST-4 for having thicker culms (Figure 8A). When the fourth internode transverse sections were observed under the microscope, those from the culms of *smos1* and LRC1 possessed smaller but increased number of cells (Figure 8B), resulting in 1.8 and 1.4 (*smos1*) times, and 1.9 and 1.5 (LRC1) times culm thickness compared to T65 and ST-4, respectively (Figure 8C). When culm diameter, another parameter determining the SM was measured, *smos1* and LRC1 showed a significantly larger diameter compared to T65 in all the internodes analyzed (Figure 8D). *smos1* and LRC1 showed a similar or slightly higher diameter compared to ST-4.

As was expected from their culm morphology, SMs of the fourth internodes of *smos1* and LRC1 were 2.3 and 2.7 times higher than that of T65, respectively (Figure 8E) and although not significant, the SM of LRC1 was higher than that of *smos1*. This might be the reason why LRC1 showed a slightly higher BMB at the fourth internode relative to *smos1* (Figure 7B).

As for the BS of each plant (Figure 8F), BS of *smos1* (and LRC1) was much lower than that of T65 (Figure 8F). This suggests that

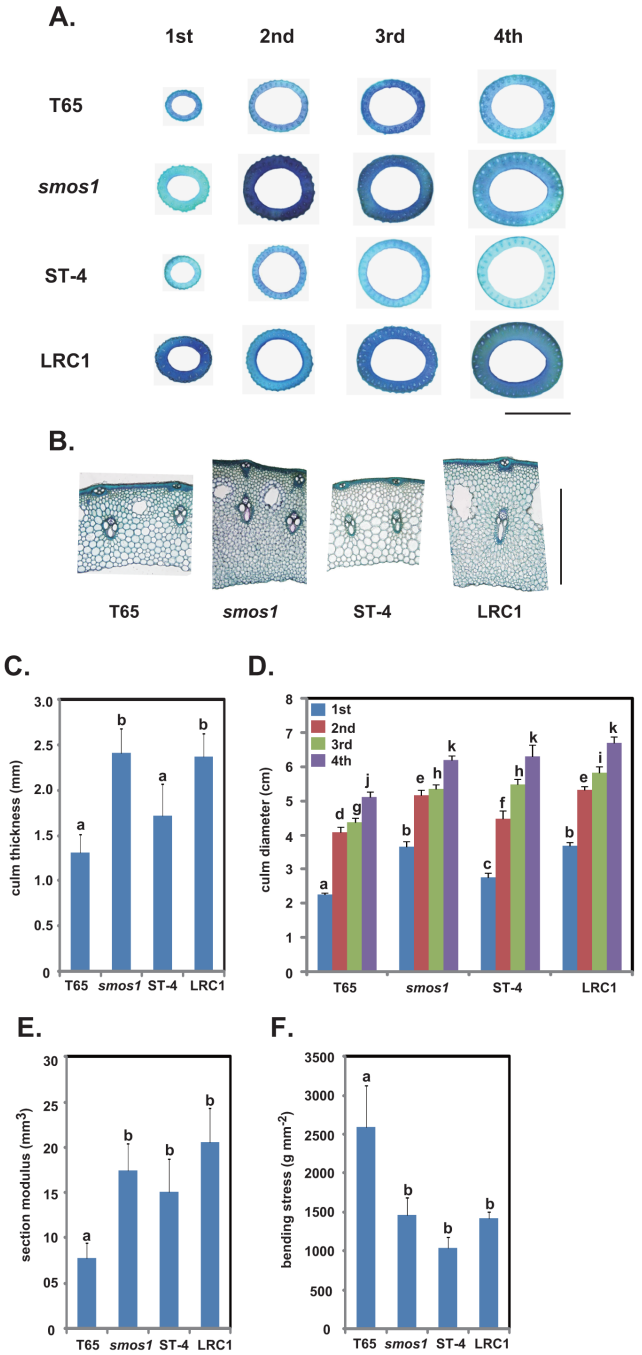

Figure 8. Culm morphology and culm strength of T65, *smos1*, ST-4, and LRC1. (A) Culm cross-sections from the uppermost (1st) to the fourth (4th) internodes of the main culms. Bar = 5 mm. (B) Cross-sections of the fourth internode showing the culm thickness of each plant. Bar = 1 mm. (C) Culm thickness of the fourth internodes of each plant (n = 3). (D) Culm diameter measured from the uppermost to the fourth internode of the main culms (n = 3). (E) Section modulus and (F) bending stress of each plant (n = 3). Tukey's test was conducted for panels (C) to (F).
doi:10.1371/journal.pone.0096009.g008

there might be a trade-off between SM and BS, and that pronounced increase in culm thickness and diameter negatively affects culm quality that in turn decreases BS (yet to be proven).

Taken altogether, the increased breaking-type lodging resistance of *smos1* and LRC1 results from the higher SM conferred by their thick and wide culms, which are lacking in T65.

Discussion

In the present study, we showed that some mutants with stiff culm have the potential to markedly increase breaking-type lodging resistance (Figure 2A). However, as far as we know, high BMB mutants have not been utilized in breeding programs up to now. This is probably due to the various unfavorable traits that are often associated with these lines, such as low tiller number, low grain yield, and some morphological defects. To assess the possibility of utilizing mutants with improved culm breaking resistance in breeding, we chose *smos1* as a representative mutant line possessing increased lodging resistance but with reduced tillers and low grain yield. As a crossing partner, we chose ST-4, a high tillering and high grain-yielding line that has inferior breaking-type lodging resistance, to investigate the possibility of producing rice with high lodging resistance and high grain yield.

Selecting rice line with stiff culm and restored tiller number

The incomplete restoration of the tiller number of LRC1 to that similar to ST-4 suggests that it is difficult to separate traits related to culm thickness (or size) and tiller number, and that the two traits may be pleiotropic effects of the same mechanism(s). Such negative correlation between tiller number and culm size has also been reported. For instance, *fine culm 1* (*fc1*) mutant shows high tiller number which results from diminished strigolactone signaling, has thin culms [14]. Similarly, *high-tillering dwarf 3* mutant possesses small culm diameter although its mechanism is unknown [15]. These observations suggest that there is a trade-off between tiller number and culm size.

Even so, the significant improvement of *smos1* in terms of lodging resistance is an attractive trait that can be harnessed in rice breeding programs. One possible way to overcome the negative effect of low tiller number is to increase grain weight per panicle. In Figure 6F, the observation that LRC1 had a significantly higher grain weight per panicle (5.3 g) than any of its parents (ST-4, 2.6 g; *smos1*, 3.1 g) or T65 (3.3 g) shows that provided a suitable crossing partner is used with *smos1* and selection of best-performing progenies is done, good candidates with thick culm and a reasonable grain yield can be obtained.

Using lines with unfavorable traits– a paradigm shift in rice breeding

Although numerous researches have been devoted to increasing rice grain production, a substantial increase in yield has been slow in coming after the ''Green Revolution'' [3]. This calls for new strategies to overcome the current rice production bottleneck and possibly bring forth a Second Green Revolution. As shown in this study, a mutant line (*smos1*) that have been ruled out as a possible breeding line in the past due to its unfavorable trait surprisingly gave rise to a lodging resistant and high yielding progeny (LRC1) after crossing with ST-4. This means that even lines with unfavorable traits from a certain viewpoint can serve as important bioresources and that a paradigm shift in the way we look at them is important.

Author Contributions

Conceived and designed the experiments: KH HK TH MM. Performed the experiments: AO TH YS KA. Analyzed the data: KH AO TH YS KA MM. Contributed reagents/materials/analysis tools: HK AO TH YS KA. Wrote the paper: KH RO MM.

References

1. Hargrove TR, Cabanilla VL (1979) The impact of semi-dwarf varieties on Asian rice-breeding programs. BioScience 29: 731–735.
2. Dalrymple DG (1986) Development and spread of high-yielding rice varieties in developing countries. International Rice Research Institute.
3. Khush GS (1999) Green revolution: preparing for the 21st century. Genome 42: 646–655.
4. Kono M (1995) Physiological aspects of lodging. In: Matsuo T, Kumazawa K, Ishii R, Ishihara K, Hirata H, eds. Science of the rice plant, Volume 2, Physiology. Tokyo: Food and Agriculture Policy Research Center 971–982.
5. Hoshikawa K, Wang SB (1990) Studies lodging in rice plants. I. A general observation on lodged rice culms. Jpn J Crop Sci 59: 809–814.
6. Islam MS, Peng S, Visperas RM, Ereful N, Bhuiya MSU, et al. (2007) Lodging-related morphological traits of hybrid rice in a tropical irrigated ecosystem. Field Crops Res 101: 240–248.
7. Watanabe T (1997) Lodging resistance. In: Matsuo T, Futsuhara Y, Kikuchi F, Yamaguchi H (eds) Science of the rice plant, vol 3, Genetics, vol 3. Food and Agriculture Policy Research Center, Tokyo, 567–577.
8. Kashiwagi T, Hirotsu N, Madoka Y, Ookawa T, Ishimaru K (2007) Improvement of resistance to bending-type lodging in rice. Japan Journal of Crop Science 76: 1–9.
9. Okuno A, Hirano K, Asano K, Takase W, Masuda R, et al. (2014) New approach to increasing rice lodging resistance and biomass yield through the use of high gibberellin producing varieties. Plos One 19; 9: e86870.
10. Aya K, Hobo T, Sato-Izawa K, Ueguchi-Tanaka M, Kitano H, et al. (2014) A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. Plant Cell Physiol. 2014 PMID: 24486766.
11. Grafius JE, Brown HM (1954) Lodging resistance in oats. Agronomy Journal 46: 414–418.
12. Ookawa T, Ishihara K (1993) Varietal difference of the cell wall components affecting the bending stress of the culm in relation to the lodging resistance in paddy rice. Japan Journal of Crop Science 62: 378–384.
13. Ookawa T, YasudaK, Kato H, Sakai M, Seto M, et al. (2010) Biomass Production and Lodging Resistance in 'Leaf Star', a New Long-Culm Rice Forage Cultivar. Plant Prod Sci 13: 56–64.
14. Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi-Tanaka M, et al. (2003) The OsTB1 gene negatively regulates lateral branching in rice. Plant J. 33: 513–520.
15. Zhang B, Tian F, Tan L, Xie D, Sun C (2011) Characterization of a novel high-tillering dwarf 3 mutant in rice. J Genet Genomics 38: 411–418.