Maize *brachytic2* (*br2*) suppresses the elongation of lower internodes for excessive auxin accumulation in the intercalary meristem region

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**Abstract**

**Background:** Short internodes contribute to plant dwarfism, which is exceedingly beneficial for crop production. However, the underlying mechanisms of internode elongation are complicated and have been not fully understood.

**Results:** Here, we report a maize dwarf mutant, *dwarf2014* (*d2014*), which displays shortened lower internodes. Map-based cloning revealed that the *d2014* gene is a novel *br2* allele with a splicing variation, resulting in a higher expression of *BR2-T02* instead of normal *BR2-T01*. Then, we found that the internode elongation in *d2014*/*br2* exhibited a pattern of inhibition-normality-inhibition (transient for the ear-internode), correspondingly, at the 6-leaf, 12-leaf and 14-leaf stages. Indeed, *BR2* encodes a P-glycoprotein1 (PGP1) protein that functions in auxin efflux, and our in situ hybridization assay showed that *BR2* was mainly expressed in vascular bundles of the node and internode. Furthermore, significantly higher auxin concentration was detected in the stem apex of *d2014* at the 6-leaf stage and strictly in the node region for the ear-internode at the 14-leaf stage. In such context, we propose that BR2/PGP1 transports auxin from node to internode through the vascular bundles, and excessive auxin accumulation in the node (immediately next to the intercalary meristem) region suppresses internode elongation of *d2014*.

**Conclusions:** These findings suggest that low auxin levels mediated by BR2/PGP1 in the intercalary meristem region are crucial for internode elongation.

**Keywords:** Auxin efflux, *br2*, Dwarfism, Internode elongation, Intercalary meristem, Maize

**Background**

Dwarfism or semi-dwarfism confers a number of advantages to crop varieties, such as increased lodging resistance, denser growth, and higher harvest index, which are extraordinarily beneficial for crop production [1–3]. For crops, height is usually determined by the internodes’ number and length [4]. Numerous dwarf mutants have been characterized by short internodes, which have been attributed to impaired internode elongation. Therefore, increasing our knowledge of internode elongation will aid the improvement of crop yield.

Stem/internode elongation is mainly controlled by several plant hormones, including gibberellins (GAs), brassinosteroids (BRs), strigolactones (SLs), and auxins [5]. Defects in the biosynthesis or the signaling of these hormones can cause dwarf phenotypes [6–10], although each hormone might play a different role in stem/internode elongation, irregularities of which will lead to...
various mutant phenotypes. In maize, there are several GA-related mutants that display extremely reduced height with uniformed short internodes, such as dwarf1(d1), d3, d5 and anther ear1 (anl), as well as dominant mutants D8 and D9 [11–13], all of which influence internode elongation throughout the growth period, accompanied by a certain degree of yield loss. Most BR-associated mutants exhibit multiple defective phenotypes in addition to dwarfism [14–17]. For example, the maize nana plant2 (na2) mutant displays suppression of tillers, altered leaf morphology, and andromonoecy [18]. Unlike GAs and BRs, the reduction in plant height of SL mutants may be an indirect effect of increased tillers because the deficiency in SLs enhances cell division in axillary meristems (AMs) [19, 20], which ultimately leads to a redirection of the nutrition toward tiller growth instead of internode elongation.

Auxin biosynthesis is performed through multiple Trp-dependent or Trp-independent pathways, mainly involving YUCCA (YUC) and TAA1/TAR1/TAR2 genes [21]. Quadruple yuc1 yuc4 yuc10 yuc11 mutants do not develop a hypocotyl and root meristem [22]. Similarly, taa1 mutants in Arabidopsis display a defective hypocotyl and root [23] and taa1 tar1 tar2 triple mutants lacked roots and were seedling lethal [24]. In maize, the vanishing tassel2 (vt2) gene is an ortholog of Arabidopsis TAA1, and vt2 mutants exhibit no tassel branches or spikelets, as well as a semi-dwarf phenotype with fewer leaves [25]. Many organs, including the stem/internode, are damaged in auxin biosynthesis mutants. The SCFTIR1/AFB-mediated proteolysis of Aux/IAA proteins is the major auxin signaling pathway, which is clearly responsible for many auxin actions [26, 27]. Most mutants in these components have a similar seedling lethal phenotype [28–30]. In addition, synthesized auxin is often directionally transported by auxin transporters to specific tissues, where it acts a potent signal that triggers a plethora of developmental responses [31]. The maize br2 and sorghum orthologue dwarf3 (dw3) exhibit reduced auxin transport and shortened statures [32], and auxin transport is crucial for regulating internode elongation.

Actually, several dwarf mutants described above exhibited various abnormal patterns of internode elongation and other morphological characteristics, which suggests that their underlying mechanisms should be discriminat-

The results of the maize d2014 dwarf mutant
A maize dwarf mutant, d2014, arose spontaneously from the HL9047 (WT) inbred line in 2014, and its self-crossed progenies steadily presented with uniform short stature with erect leaves (Fig. 1a). The plant height of d2014 was reduced by 58.61 cm, whereas its ear height was 44.59 cm less than that of WT (Table 1). This showed that the reduced height of lower internodes mainly contributes to the d2014 dwarf phenotype. Additionally, other traits of d2014, such as ear length, ear width, ear weight, hundred-grain weight, total tassel length, tassel branch number, tassel stage, and total leaf number were altered slightly in comparison with WT (Fig. 1b; Table 1). Especially for the ear traits, small variations were not enough to lead to yield loss. Therefore, the d2014 mutant might be useful in maize breeding programs.

To ascertain the genetic basis of dwarfism for d2014, three F2 and three BC1 populations derived from d2014 × HL5038, d2014 × HL5054 and d2014 × F19 were generated. F2 and BC1 populations presented tall or short individual separation. Through χ2 tests, the plant height segregated in a 3:1 ratio (tall/short) in all three F2 populations and in a 1:1 ratio (tall/short) in three BC1 populations (Additional file 2: Table S2). These results indicated that this dwarf phenotype of d2014 was genetically controlled by a single recessive gene.

The d2014 gene is a br2 allele
To clone the dwarf d2014 gene, we carried out positional cloning using the (d2014 × HL5054) × d2014 BC1 population. Firstly, 300 BC1 individuals that had a similar dwarf phenotype as d2014 were identified and genotyped by 150 pleomorphic simple sequence repeat (SSR) makers, and then the d2014 gene locus was defined to a 40.05 Mb genetic interval between marker umc1281 and umc1278 (Fig. 2a). Subsequently, 768 dwarf plants were genotyped for fine mapping by newly developed InDel molecular makers, and the d2014 gene was narrowed to a smaller segment flagged by the two markers a4 and a15, which are 2.85 Mb apart (Fig. 2a). Finally, 2000...
recessive individuals were used to determine the candidate region of about 510 Kb that contained the d2014 gene (Fig. 2a).

Database analysis showed that BR2 and six other candidate genes are within this 470 Kb region (http://ensembl.gramene.org/biomart/martview/; Fig. 2b). Genomic DNA sequences of these genes from the d2014 mutant and WT were compared, and the results showed that only the BR2 sequences in d2014 had two variant regions, including a 546 bp insertion in intron 3 (C2073) and a 233 bp deletion (A6568 to C6800) in exon 5 (Fig. 2b). The inserted sequence in intron 3 seemed not to be characterized as transposable elements, while the missing region in exon 5 would result in the deletion of 92 amino acids and a frameshift affecting the translation of 29 additional amino acids (Fig. 2c). Moreover, a known br2 mutant (114F) was crossed with d2014 and WT, respectively. Significant differences in plant growth and development between the two hybrids were observed. At the 14-leaf stage, the morphological height of hybrid 114F × d2014 was significantly lower than that of hybrid 114F × WT, and the internode length of the former was strongly shortened in comparison with hybrid 114F × WT (Additional file 3: Figure S1). Over all, these results confirmed that the d2014 gene was a br2 allele with a unique variation.

The d2014/br2 mutation alters the normal splicing of BR2 gene

Generally, a genetic mutation would cause the variation of natural expression pattern or protein function, resulting in phenotypic defects. Database analysis showed that BR2 had two transcripts: BR2-T01 (4672 bp) and BR2-T02 (2061 bp) (https://www.maizegdb.org/). Actually,
Table 1 Phenotypic comparison between d2014 and WT

| Traits                        | d2014                  | WT         | Variation |
|-------------------------------|-------------------------|------------|-----------|
| Plant height (cm)             | 163.02 ± 2.00**         | 221.63 ± 2.17 | −58.61    |
| Ear height (cm)               | 35.97 ± 1.53***         | 80.56 ± 0.67 | −44.59    |
| Total tassel length (cm)      | 45.50 ± 5.67            | 47.37 ± 6.33 | −1.87     |
| Tassel branch number (cm)     | 11.67 ± 2.07            | 13.12 ± 4.00 | −1.45     |
| Tassel stage (day)            | 50 ± 0.32               | 50 ± 0.12   | 0         |
| Total leaf number             | 20.49 ± 0.45            | 20.4 ± 0.38 | 0.09      |
| Leaf number above the ear     | 7.06 ± 0.23             | 7.04 ± 0.23 | 0.00      |
| Ear weight (g)                | 122.12 ± 4.44           | 133.25 ± 4.89 | −11.13    |
| Leaf number above the ear     | 7.06 ± 0.23             | 7.04 ± 0.23 | 0.00      |
| Ear length (cm)               | 14.13 ± 0.49            | 15.04 ± 0.43 | −0.91     |
| Ear width (cm)                | 4.59 ± 0.26             | 5.16 ± 0.20 | −0.57     |
| Grain length (cm)             | 1.09 ± 0.02             | 1.12 ± 0.03 | −0.03     |
| Grain width (cm)              | 0.92 ± 0.01             | 0.89 ± 0.02 | 0.03      |
| Ear weight (g)                | 122.12 ± 4.44           | 133.25 ± 4.89 | −11.13    |
| Hundred-grain weight (g)      | 32.35 ± 0.50            | 32.23 ± 0.43 | 0.12      |

Values indicate mean ± SD. *** represents significant difference between d2014 and WT at 0.001 level.

BR2-T01 is composed of 5 exons, while BR2-T02 only has 4 exons excluding the exon 5 (Fig. 3a). In addition to the 5’UTR, 3’UTR and exon 5, a vital region with an additional 15 bp in BR2-T02 exon 4 is different from that in BR2-T01 exon 4 (Fig. 3b). To verify the existence of these two transcripts in vivo, their cDNA sequences were amplified and compared. For BR2-T01, the band size of d2014 was distinctly less than that of WT (Fig. 3c), which was in agreement with a fragment deletion in d2014. Sequencing the band revealed the previously predicted 233 bp deletion. Meanwhile, there was also a similar band of about 2000 bp for BR2-T02 in d2014 and WT (Fig. 3c), the sequences of which were identical. This suggested that the two transcripts truly existed in vivo.

In view of this, it was necessary to analyze the effect of d2014/br2 mutation on the two transcripts. To define the expression patterns of BR2-T01 and BR2-T02, qRT-PCR was carried out through specific quantitative primers. During the three stages of 6-leaf, 10-leaf and 15-leaf, the expression levels of BR2-T01 in d2014 were all lower than in WT, whereas BR2-T02 was significantly up-regulated in d2014 during the three stages (Fig. 3d). These data suggested that the natural expression of BR2-T01 is crucial for WT normal growth, and the down-regulated BR2-T01 leads to the defective traits of d2014. Interestingly, the total BR2 expression level was roughly similar between d2014 and WT (Fig. 3d). These results revealed that the d2014 mutation alters the normal splicing of BR2 gene, causing a higher expression of BR2-T02 instead of normal BR2-T01.

Analysis of BR2/PGP1 and auxin efflux in yeast

In maize, BR2 encodes an auxin transporter, PGP1, which functions in auxin export from intercalary meristems [34]. In order to investigate whether the protein BR2/PGP1 (T01 and/or T02) is responsible for auxin transport at the cellular level, we functionally expressed these two variants in yeast. Fluorinated indole derivate (5-FI), a toxic analog of indole-3-acetic acid (IAA, namely auxin), is cytotoxic to yeast and has been used to investigate auxin transport [35, 36]. Yeast mutant strain gef1 lacks endogenous chloride channel protein [37], which results in 5-FI accumulation in gef1 cytoplasm after the undissociated 5-FI molecules entering cells by passive diffusion, leading to gef1’s hypersensitivity to 5-FI. Using the yeast mutant strain gef1, we found that both of WT-PGP1-T01 and d2014-PGP1-T01 provided certain resistance against 5-FI compared to the vector control (Fig. 4a). The result indicated that the protein PGP1-T01 exported 5-FI to the outside of the yeast cell, namely, PGP1-T01 was responsible for auxin export. Interestingly, PGP1-T02 also exhibited slight but significant resistance against 5-FI in the yeast mutant strain gef1 (Fig. 4b), indicating its functions in auxin export. In terms of alternative splicing, there are mainly two types: front mutation (T01 and T02, both defective) and back mutation (T01, defective; T02, normal). When the two proteins, PGP1-T01 and PGP1-T02, play similar roles in auxin’s export, the second type of mutation would display a mild phenotype, which opens the possibility that there is a variation of br2 mutant with diverse defects.

Defects of internode elongation in the d2014 mutant

A typical characteristic of br2 mutants is that dwarf stature derives from shortened lower internodes [32, 34]. Of the d2014/br2 mutant, the length of certain internodes below the ear was dramatically reduced; additionally, the ear-internode was also shortened, whereas the internodes above the ear were approximately normal (Fig. 5a). Meanwhile, we found that other br2 mutants, br2-114F and br2-117A, also displayed an abnormal ear-internode phenotype similar to d2014 (Fig. 5b). These data showed that the loss-of-function alleles of br2 specifically regulated the elongation of certain internodes.

Since the total leaf numbers and tasseling stage of WT and d2014 (Table 1) are nearly the same, thus, their developmental phase should be paralleled. To clarify how the mutation of br2 affects internode elongation, we carried out a dynamic comparison of stem growth between d2014 and WT. Plenty of individuals were planted and leaf numbers were labeled to ensure the same developmental stage. Before the 12-leaf stage, the 6th, 7th, 8th, 9th, 10th, and 11th internodes in d2014 were scarcely elongated (Fig. 6a, b, c, d), and the stem height of all these internodes was significantly lower than that in WT (Additional file 4: Figure S2A). From the 12-leaf stage to the 14-leaf stage, the 11th and 12th internodes in d2014 grew rapidly compared to previous patterns, and their lengths increased (Fig. 6d, e). Moreover, the length of...
the 11th, 12th and 13th internodes (ear-internode) in d2014 was similar to that of WT at the 14-leaf stage (Fig. 6e). Namely, the 5 lowest internodes in d2014 were shortened seriously (Additional file 4: Figure S2B). Strangely, after the 14-leaf stage, the 11th, 12th and 13th internodes in d2014 grew slowly in comparison with WT (Fig. 6e, f), and the elongation of the ear-internode was sharply restrained (Fig. 6g). Nevertheless, the patterns of upper internode elongation in both d2014 and WT were exactly alike after the 16-leaf stage (Fig. 6g, h), which led to nearly paralleled length of the upper internodes (Additional file 4: Figure S2C). All these results revealed that defects of internode elongation in mutant d2014 are disparate at different developmental stages; especially, at the 6-leaf, 12-leaf and 14-leaf stages, the internode elongation in d2014 exhibits variation pattern of inhibited-normal-inhibited transiently.

**Effect of the d2014 mutation on cell morphology in shortened internodes**

At an early developmental stage (before 12-leaf stage), the elongation of the lower internodes in d2014 was
seriously inhibited. At the 6-leaf stage, the first over-
ground internode (the 6th internode) was elongating.
Though it seemed to be very short (Fig. 6a), there was a
trending difference between WT and d2014. In order to
understand the cell morphology of these shortened in-
ternodes in d2014, we observed the young 6th internode
at the 6-leaf stage. Cross-sections showed that d2014
had regular parenchyma cells similar to WT, and their
cell sizes were comparable, whereas the numbers of vas-
cular bundles in d2014 had been significantly reduced
(Fig. 7a, c). Longitudinal sections showed that the length
of parenchyma cells was not changed between d2014
and WT, but the interval of two adjacent vascular bun-
dles in d2014 was larger than that in WT (Fig. 7b),
which meant that there was a reduced vascular bundle
number for d2014. Thus, the d2014 mutation originally
affects the formation of vascular bundle in shortened
internode cells.

Location of BR2 expression in the stem
Since the mutant d2014/br2 displays defects in internode
elongation, we predicted that BR2 is expressed in these
tissues. However, the expression pattern of BR2 has not
been precisely defined previously. To figure out the loca-
tion of BR2 expression in the stem, we performed in situ
RNA hybridization at the 10-leaf stage. In longitudinal
sections of the stem apex, BR2 expression was clearly lo-
calized in each node (Fig. 8a). The upper and lower
nodes revealed clear signals in the single internode on
the ear (Fig. 8b). In cross sections of the longest inter-
node, BR2 mRNAs were observed in a mass of vascular bundles (Fig. 8c). A single internode was selected for
control experiments, in which the sense probe produced
no signal (Fig. 8d, e). In maize stems, the vascular bun-
dles interlace in nodes and then tilt into internodes with multiple branches, which finally form a network pipe from top to bottom. Thus, BR2 expression was
mainly localized in vascular bundles of the node and
internode. Xing indicated that the subcellular
localization of QPH1 (BR2) is exclusively in membrane
[33]. There is no variation in the signal peptide region
d2014/br2, thus, mutation should not influence the
subcellular localization of d2014/br2. In WT, the ex-
pression level, localization and transport activity of BR2
are normal, relatively; while under other circumstances
(for example br2 mutation), developmental defects
occur. Overall, these data indicated that PGP1 functions
in auxin export from vascular bundles in stem, then
regulating internode elongation.

Quantitative analysis of auxin in the d2014 stem
AUXIN transport influences the distribution and local
concentration of auxin [38]. In the prior studies, br2
mutation influences auxin transport and local concentra-
tion, which are responsible for the dwarf phenotype
[32–34]. The defective auxin transport in d2014 stems,
mediated by BR2/PGP1, would cause the auxin concen-
tration variation in the stem and then influence the
internode elongation. To investigate the links between the two, we detected the auxin concentration in several key periods of internode elongation variation (as mentioned above). At the 6-leaf stage, auxin concentration in the d2014 stem apex was significantly higher than that in WT (Fig. 9a). We also measured the auxin concentration in the stem apex of d2014 and WT at the 12-leaf and 14-leaf stages. However, auxin concentration was gradually reduced compared with the former period (Fig. 9a). Moreover, little difference was observed between d2014 and WT at these two stages (Fig. 9a). These showed that the d2014/br2 caused higher auxin level in the stem apex at the early development stages, at least at the 6-leaf stage, and correspondingly, the internode elongation of d2014 was suppressed. In addition, we examined the auxin levels of the single ear-internode at the 14-leaf stage, including two parts: the node and the internode region. Interestingly, the auxin level of the nodes in d2014 was significantly higher than the nodes in WT, as well as the internode region itself (Fig. 9b), while in WT, there was no significant difference (Fig. 9b). Furthermore, the auxin levels of the two parts exceeded the stem apex at the 14-leaf stage (Fig. 9a; b). These data revealed that auxin at the 14-leaf stage was increased for ear-internode and was detained in the node region of d2014. Notably, the ear-internode in d2014 began to grow rather slowly after the 14-leaf stage and finally was extremely shortened. Taken together, these data showed that the d2014/br2 caused increasing in auxin levels in the shortened lower internodes (stem apex at the 6-leaf stage) and ear internodes (node region at the 14-leaf stage), which were responsible for the defects of internode elongation.

Discussion
d2014 gene, with a splicing variation, is a potentially useful br2 allele
In maize, there have been many reports on the identification of br2 mutants, which involved a variety of the mutation loci (Additional file 5: Figure S3). At first, the BR2 gene were cloned and confirmed by transposon tagging with Mutator (Mu), such as br2–6, br2–7 and br2–9 with Mu insertion in exon 1, as well as br2–3 with Mu insertion in intron 4 [32]. Subsequently, several other br2 alleles, such as one SNP variant (G/T5295) in exon 5 [33] and a 241 bp deletion (G6367 to C6617) in exon 5
were identified to result in the partial loss of BR2/PGP1 protein function. All the above \(br2\) mutants were derived from multiple inbred lines with a different genetic background. Nevertheless, they all presented shorter lower internodes, but nearly normal upper internodes. Without exception, our \(d2014br2\) displayed similar features, which were verified by allelism test with \(br2-114F\). Apart from the above phenotypes, we also found that the \(br2\) mutants (\(d2014\), \(br2-114F\) and \(br2-117A\)) displayed a shortened ear-internode. Therefore, it is certain that this \(br2\) allele is responsible for the shortened internodes.

In the maize B73 reference genome, \(BR2\) has two predicted splicing variants: \(BR2-T01\) and \(BR2-T02\). In this study, we identified a new \(br2\) allele (\(d2014\)), which contained a 546 bp insertion in \(BR2\) intron 3 (\(C_{2073}\)) and a 233 bp deletion (\(A_{6568}\) to \(C_{6800}\)) in \(BR2\) exon 5. Sequencing of the cDNA confirmed the presence of the two \(BR2\) splicing variants, and \(BR2-T02\) was highly expressed in \(d2014\) while \(BR2-T01\) expression level was very low, which was contrary to that in WT. Similarly, the two \(BR2\) splicing variants have been verified molecularly in a dwarf \(br2-NC238\) allele mutant [40]. The insertion of a novel transposon in \(BR2\) intron 4 alters the normal splicing of the gene, which results in abundant expression of \(BR2-T02\) in \(br2-NC238\) instead of \(BR2-T01\) in tall NC238 plants. Generally, insertion, deletion, or single-base replacement of the exon region may result in the defective protein with altered function; while the variation of the intron region might affect gene expression pattern through changing the mRNA splicing. Therefore, we concluded that the insertion of 546 bp in intron 3 altered the normal splicing of \(BR2\) gene, which was the main reason for the dwarfing of \(d2014\). Overall, \(d2014\) with a splicing variants has the enriched types of \(br2\) allele.

Some studies have shown that some different splicing variants perform equivalent functions to the constitutive form, even when lacking amino acid sequences or protein domains [41]. The \(BR2\) gene encodes an auxin transport protein, PGP1, which is an ATP-binding cassette (ABC) transporter [42]. In general, full-size ABC transporters consist of two similar halves, each containing a transmembrane domain (TMD) and a nucleotide binding domain (NBD) [43]. The so-called half-size ABC transporters only have a half (one TMD and one NBD) and require dimerization for their transport activity [43]. An example
is ECERIFERUM5, which is a half-size ABC protein and functions in wax export to the plant cuticle [44]. Here, through the analysis of protein domains by (http://smart.embl-heidelberg.de/smart), we found that PGP1-T01 was a full-size ABC transporter, while PGP1-T02 contained exactly a half, making it a half-size ABC transporter (Additional file 6: Figure S4). Diana Santelia and Markus Geisler examined the auxin transport properties (influx or efflux) of ABCB protein by heterogeneous expression in yeast [35, 36]. Since the strong promoter was used to induce the overexpression of ABCB protein, it would not accurately evaluate the auxin transport activity. Similarly, our heterogeneous expression assays using strong promoter identified the transport properties of the two proteins PGP1-T01/T02, which indicated that PGP1-T01 (even including the defective d2014-PGP1-T01) and PGP1-T02 have played similar roles in auxin efflux. Previously, Multani and Knöller have confirmed that br2 mutation leads to the decrease of auxin flow from top to bottom, which is responsible for the dwarf phenotype [32, 34]. In our study, the expression of br2-T01 in d2014 was significantly lower than that of BR2-T01 in WT (normal
status), indicating that the normal auxin transport activity in \textit{d2014} was impaired. Nevertheless, our phenotypic analysis showed that \textit{d2014} was a mild dwarf mutant with nearly no other undesirable traits, which should be attributable to the compensation of functional PGP1-T02. Actually, in the F\textsubscript{2} population of \textit{br2-qph1} and \textit{br2-117A}, plant height and ear height segregated in a 3:1 ratio (\textit{br2-qph1}/− plants to \textit{br2-117A}/\textit{br2-117A} plants), which indicated that \textit{br2-qph1} was dominant to \textit{br2-117A} [33]. Namely, different \textit{br2} alleles would give rise to certain defects in varying degrees. Therefore, our work shows a useful \textit{br2} allele that has beneficial potential for maize improvement by moderately reducing the plant height while not affecting the yield.

\textbf{BR2/PGP1 functions in auxin efflux from the vascular bundles of the node and internode}

Auxin is often synthesized in the apex region and is directionally transported to the lower region [45, 46]. This directional auxin flow is attributable to membranous auxin transporters. So far, three major families of auxin transporters have been identified: AUXIN-RESISTANT1 (AUX1)/AUX1-LIKEs (LAXs) for auxin influx and PIN-FORMEDs (PINs) and several PGP proteins for auxin efflux [47]. Some studies indicated that ABCB efflux transporters might limit auxin reuptake at efflux sites for assisting PIN-oriented auxin flow [48]. In addition, several studies suggested that ABCBs may play a role in local auxin loading of long-distance auxin transport [49, 50].

BR2/PGP1 is an orthologue of \textit{Arabidopsis thaliana} ABCB1/PGP1, which is expressed in shoot and root apices and functions primarily in auxin efflux from meristematic cells into long-range auxin transport streams [34, 51, 52]. Previously, Knöller applied \textsuperscript{3}H\textendash\textsuperscript{IAA} to the upper stem of maize (B73 vs \textit{br2}), and found that several \textsuperscript{3}H\textendash\textsuperscript{IAA} accumulation peaks were detected near the nodes; meanwhile, the moving front was reduced and
free auxin levels in the node of br2 mutant are significantly higher than in adjacent internodes [34]. Consistently, in our d2014/br2, higher auxin levels were detected in the stem apex (including multiple nodes) at the 6-leaf stage and in the node region of the ear-internode at the 14-leaf stage. In a word, the br2 mutation caused auxin accumulation in the node regions. Notably, our in situ hybridization assay showed that BR2 is primarily expressed in vascular bundles (VBs) of the nodes and internodes. In line with this, Anne Sophie Knoller indicated that BR2 is mainly expressed in the nodal region (dense VBs) [34]. In this context, we propose that BR2/PGP1 functions in auxin efflux from the node to the internode through vascular bundles. Thus, auxin efflux is reduced from the nodes to the internodes in br2 mutant, and auxin level is higher in br2 node regions.

Excessive auxin levels in the intercalary meristem region suppress internode elongation

Internode elongation is controlled by several factors, mainly the plant hormones. In this study, we found that the variation of auxin level in d2014 stem influences internode elongation. At the 6-leaf stage, the elongation of the lower internodes began to be severely suppressed in d2014, along with higher auxin levels in the stem apex compared with WT. As the auxin levels of the stem apex gradually decreased at the 12-leaf and 14-leaf stages, internodes in d2014 grew rapidly. At the 14-leaf stage, additionally, the auxin level of the node region next to the ear in d2014 was much more than that in WT, corresponding to the irregular and shortened ear-internode. In short, d2014/br2 and WT have the same genetic background except the br2 locus, causing the shortened internodes, as well as higher auxin levels in their node regions, which suggested that higher auxin levels in d2014 stems (node regions) suppress the internode elongation.

Generally, auxin has fundamental roles in rapid stimulation of cell expansion for promoting growth [53, 54]. This seems to be contradictory with what we found in this study. Nevertheless, similar scene that auxin inhibits growth is fit for apical dominance in plant. Growing shoot apexes produce an inhibitory hormone, auxin, which moves downwards within the stem and inhibits the growth of axillary buds (including the AM) next to

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**Fig. 8** In situ localization of BR2 mRNA in stem at the 10-leaf stage. **a** Longitudinal section of stem apex. The arrows represent each node in stem apex. **Bar** = 0.1 cm. **b** Longitudinal section of an intact internode. IN, internode; N, node. **Bar** = 0.3 cm. **c** Transverse section of longest internode. VB, vascular bundle. **Bar** = 0.2 cm. **d, e** Hybridization of a single internode with a BR2 sense probe for longitudinal section and transverse section, respectively. **Bar** = 0.2 cm.

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**Fig. 9** The change of auxin concentrations in the stem at different stages. **a** Auxin levels of stem apex in d2014 and WT at the 6-, 12-, and 14-leaf stages. **b** Auxin levels of a single ear-internode including node and internode region at the 14-leaf stage. **** indicates significant difference at 0.01 level by Student’s t-test. ns, not significant.
the node [55, 56]. Recent studies have revealed that a local auxin minimum is critical for AM formation or initiation [57–59]. Correspondingly, the stem of some plants, including maize, contains intercalary meristems that support stem growth independently of the shoot apex [5, 60]. Intercalary meristems are located in the internodes immediately above a node, by which the internode can elongate [61]. Moreover, our cytological analysis showed that the d2014 mutation causes a reduced number of vascular bundles, which should be related to intercalary meristem functions. In concordance with this, Multani has also observed the cellular architecture of the br2 internodes, and their data showed that the VB numbers of 1th over-ground internode (6-week-old) are reduced [32]; at the same time, Xing found that the VB numbers of 6th over-ground internode (19-leaf stage) are altered (reduced without specific statistics) in br2 mutants [33], which indicated that less-elongated internodes in br2 have reduced VB numbers. Some studies have shown that cell division or differentiation dependent on auxin transport flow is crucial for the formation of vascular bundles in the leaf [62]. Therefore, we speculate that a low auxin level is indispensable for intercalary meristem functions and, in reverse, internode elongation is suppressed when excessive auxin is arrested in intercalary meristem region.

All the br2 mutants presented shortened lower internodes, as well as ear-internode, but nearly normal upper internodes. Through dynamic observation of internode elongation, we found that the variation of internode elongation in d2014 is closely related to the 6-leaf, 12-leaf and 14-leaf stages. Notably, at the 6-leaf stage, the first internode (6th internode) above the ground was visible, and more internodes elongated subsequently (Additional file 7: Figure S5A); whereas, the juvenile tassel came into view at the 12-leaf stage (Additional file 7: Figure S5B); in addition, the ear-internode, at the 14-leaf stage, was undetectable until the upmost AM emerged (Additional file 7: Figure S5C). These three stages involve the growth and development of shoot apical meristem (SAM), inflorescence meristem (IM) and AM, which is associated with the synthesis of auxin. In context, we present a model to illuminate how loss-of-function of br2 alleles uniquely regulates internode elongation (Fig. 10). At early stages, vigorous auxin is synthesized in the SAM region, and then excessive auxin is arrested in the intercalary meristem region of the lower internodes, and finally, elongation of the lower internodes is suppressed. Along with SAM translating to IM, declining auxin is generated in the apex region, and the upper internodes grow normally. For the severely shortened ear-internode, the chief culprit is a new auxin source derived from the developmental AM, which promotes abundant auxin being restricted in the intercalary meristem region of the ear-internode. Overall, the BR2/PGP1-dependent low auxin level in the intercalary meristem region is crucial for internode elongation.

**Conclusions**

Plant height is one of most important agronomic traits in crop breeding. Maize BR2/PGP1 regulates the plant height by promoting auxin efflux from node to internode and avoiding excessive auxin accumulation in the intercalary meristem region to suppress the internode elongation. These findings are of great significance to the decryption of genetic mechanism of plant height and the improvement of maize yield.

**Methods**

**Plant materials**

The d2014 is a dwarf mutant derived from the HL9047 inbred line (as WT), which was bred by our pedigree
method after 9-generation selection. Three different tall stature inbred lines (HL5038, HL5054 and F19) were selected from our high generation breeding materials to construct F2 and BC1 populations with d2014. In addition, the br2 mutants were requested from the Maize Genetic Cooperation Stock Center (stock number: 114F and 117A), the br2-114F and br2-117A were identified and amplified by our group for br2 allelism test. All the plants were permissively cultivated in the standard experimental field of Sichuan Agricultural University in Sichuan during the summer and in Yunnan during the winter.

**Positional cloning of the d2014 gene**
The BC1 population (HL5054 × d2014) × d2014 was used, and 6000 individuals were prepared to define the d2014 gene locus. Indeed, using the simple sequence repeat (SSR) makers obtained from the MaizeGDB Database and Indel molecular makers acquired from Liu Jian research group in Sichuan Agricultural University, d2014 gene was mapped to chromosome 1 bin 1.06, a ~ 510 Kb region with BR2 and six other predicted genes. To identify the mutation site(s) of d2014 gene, we amplified the DNA sequences of these genes and compared them between d2014 and WT. Meanwhile, we used the br2 mutant 114F to examine the allelism between d2014 gene and BR2.

**RNA isolation and quantitative real-time PCR (qRT-PCR) analysis**
At the 6-leaf, 10-leaf and 15-leaf stages, stem apexes (about 0.5 cm) in d2014 and WT were flash-frozen by liquid nitrogen and stored at −70 °C. Thereinto, the stem apexes are the top internodes except the juvenile tassel at the 10-leaf and 15-leaf stages. Total RNA was extracted using Trizol reagent, and the quantity and purity of RNA was determined by a NanoDrop 2000. Cells were 10-fold, 100-fold, 1000-fold diluted, and 2.5 μl of each was spotted on YPDA medium containing 0 μM or 250 μM 5-FI [36] with the corresponding vector control and the yeast mutant strain ge[el]. For leucine-deficient type ge[el], single colonies were screened in synthetic minimal medium without leucine (SD-Leu−). Lastly, transformants grown in SD-Leu− to OD600 = 0.8 were washed and resuspended in water to OD600 = 1.0. Cells were 10-fold, 100-fold, 1000-fold diluted, and 2.5 μl of each was spotted on YPDA medium containing 0 μM or 250 μM 5-FI [36] with the corresponding vector control and the yeast mutant strain itself as controls. Pictures were taken after 3–6 days of growth at 28 °C. All primer sequences in homologous recombinant are listed in Additional file 1: Table S1.

**Dynamic observation of internode elongation**
Each 700 d2014 and WT individuals were planted in a specially designed plot. The plot consisted of 3-m-long rows separated by 1 m space between each row. Five-row interval planting was performed for d2014 and WT with 10 replicates, and 14 individuals were planted in each row. The 5th leaf and 10th leaf of uniform individuals were labelled in the field. In general, the first internode above the ground is visible when the 6th leaf is unfolded at the 6-leaf stage. Thus, we measured each visible internode length from the 6-leaf stage to the maturation stage, including the 6, 8, 10, 12, 14, 16, 18, and 20-leaf stages. At each stage, 10 leaf-labelled individuals in adjacent rows were investigated, and the average value was the phenotypic value of internode corresponding to the leaf number.

**Histocytological analysis**
A plenty of WT and mutant individuals were planted (as described above). At the 6-leaf stage, the first overground internode (the 6th internode) elongated. We
randomly picked three individuals of WT and mutant, respectively, as three biological replicates for cytological observation of the internodes. The whole 6th internode was cut except for two terminals (the node regions) to make a paraffin section. All samples were vacuumed quickly and fixed in FAA composed of 5% of formaldehyde (40% v/v), 5% of acetate, and 90% of 75% alcohol (v/v) overnight. The paraffin sections were made as described previously [67]. Firstly, the transection was made from the middle part (about 0.1 cm from the upper part). Next, the longitudinal section was made from the rest of the upper part. The cell morphology was observed through an optical microscopy. Based on the same area (the middle region) and the same magnification (20 ×), we counted all the vascular bundles (VBs) for the transection.

In situ hybridization analysis
At the 10-leaf stage, tissues of WT were prepared, including the stem apex (about 0.5 cm segment from the tip), longest internode and single internode (about 1 cm). In situ hybridization for BR2 was performed as described previously [63]. An antisense RNA probe labeled by the Digoxin marker was uniquely designed in the BR2 exon 1 region, and sequences are listed in Additional file 1: Table S1. To reduce the disturbance of the background signal, pilot experiment was conducted to optimize the probe dosage. In addition, a single internode was selected for the control test with a sense probe (Additional file 1: Table S1).

Endogenous auxin concentration analysis
The stem apex segment (about 0.5 cm) was prepared in several periods, including 6-, 12-, and 14-leaf stages. All tissues were flash-frozen by liquid nitrogen and stored at −70 °C. The measurements of endogenous auxin were performed through liquid chromatography tandem mass spectrometry (LC-MS). Three repeats with three plants in each replicate were performed. Furthermore, at the 14-leaf stage, the ear-internode was divided into two parts, including the basal node segment (about 0.2 cm) and the middle internode segment (about 0.2 cm), the auxin concentration of which was also measured as above.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-019-2200-5.

Additional file 1: Table S1. Probe sequences for BR2 in situ hybridization and primers used for sequencing and qRT-PCR as well as homologous recombinant.

Additional file 2: Table S2. Plant height separation performance in F2 and BC1 populations.

Additional file 3: Figure S1. The br2 allele test for d2014.

Additional file 4: Figure S2. Comparison of certain internode length between d2014 and WT at the 12-leaf, 14-leaf, and 20-leaf stages.

Additional file 5: Figure S3. Different mutation sites of br2 alleles.

Additional file 6: Figure S4. The analysis of protein domains of PGP1-T01 and PGP1-T02.

Additional file 7: Figure S5. The morphological characteristics of the stems of d2014 and WT.

Abbreviations
S-Ft: 5-Fluorindole; ABC: ATP-binding cassette; AMs: Axillary meristems; an1: Anther ear1; ATCC: American Type Culture Collection; AUX1: AUXIN-RESISTANT1; br2: Brachytic2; BRs: Brassinosteroids; d1: Dwarf1; d2014: Dwarf2014; dv3: Dwarf3; GAs: Gibberellins; Iw: Inflorescence meristem; LAXs: AUX1-LIKEs; LC-MS: Liquid chromatography tandem mass spectrometry; Mu: Mutator; na2: Nana plant2; NBD: Nucleotide binding domain; NLS: Nuclear localization signal; PGP1: P-glycoprotein1; PINs: PINFORMEDs; qRT-PCR: Quantitative real-time PCR; SAM: Shoot apical meristem; SLs: Strigolactones; SSR: Simple sequence repeat; TMD: Transmembrane domain; VBs: Vascular bundles; vt2: Vanishing tassel2; WT: Wild type.

Acknowledgements
This work was financially supported in part by National Key Laboratory of Wheat and Maize Crop Science in Henan Agricultural University and the National Natural Science Foundation of China (No.31571684). We thank Chengdu Diversity Co. Lit (Chengdu Sichuan) for the measurement of the endogenous hormone auxin.

Authors’ contributions
YBH and JHT designed the research. XGZ, XBH, YHL, LJZ, QY, HJZ, XRH, FLZ, and LC performed the experiments. XGZ and XBH wrote the manuscript, JJZ, YBH and JHT designed the research. XGZ, XBH, YHL, LJZ, QY, HJZ, XRH, FLZ, and LC performed the experiments. XGZ and XBH wrote the manuscript, JJZ, YFH, HML, YPL, GWY and HHH revised the manuscript. All authors read and approved the final manuscript.

Funding
This work was financially supported in part by National Key Laboratory of Wheat and Maize Crop Science in Henan Agricultural University and the National Natural Science Foundation of China (No.31571684). The funding body was involved in the material creation and designing the study.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Page 14 of 16
Zhang et al. BMC Plant Biology (2019) 19:589
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