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RESEARCH PAPER

Brachytic2/*Zm*ABCB1 functions in IAA export from intercalary meristems

Anne Sophie Knöller, Joshua J. Blakeslee†, Elizabeth L. Richards, Wendy Ann Peer and Angus S. Murphy*

Purdue University, Horticulture and Landscape Architecture Department, 625 Agriculture Mall Drive, West Lafayette, IN 47907, USA

† Present address: Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, USA.
* To whom correspondence should be addressed: E-mail: murphy@purdue.edu

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Abstract

Dwarfism traits in *Zea mays* are regulated by multiple factors including the hormone auxin. Dwarf *brachytic2* (*br2*) mutants harbour lesions in the gene encoding an orthologue of *Arabidopsis thaliana* ABCB1 which functions in auxin efflux out of meristematic regions in the shoot and root. *br2* mesocotyls and coleoptiles exhibit reduced auxin transport. However, the dwarf stature of *br2* derives from shortened lower internodes whilst the upper portion of the plant is completely normal. As such, it is counter-intuitive to attribute *br2* dwarfism exclusively to reduced auxin export out of the shoot apex. *Arabidopsis abcb1* mutants exhibit only minor reductions in auxin transport and plant height unless combined with mutations in the ABCB19 auxin transporter. Phylogenetic modelling analysis excludes the possibility that BR2 is more closely related to ABCB19 which has three more closely related orthologues in maize. BR2 is expressed in nodal meristems, and analyses of auxin transport and content indicate that BR2 function in these grass-specific tissues is analogous to ABCB1 function in the shoot and root apex of *Arabidopsis*. These results indicate that ABCB1/BR2 function is conserved between dicots and monocots, but also suggests that this function must be understood in the context of the segmental organization of grass plants.

Key words: ABC transporter, *Arabidopsis*, auxin, dwarfism, maize.

Introduction

Dwarfism traits have been particularly beneficial for crop production, and the introduction of dwarf varieties of wheat and rice served as a cornerstone of the 'Green Revolution' of the late 20th century. During this period, the generation of high-yield varieties significantly increased cereal production, especially in Latin America and India (Gaud, 1968). High-yield varieties have higher nitrogen uptake capacities and exhibit increased overall growth and cell elongation. However, taller plants are more likely to lodge in response to heavy rainfall and wind, and the heavier inflorescences of high-yield elite breeds make them more susceptible to lodging. To ameliorate this negative characteristic, dwarf and semi-dwarf traits were identified and subsequently bred into these high-yield lines (Gale and Youssefian, 1985; Salamini, 2003). The most useful dwarfing traits are associated with defects in the biosynthesis or perception of the growth hormone gibberellic acid, or GA (Fig. 1; Peng *et al.*, 1999; Monna *et al.*, 2002). However, manipulation of GA biosynthesis and signal transduction has been a less effective strategy in *Zea mays* and *Sorghum bicolor*, as GA mutants in these species generally exhibit defects in the reproductive organs (Evans and Poethig, 1995; Miralles *et al.*, 1998).

Manipulation of auxin transport appears to be a more effective way to generate productive dwarfs in maize and sorghum (Fig. 1). The sorghum mutant *dwarf3* (*dw3*) harbours a defect in the gene encoding the ATP binding cassette type B (ABCB) auxin transporter SbABCB1, and a maize orthologue, *brachytic2* (*br2*), exhibits reduced seedling auxin transport, reduced mature stalk height due to shortened lower internodes, thicker stems, and altered stalk vasculature (Multani *et al.*, 2003). Brachytic2 is an orthologue of *Arabidopsis thaliana* ABCB1, which has been extensively studied in *Arabidopsis* (reviewed in Titapiwatanakun and

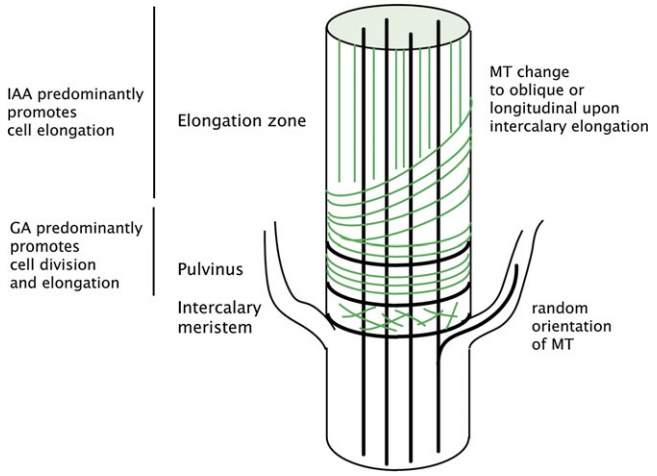

Fig. 1. Model for internode elongation in monocots. Auxin and gibberellic acid (GA) interact during cellular elongation. Both auxin and gibberellic acid promote the reorientation of microtubules (MT) from random to transverse, and suppress cell wall peroxidase activity, thereby promoting cell wall extensibility.

Murphy, 2009). ABCB1/PGP1 is expressed in shoot and root apices (Sidler *et al.*, 1998) where it functions primarily in exporting IAA from meristematic cells into long-distance polar auxin transport streams (Noh *et al.*, 2001; Geisler *et al.*, 2005; Yang and Murphy, 2009). Although *Arabidopsis* ABCB1 shares a 62% amino acid identity with maize BR2, *Arabidopsis abcb1/pgp1* mutants exhibit only a slight reduction in plant stature unless combined with a mutation in the gene encoding the ABCB19/PGP19 auxin transporter which plays a more global role in long-distance auxin movement (Noh *et al.*, 2001; Blakeslee *et al.*, 2007).

Sequence similarity and biochemical evidence support functional conservation of BR2 and *Arabidopsis* ABCB1. However, the differences observed between *Arabidopsis abcb1* and maize *br2* growth phenotypes suggest that while BR2/ABCB1 function in meristematic tissues is conserved, BR2 functions in intercalary as well as apical meristems. Although both monocots and dicots have a shoot apical meristem, in monocots the shoot apical meristem divides during development to form an additional meristem below the apex. This process is continuous until the onset of flowering and creates the intercalary meristems located in the nodes of a grass culm. Further, the vasculature in dicot stems is located in the central cylinder in a circular order, but is scattered in monocot stems. Depending on the species, the stem can also be hollow, consisting of leaf sheaths inserted into each other. The anatomical features of monocots could thus contribute to the more severe phenotypes observed in *br2* mutants.

As the maize genome was incomplete at the time of the initial characterization of *br2* (Multani *et al.*, 2003), it is also possible that BR2 is functionally more analogous to *Arabidopsis* ABCB19 than ABCB1 or that maize lacks an ABCB19 equivalent. A combination of phylogenetic, phenotypic, and physiological analyses is used here to show that BR2 is the closest orthologue of *Arabidopsis* ABCB1 and that ABCB1 function in meristematic tissues is conserved in maize and directly underlies the phenotype of the *br2* mutant.

Materials and methods

Phylogenetic analysis and synteny mapping

ABCB protein sequences were derived from TAIR (http://www.arabidopsis.org/), TIGR rice (http://rice.plantbiology.msu.edu/), NCBI (\\http://www.ncbi.nlm.nih.gov/), The Maize Sequence Organization (http://www.maizesequence.org/index.html), the Plant Genome Duplication Database (PGDD, http://chibba.agtec.uga.edu/duplication/), and the *Physcomitrella* genome organization (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1. home.html). The protein alignment file was generated with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html), using the Neighbor–Joining method. The output was set to pir and the format of the resulting file was manually changed to NEXUS format. The phylogenetic tree was generated with Mr Bayes (http://mrbayes.csit.fsu.edu/). A mixed amino acid model was used. The number of generation was set to 30 000 000; however, the 'Stopwatch' command was enabled so that iteration stops once the standard deviation of split frequencies falls below 0.01, which was the case after about 2 500 000 generations. Sample frequency was set to 1000. Genome markers were derived from TIGR rice (http://rice.plantbiology.msu.edu/) and MaizeGDB database (http://www.maizegdb.org/). For further analysis of syntenous regions, the Plant Genome Duplication Database (PGDD, http://chibba.agtec.uga.edu/duplication/) was consulted.

Plant material and growth conditions

Plants were grown in 20 cm pots filled with 1:1 v/v Turface:Pro-Mix growth media.

Greenhouse conditions were set to 23.9/18.3 °C day/night; RH 85/70% day/night (summer), 60/50% day/night (winter); natural light supplemented to 16 with HID lights, 100 μmol m^{-2} s^{-1} at the benchtop.

Maize stem sectioning

Maize was grown for 7 weeks and upper nodes (node 6 and above) were hand-sectioned using a razor blade. Sections were analysed under a binocular microscope. Nodes of eight individual plants were analysed.

Auxin transport and free IAA analysis

[^{3}H]-IAA (5 μl, 20 Ci $mmol^{-1}$, 10 μM solution in lanolin paste) was applied to the tip of 6-week maize plants. Stems were dissected in 1 cm segments, measured from the tip. This experiment was repeated 13 times with at least three plants in each replicate. Plant material was harvested simultaneously with the auxin transport assays from untreated plants. Free IAA measurements were performed as in Kim *et al.* (2007. Three repeats with three plants in each replicate were performed.

RNA extraction

Wild-type maize (*Zea mays*) was grown in soil for 6–8 weeks until four nodes were visible. The plant material was harvested by cutting the stem directly below the first node. After stripping the leaves, the stem was cut in 1 cm segments and frozen immediately in liquid nitrogen, ground using a mortar and pestle, and stored at –80 °C until RNA extraction. RNA extraction was performed with 100 mg tissue using Invitrogen plant RNA extraction reagent (Life Technologies, Carlsbad, CA, USA) supplemented with 20 μl β-mercaptoethanol ml^{-1} following the manufacturer's instructions. After the isopropanol precipitation, an additional LiCl precipitation was performed (2 M LiCl final concentration, incubation at

–20 °C for 20 min, centrifugation at 10 000 *g* at 4 °C for 20 min) to ensure complete DNA removal. This experiment was repeated twice, with three plants in each replicate.

DNase treatment, reverse transcription, and PCR

For cDNA synthesis, ~3.5 µg total RNA was treated with 0.5 U RQ1 DNAse (Promega) for 30 min at 37 °C in a total volume of 15 µl, then DNAse was inactivated with 1 µl DNAse stop solution and incubation at 80 °C for 10 min. First-strand cDNA was synthesized using oligo dT and SuperScript III reverse transcriptase (Life Technologies) according to the manufacturer's protocol. For PCR reactions (30 cycles, 94 °C 30 s, 55 °C 30 s, 72 °C 30 s) the following primers were used: BR2_2485_for (GACCCGCGGTACATGAAG), BR2_2771_rev (TTCTGGACGATGACGGAGAT), BR2_2190_for (CATCATGACGCGCAACTC), BR2_2482_rev (GCGCGTAGTAGACGCTGAG), act81/83_498_for (CCTGACTGACAGCCTGATGA), act81/83_802_rev (CGCATTTCATGATGGAGTTG).

Gibberellin quantitations

GA_{20} and GA_1 were extracted in 80% methanol from 1 g in the presence of 25 ng $^2H_2$ GA_1 and GA_{20} standards, purified by Oasis-MAX SPE (Waters, Milford, MA, USA), and analysed with an Agilent 5975 GC-MS after derivatization as in Proebsting *et al.* (1992).

Results and discussion

Phylogenetic relationship of monocot and dicot ABCB proteins

The ABC transporter superfamily in plants expanded to a greater extent than in animals, and an unrooted tree of *Arabidopsis* and rice ABC transporters shows varying degrees of duplication events in different clusters (Verrier *et al.*, 2008). The two principal *Arabidopsis* auxin efflux transporters, ABCB1 and ABCB19, each have a single homologue in rice (ABCB22 and ABCB14, respectively). By contrast, the cluster containing the reversible auxin transporter ABCB4 (Terasaka *et al.*, 2005; Cho *et al.*, 2007; Wu *et al.*, 2007; Yang and Murphy, 2009) displays a greater number of duplication events in both rice and *Arabidopsis*. A more thorough analysis of monocot and dicot ABCB sequences using a rooted tree including sequences from the moss *Physcomitrella patens* was developed. The tree was rooted using ABCC1 (MRP1) from *Arabidopsis*, which is a member of the related ABC subfamily C. The phylogenetic analysis included sequences from *Arabidopsis thaliana*, *Carica papaya*, *Populus trichocarpa*, *Vitis vinifera*, *Oryza sativa*, *Sorghum bicolor*, *Brachypodium distachyon*, *Zea mays*, and *Physcomitrella patens*. Maize ABCB sequences were identified from the released genome sequences (http://www.maizesequence.org/index.html) as well as from the NCBI high-throughput database containing genomic sequences that have not been fully assembled. These sequences were aligned with the closest rice gene in order to determine intron–exon borders and then translated into amino acid sequences. Intron–exon ordering was also examined to ensure correct assembly of the gene models and predicted amino acid sequences.

A preliminary phylogenetic analysis of plant ABCB transporters utilized *Arabidopsis* and rice sequences, the bacterial ABC transporters sav1866 and MRP1 as outgroups, and the TAP/HMTs (Transporter associated with Antigen Presentation/Heavy Metal Tolerance factor) family of transporters. These transporters differ from the MDR/PGP subgroup of ABCBs, as they are 'half-transporters' that require dimerization for proper function (Verrier *et al.*, 2008). However, in this analysis, very low bootstrap values of 0.59–0.68 were generated for a number of clades, and closer association was observed between the TAPs and the bacterial half transporter sav1866 than with MRP1. As such, the TAP/HMT proteins were removed from the analysis. Further, rice ABCB20 and ABCB21 and all identified homologues in other species that exhibit greater similarity to bacterial half transporters were removed.

The resulting tree (Fig. 2) can be organized into five clades (I–V). All five clades have 2–3 *Physcomitrella* sequences that root these clades. Clades I, II, IV, and V can be further divided into subclades (labelled a and b), suggesting an early gamma polyploidization event that preceded the split of monocots and dicots. The majority of the branch points of the tree had bootstrap values of 1.0, and the lowest observed value was 0.57. *Physcomitrella* genes are duplicated in all five clades. In some cases, an additional third homologue was found, consistent with the whole-genome duplication observed in *Physcomitrella* (Rensing *et al.*, 2008).

The principal auxin transporters in *Arabidopsis*, ABCB19 and ABCB1, are located in clades II a and b, respectively (Fig. 3). BR2 groups together with *Arabidopsis* ABCB1 and rice ABCB22 in clade IIb. Further, the BR2/ZmABCB1 locus lies in a region syntenous to OsABCB22 (see Supplementary Fig. S1 at *JXB* online), suggesting that BR2 is indeed orthologuous to *Arabidopsis* ABCB1. All monocots examined have two homologues of ABCB19, suggesting that this duplication preceded speciation. Maize has an additional third homologue. However, clade IIa lies outside the *Physcomitrella* root, suggesting that either (i) the duplicated ABCB1/19 precursor underwent subfunctionalization in the lineage of higher plants but was lost in mosses or (ii) the duplication event producing ABCB1 and ABCB19 took place in higher plants and the grouping of subclade IIa outside the *Physcomitrella* root is due to a stochastic error. The second possibility cannot be excluded without more extensive genomic information from bryophytes, mosses, and ferns. However, subclade IIb contains the ABCB19-like auxin transporters that function in long-distance transport found only in higher plants, and *Physcomitrella* has three 'short' PINs that mediate intracellular auxin homeostasis, but no 'long' PINs that function in polar auxin transport (Mravec *et al.*, 2009). This suggests that an ABCB19 precursor in mosses was subfunctionalized.

Arabidopsis ABCB19 has two homologues in rice, ABCB14 and ABCB16. Both are located on chromosome 4. In maize, three sequences with high similarity to ABCB19 were identified. Two of them, ZmABCB10-1 (chromosome 10) and ZmABCB2-1 (chromosome 2), group together with OsABCB16, while the other, ZmABCB10-2 (chromosome 2), groups with OsABCB14. This could either be the result of a tandem or whole-genome duplication, which is reported to have taken place in maize (Schnable *et al.*,

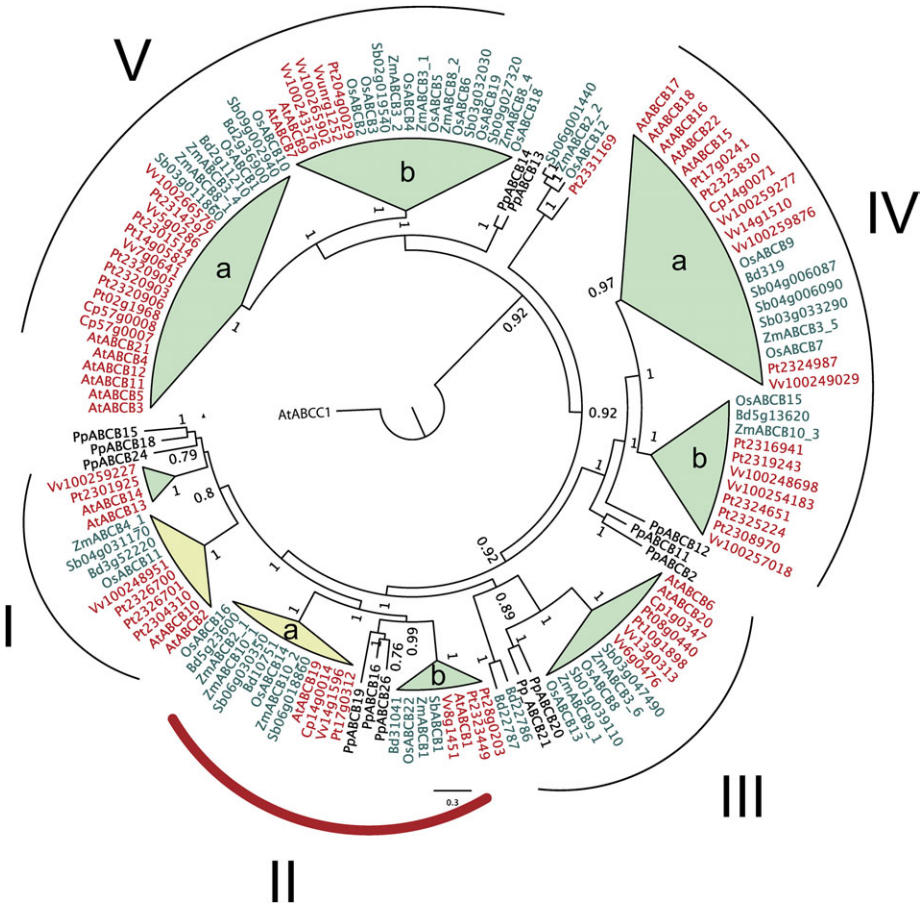

Fig. 2. Phylogenetic tree of plant ABCB transporters, including sequences from *Arabidopsis*, *Vitis*, *Populus*, *Carica*, *Sorghum*, *Zea*, *Oryza*, *Brachypodium*, *and Physcomitrella*. The phylogenetic tree can be divided into five clades (I–V). Branch numbers represent bootstrap values. Species names are colour coded for monocots (green) and dicots (red). Subclade Ib and IIa lie outside the *Physcomitrella* root (yellow shaded subclades).

2009). Synteny mapping shows that both chromosomes 2 and 10 in maize have large regions that are syntenous to rice chromosome 4 (see Supplementary Fig. S1 at *JXB* online). Thus, it is possible that maize had or has four homologues of *Arabidopsis* ABCB19, although only three homologues have been identified in gene models to date. Alternatively, the fourth homologue may have been lost or is not contained in the duplicated region. Another possibility is that the Mu8 transposon sequence that contains a fragment of the *BR2*/*ZmABCB1* gene (Multani *et al.*, 2003) interferes with expression of the maize orthologues of *Arabidopsis ABCB19*, which appear to encode functionally related auxin transporters. However, recent identification of new alleles of *br2* that do not involve a Mu8 transposon eliminates this possibility. Taken together, these results suggest that ZmABCB1 is orthologueous to *Arabidopsis* ABCB1 and that the severe dwarf phenotype of *br2* results from the monocot-specific distribution of BR2 in the monocot clade.

BR2 *functions in IAA re-export from intercalary meristems*

When [^{3}H]-IAA is applied to the upper stalk of maize B73 inbred plants, a front of labelled IAA moving through the stalk can be followed as it moves toward the root over time (Fig. 4A). In *br2*, several [^{3}H]-IAA accumulation peaks were detected in the vicinity of the nodes, and the moving front was reduced, suggesting that BR2 indeed facilitates IAA transport across the nodal/intercalary meristem region. As polar auxin transport was maintained and the amplitude of these peaks decreased at each node, it appears that shoot-to-root polar fluxes, thought to be maintained by PIN1 and ABCB19 paralogues, are still functional. Further, these results are consistent with a role of BR2 in re-export of auxin derived from the shoot apex and leaves from the intercalary meristem and node.

Free IAA measurements showed that IAA levels decreased progressively and that the IAA content in nodes and the adjacent internodes of B73 were not significantly different (Fig. 4B, P >0.05). However, in *br2*, free IAA levels in nodes were significantly higher than in adjacent internodes (P <0.01), particularly in the upper portion of the stalk, suggesting that the shoot apex is the primary source of auxin during later development. In the lower nodes, IAA levels were reduced in both nodes and internodes and, as such, differences were not significant. These results again support BR2 facilitation of IAA reloading in the intercalary meristem.

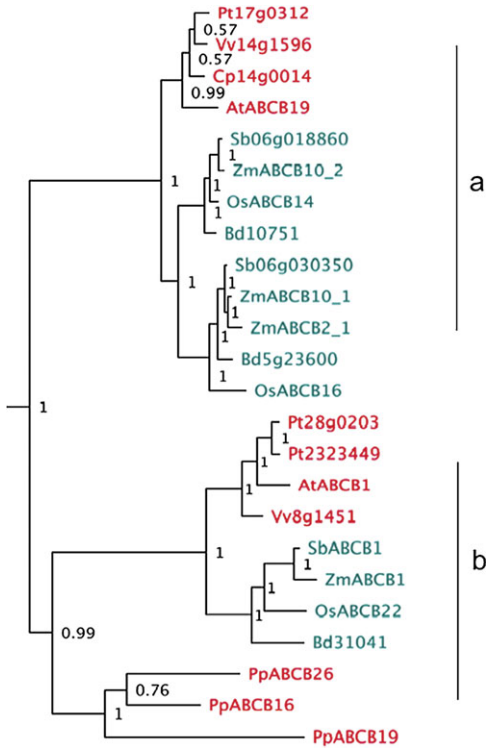

Fig. 3. Phylogenetic relationship of plant ABCB transporters in Clade II. Clade II contains the two principal auxin transporter AtABCB1 and AtABCB19 in two subclades (a and b). Branch numbers represent bootstrap values. Species names are colour coded for monocots (green) and dicots (red).

BR2 *is expressed in intercalary nodes, but not internodes*

The proposed function of BR2 in auxin loading at the nodes requires that *BR2* is expressed in these tissues, and the expression pattern of *BR2* had not been determined previously. However, extraction of high quality RNA from the lignified maize stem poses a major technical challenge. Sectioning of nodal regions for RNA *in situ* hybridization is similarly difficult. Unlike leaves, reproductive tissues, and roots, the nodal region is extremely hard and difficult to grind without thawing of the tissue. In order to obtain the highest quality of RNA possible, several methods of RNA extraction were tested. Although a preferred method was determined, RNA quality was found to vary to an extent that quantitative real-time PCR was not meaningful (see Supplementary Fig. S2 at *JXB* online). Thus, repetitive non-quantitative RT-PCR was performed to determine whether BR2 was expressed in a particular tissue and RNA quality was assessed by RT-PCR analysis of actin transcripts. For these experiments, tissues from three nodes and internodes starting from node 3 were used. In all cases, *BR2* expression was detected in the nodes, but not in the internodes (Fig. 4C).

Altered morphology of br2 *leaves and nodes*

Leaves originating in lower truncated nodes of *br2* (leaf 3 through leaf 7) were slimmer and more curled compared with the equivalent leaves in B73 (Fig. 5A). Leaves originating in the upper nodes were broader than those in

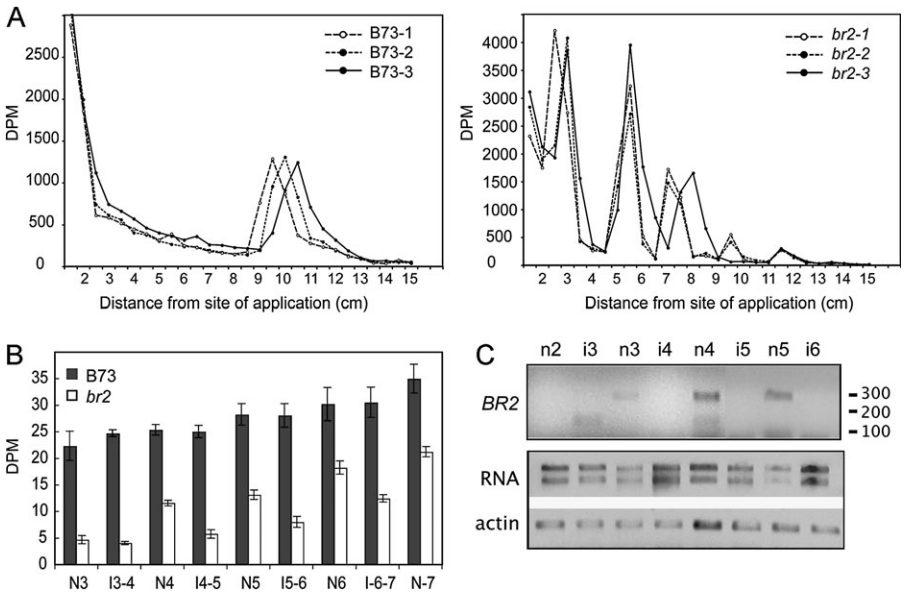

Fig. 4. BR2 functions in auxin transport in the nodal region. (A) [^{3}H]-IAA was applied to the tip of B73 and *br2* plants and 0.5 cm sections were harvested (distance measured from the top). For each experiment, a minimum of three plants were used for each genotype and the experiment was repeated 13 times. As the final heights of the plants were not uniform, the three biological repeats were plotted individually instead of calculating a mean. [^{3}H]-IAA levels in different sections of B73 plants (left), [^{3}H]-IAA levels in different sections of *br2* plants (right). (B) Free IAA determination in B73 and *br2*. Plant material was harvested as in (A). Data are means ±standard deviations. (C) *BR2* is expressed in the nodal region (n), but not in the adjacent internodes (i). Total RNA and actin controls are shown.

B73. As might be expected, the leaf width phenotypes were more pronounced under low light conditions associated with reduced maize growth. The leaf phenotypes observed in *br2* are consistent with altered auxin distribution. B73 treated with auxin transport inhibitors and the *rough sheath 2* (*rs2*) mutant, in which auxin transport is reduced due to a defect in a myb-like regulator of *KNOX* homeobox genes, exhibit similar leaf curling and size reduction (Tsiantis *et al.*, 1999).

Cross-sections of *br2* stems show increased vasculature with altered distribution (Multani *et al.*, 2003). At the nodal plexus, a portion of the vertical vasculature of the stem is redirected horizontally and forms the vasculature of the corresponding leaf. The number of vasculature bundles in the nodal plexus of *br2* was found to be higher in node 7 and above (Fig. 5B). A similar pattern appears to exist in lower nodes, but could not be thoroughly analysed due to the difficulty of cutting woody nodes longitudinally. Together, these data strongly suggest that altered auxin transport mediated by BR2 in the intercalary meristem/node region underlies the altered leaf morphology observed in *br2*. Other downstream processes such as the regulation of cell wall extensibility and lignification by peroxidases may be influenced by altered auxin movement in *br2*, as cell wall peroxidise activity has been shown to be altered in the sorghum *dwarf3*/*Sbabcb1* mutant (Schertz *et al.*, 1971).

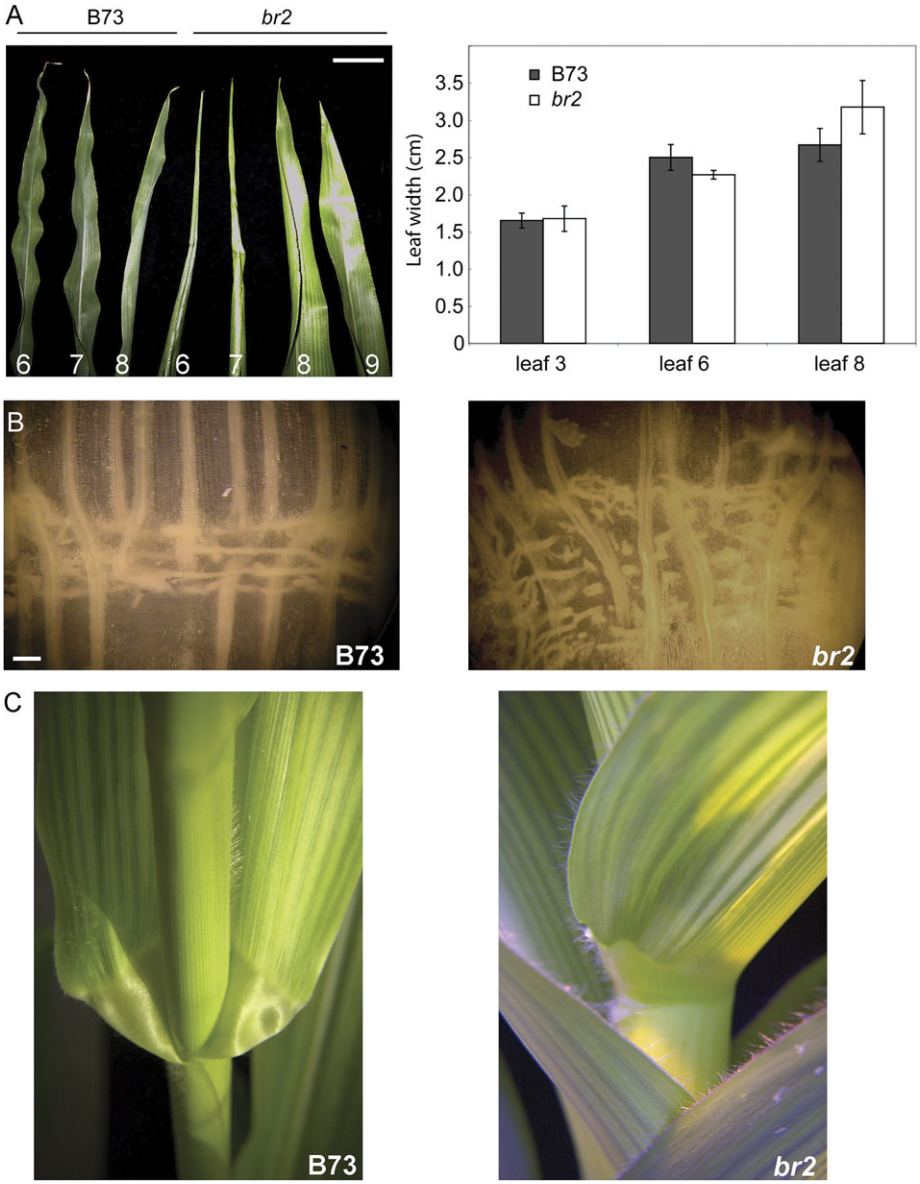

Fig. 5. The maize mutant *br2* shows altered leaf development and altered vasculature in nodes immediately above shortened internodes. (A) Upper leaves of *br2* are wider than in B73, whereas lower leaves are narrower and curled. This phenotype is more pronounced under reduced light and shorter day conditions. (B) Longitudinal section of node 7 in B73 and *br2*. Nodes above node 6 displayed a similar pattern. (C) Leaf sheath and ligule in B73 and *br2*. Size bar: (A) 3 mm; (B) 100 μm.

Interactions of auxin transport with GA signalling in br2 *internodes*

It is quite possible that reduced internode elongation in *br2* involves interactions with GA signalling, as IAA produced in the barley shoot apex is necessary for GA_1 synthesis and growth promotion in the nodal region itself (Wolbang *et al.*, 2004). However, GA application does not rescue *br2* growth phenotypes (Multani *et al.*, 2003). Further, elongation in etiolated maize seedlings has been shown to be GA-dependent (Rood *et al.*, 1986; Markelz *et al.*, 2003), but *br2* seedlings elongated in a manner similar to B73 under dark growth conditions and the GA biosynthetic mutants *dwarf2*, *dwarf3*, and *dwarf5* did not (data not shown). Although an exhaustive analysis of GA speciation was not performed, no significant differences in GA_1 or GA_{20} levels in nodes and internodes could be detected. Taken together, these results suggest that GA plays a limited role in reduced internode elongation in *br2*.

Conclusion

The results presented here indicate that the function of ABCB1 in exporting auxin from meristematic regions is conserved in monocots and dicots, despite the pronounced differences observed in loss-of-function mutants. The more pronounced phenotypes associated with loss of *ABCB1* function in maize compared with *Arabidopsis* can be attributed primarily to ABCB1-mediated auxin export in the maize intercalary meristem. However, the diversification of ABC transporters in plants (Verrier *et al.*, 2008) suggests that caution should be exercised in assuming that sequence homology between other ABCB transporters indicates conserved function. The central role that auxin transport plays in plant evolution and development suggests that functional conservation of ABCB1 and ABCB19 auxin transporters could be an exception rather than the rule.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Synteny mapping of maize chromosomes 1, 2, and 10 to rice chromosomes.

Fig. S2. Comparison of different RNA extraction techniques in maize.

Acknowledgement

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