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# BBX proteins in green plants: Insights into their evolution, structure, feature and functional diversification

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ABSTRACT

The B-box domain is conserved in a large number of proteins involved in cell growth control, differentiation and transcriptional regulation among animal and plant species. In *Arabidopsis thaliana*, some works have found that B-box proteins (BBX) play central developmental functions in flowering, light and abiotic stress signaling. Despite the functional importance of this protein family, evolutionary and structural relationships of BBX proteins have not been extensively investigated in the plant kingdom. Using a phylogenetic approach, we conducted a comprehensive evolutionary analysis of the BBX protein family in twelve plant species (four green algae, one moss, one lycophyte, three monocots and three dicots). The analysis classified 214 BBX proteins into five structure groups, which evolved independently at early stages of green plant evolution. We showed that the B-box consensus sequences of each structure groups retained a common and conserved domain topology. Furthermore, we identified seven novel motifs specific to each structure group and a valine–proline (VP) pair conserved at the C-terminus domain in some BBX proteins suggesting that they are required for protein–protein interactions. As it has been documented in mammalian systems, we also found monopartite and bipartite amino acid sequences at the C-terminus domain that could function as nuclear localization signals (NLSs). The five BBX structure groups evolved constrained by the conservation of amino acid sequences in the two B-boxes, but radiating variation into NLSs and novel motifs of each structural group. We suggest that these features are the functional basis for the BBX protein diversity in green plants.



## 1. Introduction

The B-box domain is found in more than 1500 proteins of multicellular species and some unicellular eukaryotes (Meroni and Diez-Roux, 2005). In animals, the B-box is often found together with RING finger and coiled-coil domains, forming tripartite motif proteins named TRIM/RBCC. The TRIM/RBCC family includes a large number of proteins involved in diverse cellular processes like apoptosis, cell cycle regulation and viral response (Meroni and Diez-Roux, 2005). The B-box of some TRIM/RBCC proteins functions as a protein–protein interaction domain and is required for substrate recognition (Meroni and Diez-Roux, 2005; Torok and Etkin, 2001). Other functions of the B-box domain involve localization in nuclear bodies and transcriptional regulation (Beenders et al., 2007; Borden et al., 1996). This domain is present in the N-terminus of the proteins as a single B-box or tandem repeats designated as B-box1 (B1) and B-box2 (B2) (Massiah et al., 2006, 2007; Short and Cox, 2006). Despite the functional importance of TRIM/RBCC proteins in animals, they are absolutely absent in plants.

In plants, B-box (BBX) proteins regulate plant development. For example, CO/AtBBX1 is a central player in the photoperiod control of flowering in *Arabidopsis thaliana* plants (Onouchi et al., 2000; Samach et al., 2000). *co* mutants flower late only under long days, whereas *CO*-over-expressing plants of *A. thaliana* flower early in both long and short days (Putterill et al., 1995; Suarez-Lopez et al., 2001). Other AtBBX proteins with double B-box and CCT domains, such as COL3/AtBBX4 and COL9/AtBBX7, also regulate flowering time (Cheng and Wang, 2005; Datta et al., 2006). Besides, AtBBX proteins with only B-box domains are involved in the control of plant development by light and other abiotic stress factors. The characterization of *A. thaliana* mutants has revealed that DBB1b/AtBBX19, STO/AtBBX24 and STH/AtBBX25 are negative regulators (Datta et al., 2006; Gangappa et al., 2013, in press; Indorf et al., 2007; Kumagai et al., 2008), while DBB1a/AtBBX18, STH2/AtBBX21 and STH3/AtBBX22 act as positive regulators of seedling de-etiolation processes (Datta et al., 2007, 2008; Kumagai et al., 2008). Very recently, experiments in shaded environments have demonstrated that STH2/AtBBX21 is also involved in the fine-tuning of shade avoidance responses (Crocco et al., 2010, 2011). Additionally, STO/AtBBX24 is implicated in saline stress and UV-B photomorphogenesis responses (Indorf et al., 2007; Jiang et al., 2012;

*Abbreviations:* BBX, B-box proteins; VP, valine–proline; NLS, nuclear localization signal; CO, CONSTANS; COL, CO-like.

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Nagaoka and Takano, 2003). Some BBX proteins can also act as helpers of other BBX proteins. For example, Holtan et al. (2012) have recently demonstrated that AtBBX32 is a transcriptional modulator of STH2/AtBBX21 action in the light signaling pathway.

The BBX proteins in plants are characterized by having one or two B-box domains in the N-terminus, and, in some cases, a CCT domain in the C-terminus (Griffiths et al., 2003; Robson et al., 2001). Recently, a phylogenetic analysis performed in *A. thaliana* has found that BBX proteins are encoded by a gene family of 32 members named from AtBBX1 to AtBBX32 (Khanna et al., 2009). This foundational work classified AtBBX proteins into five structure groups (I–V). The AtBBX members of structure group I (AtBBX1 to AtBBX6) contain two B-boxes in tandem (B1 and B2) and a CCT domain, being the B1 domain located N-terminal to the B2 domain and separated by 5 to 20 residues. The AtBBX members of structure group II (AtBBX7 to AtBBX13) contain B1, B2, and CCT domains, as those of structure group I, with some differences at their consensus sequences of the B2 domain (Chang et al., 2008). The AtBBX members of structure group III (AtBBX14 to AtBBX17) contain a single B-box domain in association with a CCT domain. The B-box proteins of structure group IV (AtBBX18 to AtBBX25) have the B1 and B2 domains, but not the CCT domain, whereas the protein members of structure group V (AtBBX26 to AtBBX32) carry a single B-box domain (Khanna et al., 2009). Clustering of BBX proteins into five structure groups was also supported by a recent study in rice (Huang et al., 2012).

Although the study of BBX proteins in plant development is a growing area of recent interest, the evolutionary relationships of the members of without "the" BBX family among different species have not yet been studied. Thus, in the present work, we carried out phylogenetic and structural analyses using the sequence information of 214 BBX proteins that belong to twelve green plant species (four green algae, one moss, one lycophyte, three monocot and three dicot species). Based on the evolutionary origins and structural protein changes, we discuss the functional diversity of BBX proteins of land plants.

## 2. Materials and methods

### 2.1. Identification of sequences and domains of BBX proteins across green plants

The KEGG (http://www.genome.ad.jp/kegg/) nucleotide and protein sequence databases for fully sequenced genomes were scanned for proteins related to AtBBX described in *Arabidopsis* (Khanna et al., 2009), using either BLASTp (protein databases) or tBLASTn (nucleotide databases). After initial sequence collection, the full BBX proteins of each species were used for the classification of protein sequences into paralog and ortholog clusters using sequence similarity profiles of KEGG/SSDB. This allowed enhancing the chances of finding sequences related to particular divergent BBX proteins and of better defining the range of organisms containing these proteins. The amino acid sequence data and the information of B-box domain locations were taken from KEGG, together with the annotations made by the original authors, SWISS-PROT, and KEGG itself. Each predicted BBX protein sequence was confirmed by Pfam search for the presence of a B-box signature (E-value < 0.05). Only 23 of the 30 OsBBX proteins described in rice by Huang et al. (2012) have a B-box signature with an E-value < 0.05, while the 32 AtBBX proteins described in *Arabidopsis* by Khanna et al. (2009) fulfill this criteria. The complete amino acid sequence of all BBX proteins can be found in Supplemental Data 1. The BBX proteins were renamed according to the result of the phylogenetic analysis for each species (Supplemental Data 2, 3).

### 2.2. B-box consensus sequences

The B-box motifs that belong to each structure group were detected with GLAM2 software (Frith et al., 2008), using the extracted B-box sequences of each BBX protein predicted by Pfam (E-value < 0.05). As some BBX proteins have a double B-box domain, we used the following criteria to designate them: the first B-box that appeared within the protein in the N-terminal position was called B1 and the second B-box termed B2.

### 2.3. Phylogenetic analysis

The alignment of the full length BBX protein and B-box domain sequences was performed in ClustalW (Thompson et al., 1997) using standard settings (Gonnet weight matrix, gap opening = 10 and gap extension = 0.2) and was adjusted by visual inspection. The full-length amino acid sequence alignment of BBX proteins is documented in Supplemental Data 4 and the B-box domain alignment can be found in Supplemental Data 6. The model of protein evolution that best fits the protein sequence data was selected using the program MEGA 5.01 (Tamura et al., 2011). The best-scoring model for the full-length BBX protein alignment was the Dayhoff probability model with rate variation among sites calculated as a gamma distribution (+G), and the best-scoring model for the B-box domain alignment was JTT probability model with rate variation among sites calculated as a gamma distribution (+G). Bayesian phylogenetic analyses on aligned full-length BBX sequences were performed with MrBayes v. 3.1.2 setting a MCMC algorithm (Ronquist and Huelsenbeck, 2003). Two independent runs were computed for 2,000,000 generations with a burn-in of 5000 trees in order to reach acceptable standard deviation of split frequencies. Trees were sampled from each chain every 100 generations. In addition, a neighbor-joining (NJ) tree was obtained using MEGA 5.01 (Tamura et al., 2011) (Supplemental Data 5). Maximum likelihood analyses on aligned B1 and B2 sequences were performed with MEGA 5.01 (Tamura et al., 2011). The evolutionary distances were computed using the JTT matrix-based method with a gamma distribution (shape parameter = 1). The bootstrap consensus tree inferred from 7000 replicates (Supplemental Data 7). In addition, a phylogenetic maximum likelihood tree of B1 and B2 sequences was made using the PhyML3.0 algorithm implemented through the web-based interface available at http://www.atgc-montpellier.fr/phyml/, using LG substitution model (Guindon et al., 2010) (Supplemental Data 7). Phylogenetic trees were visualized using the program MEGA 5.01 (Tamura et al., 2011).

### 2.4. Detection of conserved motifs

The MEME software (Bailey et al., 2009) was used to discover patterns in the complete amino acid sequences of plant BBX proteins. We performed a search between BBX proteins of the same structure group. Each motif was individually checked so that incorrect or insignificant matches were discarded.

## 3. Results

### 3.1. Identification and global phylogenetic analysis of BBX proteins in green plants

Previous phylogenetic analyses of BBX proteins were based on the genome sequences of *A. thaliana* (At) and *Oryza sativa* (Os) (Huang et al., 2012; Khanna et al., 2009). These foundational works generated a useful but a limited phylogenetic framework for the classification of BBX proteins in angiosperms. Our work includes twelve species and provides insight about the early diversity of this family in green plants. Besides *A. thaliana* and *O. sativa* species, we extended the phylogenetic analysis to the complete genomes of ten species including four algae [*Volvox carteri* (Vc), *Chlamydomonas reinhardtii* (Cr), *Ostreococcus tauri* (Ot) and *Ostreococcus lucimarinus* (Ol)], one moss [*Physcomitrella patens* (Pp)], one lycophyta [*Selaginella moellendorffii* (Sm)] and four additional angiosperms [*Zea mays* (Zm), *Brachypodium distachyon* (Bd), *Ricinus communis* (Rc) and *Populus trichocarpa* (Pt)]. In addition to 32 and 23 BBX proteins of *A. thaliana* and *O. sativa*, we identified 159 BBX proteins:

39 belong to *P. trichocarpa* (cottonwood), 22 to *R. communis* (castor oil plant), 23 to *Z. mays* (maize), 26 to *B. distachyon*, 15 to *S. moellendorffii* (spikemoss), 24 to *P. patens*, 2 to *V. carteri*, 3 to *O. lucimarinus*, 4 to *O. tauri* and 1 to *C. reinhardtii* (Table 1 and Supplemental Data 3). Before conducting a comprehensive phylogenetic analysis, we organized their nomenclature performing a Bayesian inference phylogenetic analysis for each individual species (maize, rice, *P. patents* and *P. trichocarpa*). We adopted the *Arabidopsis* BBX nomenclature proposed by Khanna et al. (2009). For each species, we listed the number of BBX proteins according to their position in their corresponding phylogenetic tree (Supplemental Data 2). We grouped the correspondence between the BBX nomenclature with the accession number, the protein length and the coordinates of the B-box domains (Supplemental Data 3).

In order to investigate the structural diversification of BBX proteins of green plants we carried out a global phylogenetic analysis with the full-length of 214 BBX proteins of the twelve species. Proteins were completely aligned and a Bayesian inference phylogenetic tree was constructed using MrBayes (Ronquist and Huelsenbeck, 2003; Supplemental Data 4). Based on the topology structure of the phylogenetic tree, branch lengths and clade support values, we classified BBX proteins into five structure groups based on the Bayesian tree (Fig. 1). The structure groups identified here were mostly consistent with those found in *A. thaliana* and rice (Huang et al., 2012; Khanna et al., 2009). Structure groups I and II corresponded to BBX proteins with double B-box + CCT domains (B1 + B2 + CCT). Structure group III included BBX proteins with a single B-box + CCT (B1 + CCT) domains. Structure group IV contained BBX members with double B-box (B1 + B2) and structure group V involved BBX members with a single B-box domain. The green algae OlBBX1, OtBBX2, OtBBX3 and VcBBX2 clustered together outside of the structure groups described above (Fig. 1). Some BBX proteins showed specific features within the phylogenetic pattern. For example, ZmBBX7 and BdBBX11 were grouped into structure group II but both of them lacked one B-box domain, while AtBBX26 belongs to structure group III but contains a single B-box (Fig. 1, Supplemental Data 4). These results suggest that some BBX proteins can lost a domain in a recent evolutionary event, but conserve other common characteristics of their structure group. Interestingly, the phylogenetic analysis showed that BBX proteins that belong to the same structure group were classified by amino acid similarity, and secondarily by the structure organization of the B-box and CCT domains.

### 3.2. The B-box domains evolved independently in different structure groups

To understand the evolutionary origin of the B-box domains in green plants, the aligned B1 and B2 domain sequences of the 214 BBX proteins were also used for phylogenetic analysis (Supplemental Data 6) and similar overall patterns compared with the full-length BBX protein sequences were identified: BBX proteins of the same structure group share a high sequence similarity into B1 and B2 domains (Fig. 2; Supplemental Data 7).

Some previous studies demonstrated that the B-box domains of *A. thaliana* are necessary for protein–protein interactions and transcription activity (Datta et al., 2006, 2007, 2008; Koornneef et al., 1991; Robson et al., 2001). Taking these observations into consideration, we performed an analysis to find the B1 and B2 conserved sequences for each structure group. We identified the B1 and B2 motifs belonging to each structure group using the GLAM2 software (Supplemental Data 8). This structural analysis showed a high sequence similarity between the B1 domains of double B-box structure groups (i.e., I, II and IV) and the single B-box of structure groups III and V (Fig. 3a), which is also supported by the phylogenetic tree of B-box domains (Fig. 2). Besides, the B1 consensus domain of structure group V had an amino acid less in the 7th position than in the other structure groups (Fig. 3). However by aligning the five B1 consensus domains, we found that the number of cysteine residues is conserved and that the topology among the BBX proteins of the five structure groups is retained (Fig. 3b). Although the topology of the B2 domain was retained, the B2 consensus domain showed a low percent sequence identity between structure groups I, II and IV (Fig. 3). This is consistent with the idea that the B2 domain has an early evolutionary origin compared to the B1 domain and that the B2 domain of structure group I arose independently with respect to B2 domain of structure groups II and IV (Fig. 2). Regardless of the structure group they belong to, the domain topology generated by cysteine and other amino acids is preserved between the B1 and B2 consensus sequences (Fig. 3b), suggesting that the conservation of the topology is essential for the molecular function of this domain. Interestingly, there are some evidences indicating that amino acid alterations in the consensus sequence of B1 and B2 produce dysfunctional proteins (*co-3*, *co-6*, *sth3-D20*, *sth3-D72* and *sth3-D81*; see Fig. 3b), with consequences in plant development (Datta et al., 2008; Robson et al., 2001). The fact that the consensus B-box domains are highly conserved in green plants suggests that the BBX functional diversification could be given by conserved sequences outside of the B-box domain.

### 3.3. Conserved motifs in BBX proteins outside the B-box domains

Regarding previous results, we carried out an analysis to search other conserved protein motifs flanking the B-box domains by using the 214 full-length amino acid sequences of BBX proteins. Different motifs of 10 to 40 residues were detected by the MEME suite software (Bailey et al., 2009). Seven novel motifs (M1 to M7) were detected in a conserved position, each shared by the BBX members of the same structure group (Fig. 4a; Supplemental Data 9). Motifs M1 and M2 are specific to the BBX members of structure group I (shared by 82 and 77% of the BBX members, respectively), M3 to those of structure group II (73%), M4 and M5 to those of structure group III (83 and 67%, respectively) and M6 and M7 to those of structure group IV (64 and 98%, respectively) (Fig. 4b). Outside the B-box and CCT domains, we did not find any common motif for the BBX proteins of structure group V. The M1 motif belong to BBX members of structure group I contains a conserved valine next to a proline (VP pair). Previous studies have demonstrated that the VP pair is critical for BBX protein–protein interaction (Datta et al., 2006; Holm and Deng, 1999; Holm et al., 2001). Interestingly, we found that the VP pair was also conserved at the C-terminus in 67, 64 and 24% of the BBX members of structure groups III, IV and V, respectively (Fig. 4c, Supplemental Data 10).

### 3.4. The C-terminus domain of BBX proteins has nuclear localization signals (NLSs)

In both plants and animals, different proteins are targeted to the nucleus by NLSs, usually located at the C-terminus (Dingwall and Laskey, 2001; Robbins et al., 1991). The best characterized NLSs consist of short stretches of basic amino acids and can be classified into two

**Table 1**
Number of BBX proteins in the twelve representative species of green plants.

| Taxa | Species | | No. BBX proteins |
|---|---|---|---|
| | Name | Abbreviation | |
| Green algae | *Chlamydomonas reinhardtii* | CrBBX | 1 |
| | *Ostreococcus lucimarinus* | OlBBX | 3 |
| | *Ostreococcus tauri* | OtBBX | 4 |
| | *Volvox carteri* | VcBBX | 2 |
| Lycopsida | *Selaginella moellendorffii* (spikemoss) | SmBBX | 15 |
| Mosses | *Physcomitrella patens* | PpBBX | 24 |
| Monocots | *Oryza sativa* (rice) | OsBBX | 23 |
| | *Zea mays* (maize) | ZmBBX | 23 |
| | *Brachypodium distachyon* | BdBBX | 27 |
| Eudicots | *Arabidopsis thaliana* | AtBBX | 32 |
| | *Populus trichocarpa* (cottonwood) | PtBBX | 39 |
| | *Ricinus communis* | RcBBX | 22 |

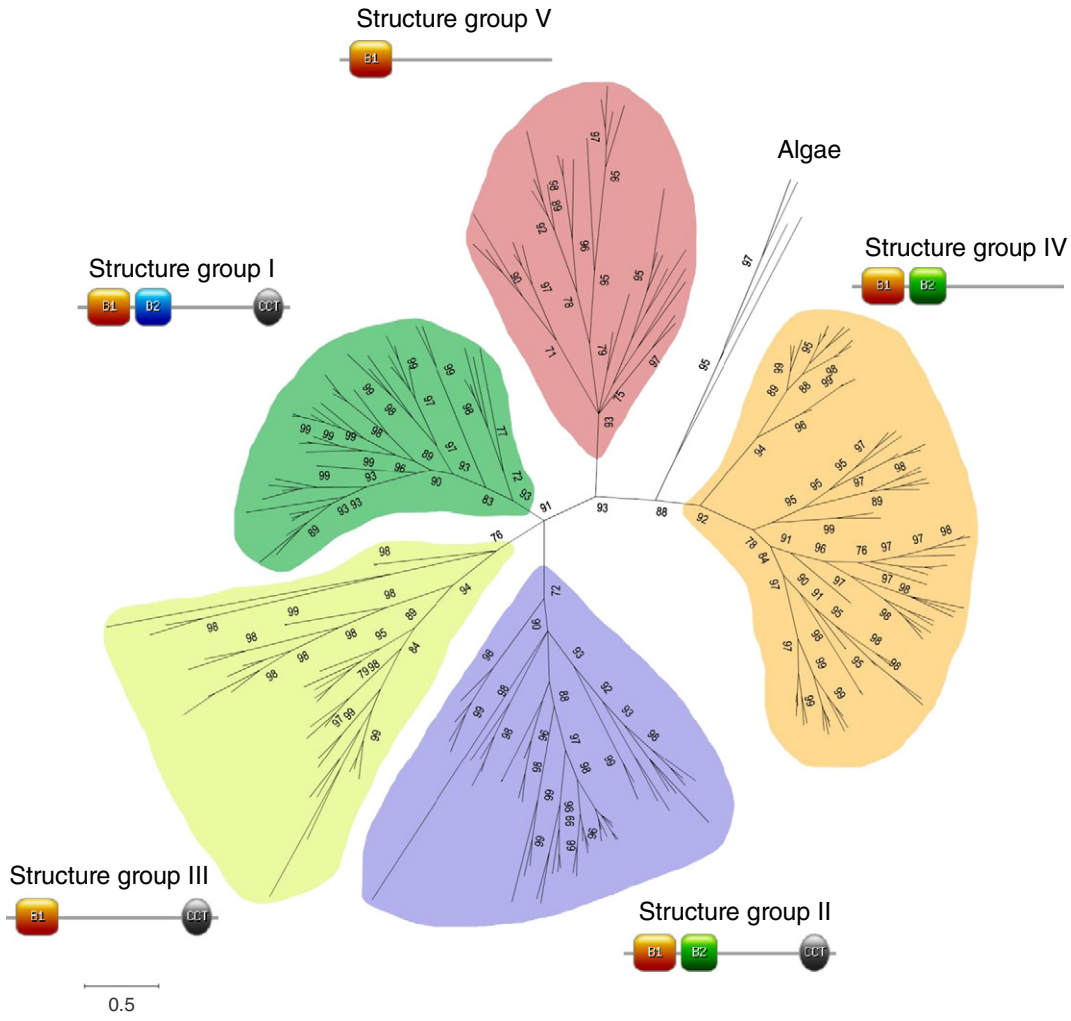



**Fig. 1.** Unrooted Bayesian inference phylogenetic tree for 214 BBX proteins. Full-length proteins of the 214 BBX members were aligned using ClustalW (Thompson et al., 1997) (Supplemental Data 4). The best-scoring model for the BBX full-length protein alignment was the Dayhoff probability model, with a rate variation among sites calculated as a gamma distribution (+G). MrBayes (Ronquist and Huelsenbeck, 2003) was used to construct a Bayesian inference phylogenetic tree with 2,000,000 generations. Bootstrap values (>50%) for this tree are shown on respective branches. The 214 BBX full-length proteins were grouped into five different structure groups (I to IV). The correspondence of each BBX protein to their particular structure group is indicated in the Supplemental Data 3. A similar tree was obtained with MEGA 5.01 (Tamura et al., 2011) using the neighbor-joining (NJ) method (Supplemental Data 5). Schematic diagram of protein domain structures is indicated for each structure group. B1, B-box domain type 1; B2, B-box domain type 2; CCT domain.

major groups: monopartite and bipartite NLSs. Monopartite NLSs contain K-(K/R)-X-(K/R) as a putative consensus sequence, where X indicates any amino acid (Lange et al., 2007). The putative consensus sequence of bipartite NLSs is (K/R)-(K/R)-X10-12-(K/R), being the two clusters of basic residues separated by a 10–12 amino acid linker (Dingwall and Laskey, 2001; Xu et al., 2010). The NLSs of plants consist of the LGKR-(K/R)-(W/F/Y) core sequence containing six amino acids (Conti et al., 1998). We performed a search for NLS motifs in the C-terminus of the 214 BBX members of the five structure groups and the LGKR-(K/R)-(W/F/Y) core sequence was absent in the BBX proteins analyzed in this study. However, we found a novel and consensus bipartite NLSs mapped into the CCT domain in 100, 89 and 93% BBX members of structure groups I, II and III, respectively (Supplemental Data 11). The bipartite NLSs consisted of an R-K-X11-R consensus sequence covering 33% in the second half sequence of the CCT domain (Fig. 5). On the other hand, 54% of the BBX members of structure group IV contain monopartite NLSs with K-K-X-R or K-R-X-R as consensus sequences; and only 16% of the BBX members have bipartite NLSs with K-R-X10-R as consensus sequences (Supplemental Data 13). Finally, 9% and 39% of the BBX members of structure group V contain monopartite (K-R-X-R) and bipartite (R-R-X10-11-R) NLSs, respectively (Supplemental Data 13).

## 4. Discussion

Despite the conspicuous presence of BBX proteins in prokaryotes and eukaryotes, the function and evolution of these proteins have not been thoroughly studied in the plant kingdom. To investigate the evolutionary relationships of the BBX protein family, in the present study we carried out a comprehensive phylogenetic and structural analyses of 214 BBX proteins from twelve representative species including green algae, a moss and a lycophyta. In spite of differences at protein structure level, the BBX proteins of divergent species are represented in the five structure groups (see Fig. 1). The presence of BBX proteins in algae like *V. carteri*, *C. reinhardtii*, *O. tauris* and *O. lucimarinus* suggests that the earliest BBX proteins in photosynthetic organisms originated ~one billion years ago (Peers and Niyogi, 2008), and after that, the BBX protein family expanded during the colonization into land plants probably in the upper Silurian at least 450 Ma ago (Kenrick and Crane, 1997). The conservation and expansion of these structure groups during the course of evolution suggest that this multi-protein family may have important physiological roles during adaptation of land plants. In fact, the biological activity of BBX proteins with B-box and CCT domains is preserved among different species of green plants.

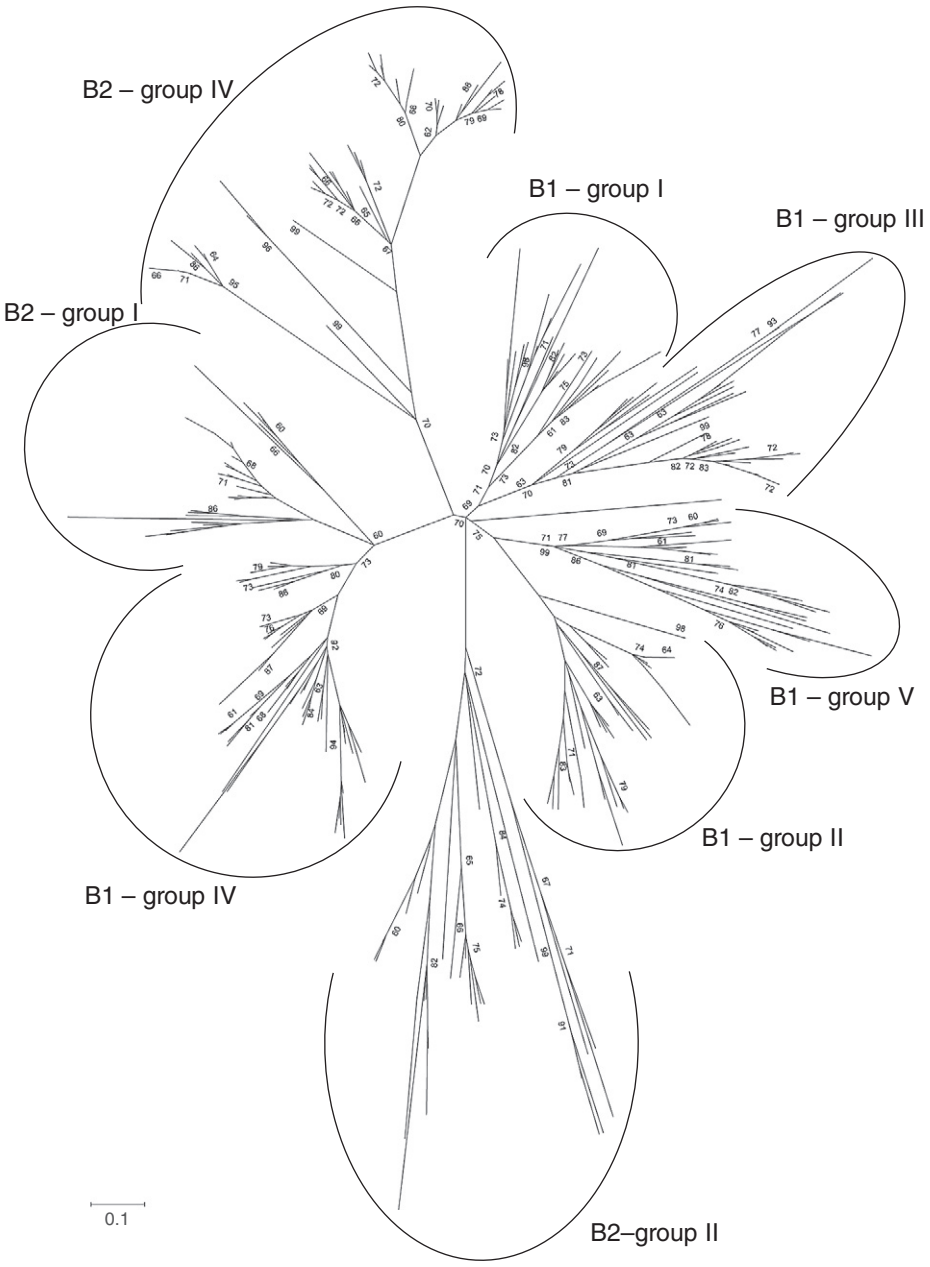



**Fig. 2.** Unrooted Bayesian inference phylogenetic tree for B-box domains. Evolutionary analyses were conducted in MEGA 5.01 (Tamura et al., 2011) using the neighbor-joining method. The evolutionary distances were computed using the JTT matrix-based method with a gamma distribution (shape parameter = 1). The bootstrap consensus tree inferred from 7000 replicates is taken to represent the evolutionary history of the taxa analyzed. Bootstrap values (>50%) for this tree are shown on respective branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 340 amino acid sequences. A similar tree was obtained using PhyMol (Guindon et al., 2010) (Supplemental Data 7).

The first BBX identified in *Arabidopsis*, AtCO/AtBBX1, is a photoperiodic flowering-time protein in which the overexpression induces early flowering, and mutations delay flowering (Putterill et al., 1995; Suarez-Lopez et al., 2001). In rice, OsCO3/OsBBX2 acts as a floral repressor in the regulation of flowering under short day (SD) conditions, whereas an orthologous counterpart of AtCO/AtBBX1, Hd1/OsBBX1, shows a dual function promoting flowering in SD and inhibits flowering in long days (Hayama et al., 2003; Kim et al., 2008). Beyond the species used in this work, other BBX proteins with B-box and CCT domains were recently isolated and characterized in green plants. Nemoto et al. (2003) showed that TaHd1-1 gene in wheat complements the bifunctional role of the rice Hd1/OsBBX1. In potato, StCO is involved in photoperiodic tuberization in a graft-transmissible manner (González-Schain et al., 2012). BvCOL1 was isolated from sugar beet and the heterologous overexpression of this gene in the *co-2* mutant of *Arabidopsis* rescued the wild-type flowering phenotype suggesting functional equivalence with AtCO/AtBBX1 (Chia et al., 2008). Unlike BBX members with B-box and CCT domain, other BBX proteins belonging to the structure groups IV and V show a wide functional diversity, which have been associated with photomorphogenetic process and abiotic stress responses. For example, AtBBX24/STO is involved in UV-B signaling pathway (Jiang et al., 2012), photomorphogenesis seedling de-etiolation and saline stress responses (Indorf et al., 2007), whereas AtBBX21/STH2/LHUS and AtBBX22/STH3 are implicated in seedling photomorphogenesis (Datta et al., 2007, 2008) and shade avoidance responses (Crocco et al., 2010), while AtBBX18 negatively regulates thermotolerance in *Arabidopsis* (Wang et al., 2013). Yamawaki et al. (2011) demonstrated that heterologous expression of some moss BBX genes of the structure

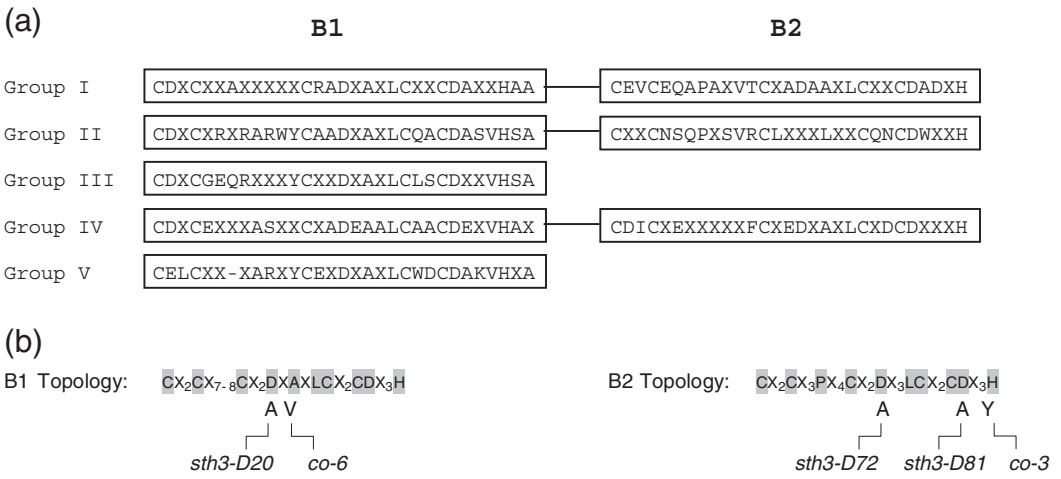
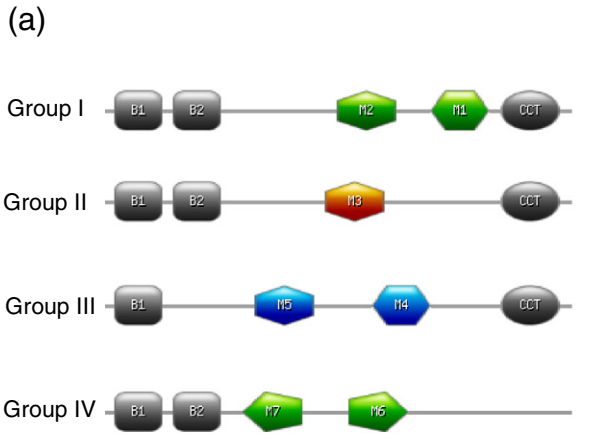



**Fig. 3.** B-box domains evolve independently in each structure group. ClustalW software (Thompson et al., 1997) was applied to align the amino acid sequences of the B1 and B2 domains belonging to structure groups I, II, III, IV and V. B1 and B2 ancestral topologies were inferred from the amino acids conserved in all structural groups. X represents any amino acid, – represents a gap. The position and type of amino acid substitution in mutant lines (*co-3*, *co-6*, *sth3-D20*, *sth3-D72* and *sth3-D81*) are indicated over the topology B-box structure.

group IV is functional for the regulation of light signaling processes in *A. thaliana* plants. These results indicate that plant developmental processes regulated by BBX proteins could be common and conserved between an ancestral lineage of moss and dicot species.

In this work we show the identity between the B-box domains of each structure group and found the most probable B1 and B2 ancestral sequences of green plants. The retention of structural topology between B1 and B2 ancestral sequences suggests that both domains have discrete molecular functions in the plant development (Fig. 3b). Previous experimental evidences indicate that an amino acid alteration in the B1 and/or B2 ancestral sequences produce dysfunctional proteins that affect plant development. Point amino acid mutations on B1 (Ala by Val, see *co-6* mutation in Fig. 3b) or B2 (His by Tyr, see *co-3* mutation in Fig. 3b) affect the proper function of the CO/AtBBX1 protein in flowering time (Koornneef et al., 1991; Robson et al., 2001). Datta et al. (2008) showed that punctual amino acid changes into the B-box domain produce dysfunctional transcription activity and also impaired protein–protein interactions. These authors demonstrated that amino acid substitutions in the B2 domain of STH3/AtBBX22 (Asp by Ala, see *sth3-D72* and *sth3-D81* in Fig. 3b) result in an impaired interaction with HY5, and the




| Motif | % BBX | Conserved sequences |
|---|---|---|
| M1 | 82% | SLSHSVSSSS[MLS][DE][VA][GA]VVP[DE][SAG] |
| M2 | 77% | [GD]D[ED][ED][ES][ED][ED][AE]EAASWLL[PL]NP[DG]KNSNN |
| M3 | 73% | [DG][FL][NC][VM]D[DE][AV]DLx[FL]ENYE[ED][LI]FG |
| M4 | 83% | F[GL]P[TS]D[MA][ED]L[AR]EFAAD[MV]E[SA]LLG |
| M5 | 67% | K[RT]SLLL[RK]L[ND]YE[AD][VI][IL][ST][AS]W |
| M6 | 64% | LT[ED]T[LV]PGW[HR]V[DE][ED][FL][LF] |
| M7 | 98% | H[QG]R[FY]LL[TL][GR][VQ][RK][VL][SGE][LF] |

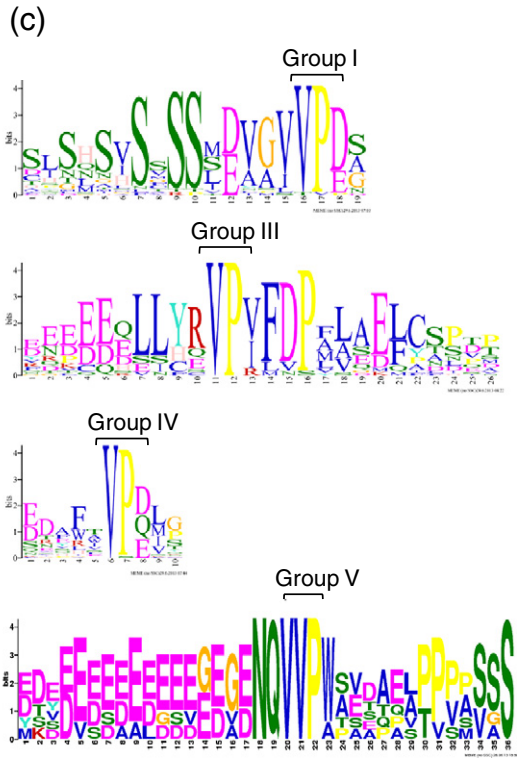



**Fig. 4.** Common domain organization between BBX proteins in each structure group. (a) Diagrams show the relative position of each novel motif according with the structure group. M1-7, motifs 1 to 7; B1, B-box domain type 1; B2, B-box domain type 2; CCT, CONSTANS domain.; (b) sequences of the seven conserved motifs (M1 to M7); the percentage of BBX proteins carrying the corresponding motif in each structure group is indicated as % BBX. X represents any amino acid. (c) Motif logos and the valine–proline (VP) pair in the structure groups I, II, IV and V.

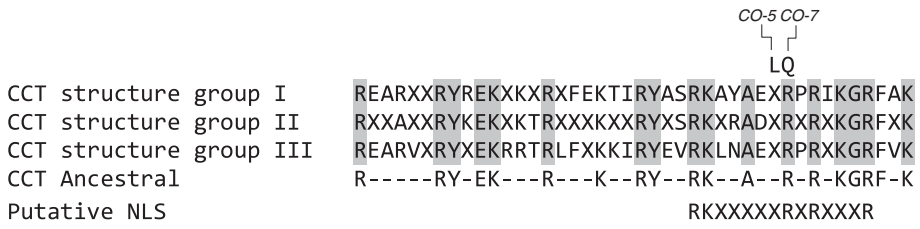



**Fig. 5.** The CCT domain contains bipartite nuclear localization signals (NLSs). Alignment of CCT consensus sequences of structure groups I, II and III. The CCT ancestral sequence was inferred from the amino acid sequence conserved in all structural groups (Supplemental Data 12). X represents any amino acid. – represents a gap. The amino acid substitutions in *co-5* and *co-7* alleles of CO/AtBBX1 are indicated over the CCT common sequence belonging to group I. The putative NLS was inferred by analogy with bipartite NLS consensus sequences of mammals and analyzing the nuclear uptake of *co-5* and *co-7*.

transcription activity is affected by amino acid substitutions in the B1 or B2 domains (Asp by Ala, see D20 in Fig. 3b; Datta et al., 2008). We hypothesize that some amino acids are conserved in the B-box sequence domain in a wide range of BBX proteins because they have played core functions in the regulation of plant development during evolution times.

Some BBX proteins have been shown to interact with COP1 and modulate its activity. In *A. thaliana*, COP1 interacts and promotes the degradation of AtBBX1/CO (Jang et al., 2008; Liu et al., 2008), AtBBX22/STH3 (Chang et al., 2011), AtBBX24/STO (Yan et al., 2011), and AtBBX25/STH1 (Holm et al., 2001). The VP pair located at the C-terminus is critical for the interaction with COP1 (Datta et al., 2006; Holm and Deng, 1999; Holm et al., 2001). Interestingly, we found that 96 from 214 BBX proteins belonging to structure groups I, III, IV and V contain a conserved VP pair at the C-terminus (Fig. 4c). In plants, COP1 acts as an E3 ubiquitin ligase to repress light signaling through the ubiquitylation and the later degradation of targeting photoreceptors and downstream transcription factors (Holm and Deng, 1999; Jang et al., 2005). The COP1 protein comprises three recognizable domains: RING finger, coiled-coil and WD40, all of which are implicated in mediating the interaction of COP1 with other proteins (Hardtke et al., 2000; Holm and Deng, 1999; Jang et al., 2005; Yang et al., 2001). On the other hand, the TRIM/RBCC family of proteins is an evolutionarily ancient group of proteins with homologs in both invertebrate and vertebrate species. TRIM/RBCC proteins are defined by the presence of the tripartite motif composed of a RING domain, one or two B-boxes and a coiled-coil region. Interestingly, some TRIM/RBCC proteins are implicated in protein ubiquitination and represent a novel class of single protein RING finger ubiquitin E3 ligases (Meroni and Diez-Roux, 2005). Since plants do not encode TRIM/RBCC proteins, Datta et al. (2007) suggested that the molecular functional equivalent of E3 ubiquitin ligase activity of animals could be the BBX-COP1 protein complex in green plants. Our phylogenetic study indicates that 45% of the BBX proteins analyzed have the VP pair and these proteins are potentially candidates to be part of the BBX-COP1 protein complexes. However, more experimental data are needed to demonstrate the role of VP pair in the protein–protein interactions.

Besides B-box and VP motifs, our phylogenetic analysis suggests that BBX proteins have other regulatory domains conserved in the BBX structural groups. Outside of the B-box and CCT domains, we found that BBX members of the same structure group share conserved motifs (M1 to M7, Fig. 4). Additionally, our analysis predicted conserved bipartite or monopartite NLS sequences at the C-terminus region in most of the BBX proteins analyzed. Bipartite NLSs with a consensus sequence type R-K-X11-R were found in most of the BBX members of structure groups I, II and III, within the second half of the CCT domain (Fig. 5). Although the BBX proteins of structure groups IV and V do not have a CCT domain, most of them have also monopartite or bipartite NLSs at the C-terminus. Interestingly, some previous works showed that predicted NLSs play a nuclear uptake function in CO/AtBBX1, COL3/AtBBX4 and STO/AtBBX24 (Datta et al., 2006; Robson et al., 2001; Yan et al., 2011). In fact, *co-5* mutation on the conserved bipartite NLS sequence of CO/AtBBX1 produces a dysfunctional nuclear uptake of CO/AtBBX1. Our analysis predicts that AtBBX24 has a monopartite NLS type K-K-X-R (Supplemental Data 13). Furthermore, Yan et al. (2011) found that a core of four amino acids (K-K-P-R) is necessary for the nuclear uptake of the AtBBX24 protein. These authors showed that a point mutation introduced in this motif (Lys-226 to Asn) was sufficient to prevent nuclear import of the AtBBX24. These evidences support the notion that the monopartite and bipartite sequences proposed here could play a nuclear uptake function for the action of BBX proteins in the nucleus. However, other direct evidence demonstrating the functional role of NLSs and M1 to M7 novel motifs remains to be found.

Based on the phylogenetic trees obtained from the full-length sequence (Fig. 1) and the B-box domain alignments (Fig. 2), we propose a hypothetic model for the BBX evolutionary trajectory in green plants. In animals, the B1 domain has a consensus type C-*X*2-C-X6-17-C-*X*2-C-X4-8-C-*X*2-3-(C/H)-X3-4-H-X5-10-H, while B2 domain has a different consensus C-*X*2-4-H-X7-10-C-X1-4-(D/C)-X4-7-C-*X*2-C-X3-6-H-*X*2-5-H (Massiah et al., 2006, 2007). In contrast to animals, here showed that the green plant B1 consensus type C-$X_2$-C-$X_{7\text{-}8}$-C-$X_2$-D-X-A-X-L-C-$X_2$-C-D-$X_3$-H and the B2 consensus type C-$X_2$-C-$X_3$-P-$X_4$-C-$X_2$-D-$X_3$-L-C-$X_2$-C-D-$X_3$-H retain the same topology (Fig. 3). This evidence suggests that the early BBX proteins of green plants originally had only one B-box domain that evolved in some cases in a duplication event (Fig. 6). This hypothesis is supported by the fact that most of green algae have a single B-box domain (Supplemental Data 3). However, the existence of an alga with double B-box domain, CrBBX1, suggests that the first B-box duplication event happened before green plants colonized the land. Later, an addition of a CCT domain at the C-terminus generated BBX proteins with double B-box and CCT domain, the early BBX members of structure group II (B1 + B2 + CCT, Fig. 6). A deletion event of the B2 domain of an early BBX member belonging to the structure group II rises to a BBX protein with a single B-box and CCT domain (B1 + CCT), a characteristic of the structure group III (Fig. 6). A duplication event of the B1 domain in a BBX protein of structure group III could have been a BBX precursor of structure group I, generating BBX proteins with B1 + B2 + CCT domains (Fig. 6). However, structure groups I and III share a common monophyletic origin, both evolved independently and acquired a different structure organization gaining novel domains (see Figs. 4a, b). In the BBX members that belong to the structure group V, the B-box amino acid consensus sequence is more similar to the B1 consensus sequence of members that belong to the structure group IV than to the B2 domain (see Figs. 2 and 3a). This suggests that an early BBX protein belonging to the structure group IV (B1 + B2) could be the ancestor of an early BBX member of structure group V (B1) after a deletion event of the B2 domain (Fig. 6). All these changes at B-box and CCT organization, which led to the origin of the different structural groups, occurred at an early stage of green plant evolution retaining the molecular function of the B-box domain to present.

Our phylogenetic analysis demonstrated that BBX proteins of the same structure group share a higher order of organization above the B-box and CCT domain similarity (Fig. 1). The five BBX structure groups evolved constrained by the conservation of amino acid sequences in the two B-boxes, but radiating variation into NLSs and novel motifs of each

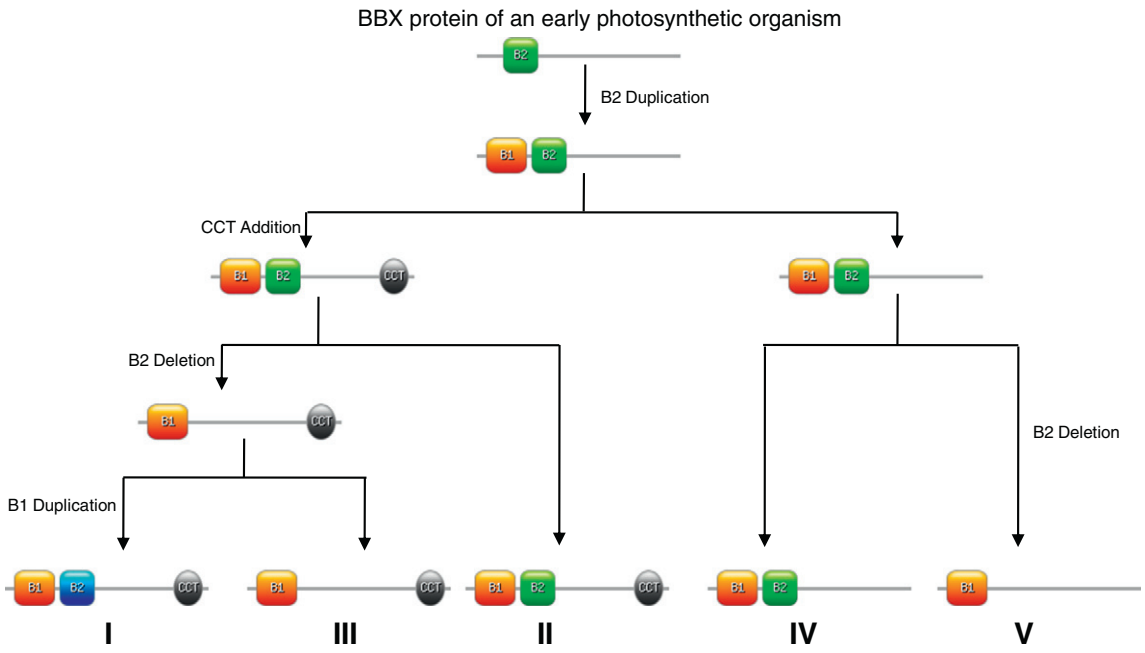



**Fig. 6.** A hypothetic model for the BBX evolutionary trajectory in green plants. The BBX evolutionary trajectory was inferred using phylogenetic analysis data of B-box domain in green plants and supported by novel motifs predicted in each structure group. B1, B-box domain type 1; B2, B-box domain type 2; CCT domain.

structural group. We suggest that these features are the functional basis for the BBX protein diversity in green plants.

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## Conflict of Interest

The authors report no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.08.037.

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