Arabidopsis thaliana BTB/POZ-MATH proteins interact with members of the ERF/AP2 transcription factor family

Henriette Weber and Hanjo Hellmann

1 Washington State University, Pullman, WA, USA
2 Freie University Berlin, Germany

In Arabidopsis thaliana, the BTB/POZ-MATH (BPM) proteins comprise a small family of six members. They have been described previously to use their broad complex, tram track, bric-a-brac/POX virus and zinc finger (BTB/POZ) domain to assemble with CUL3a and CUL3b and potentially to serve as substrate adaptors to cullin-based E3-ligases in plants. In this article, we show that BPMs can also assemble with members of the ethylene response factor/Apetala2 transcription factor family, and that this is mediated by their meprin and TRAF (tumor necrosis factor-associated factor) homology (MATH) domain. In addition, we provide a detailed description of BPM gene expression patterns in different tissues and on abiotic stress treatments, as well as their subcellular localization. This work connects, for the first time, BPM proteins with ethylene response factor/Apetala2 family members, which is likely to represent a novel regulatory mechanism of transcriptional control.

Structured digital abstract

- **MINT-7262792**: BPM1 (uniprotkb:Q8L765) physically interacts (MI:0915) with RAP2-4 (uniprotkb:Q8H1E4) by two hybrid (MI:0018)
- **MINT-7262805**: BPM1 (uniprotkb:Q8L765) physically interacts (MI:0915) with RAP2-13 (uniprotkb:Q9M8J9) by two hybrid (MI:0018)
- **MINT-7262749**: BPM3 (uniprotkb:Q2V416) physically interacts (MI:0915) with RAP2-4 (uniprotkb:Q8H1E4) by two hybrid (MI:0018)
- **MINT-7262764**: BPM3 (uniprotkb:Q2V416) physically interacts (MI:0915) with RAP2-13 (uniprotkb:Q9M8J9) by two hybrid (MI:0018)
- **MINT-7262838** and **MINT-7262882**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM1 (uniprotkb:Q8L765) by pull down (MI:0096)
- **MINT-7262911**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM2 (uniprotkb:Q9M39) by pull down (MI:0096)
- **MINT-7262935**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM3 (uniprotkb:Q2V416) by pull down (MI:0096)
- **MINT-7262945**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM4 (uniprotkb:Q9SRV1) by pull down (MI:0096)
- **MINT-7262970**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM5 (uniprotkb:Q1EBV6) by pull down (MI:0096)
- **MINT-7262992**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to BPM6 (uniprotkb:A1L4W5) by pull down (MI:0096)
- **MINT-7263095**: RAP2-4 (uniprotkb:Q8H1E4) binds (MI:0407) to RAP2-4 (uniprotkb:Q8H1E4) by pull down (MI:0096)

Abbreviations

BPM, BTB/POZ-MATH; BTB/POZ, broad complex, tram track, bric-a-brac/POX virus and zinc finger; ERF/AP2, ethylene response factor/Apetala2; GFP, green fluorescent protein; GUS, b-glucuronidase; MATH, meprin and TRAF homology; proBPM, promoterBPM; RAP2.4, related to Apetala2.4; TRAF, tumor necrosis factor receptor-associated factor; Y2H, yeast two-hybrid.
Introduction

In recent years, a novel superfamily of proteins has been described in plants that contains a conserved protein–protein interaction motif named broad complex, tram track, bric-a-brac/POX virus and zinc finger (BTB/POZ) [1–5]. This protein family is highly diverse with, for example, 80 members in Arabidopsis and 149 in rice [4,5]. The BTB/POZ domain has a length of around 116 amino acids and mediates homophilic and heterophilic interactions between the same or different proteins, respectively [6,7]. The BTB/POZ fold consists of six α-helices and three β-sheets that form a tightly interwound butterfly-shaped dimer with an extensive hydrophobic interface [8,9]. BTB/POZ proteins are often transcriptional regulators containing a C2H2 domain for DNA binding, but can also be found in combination with various other protein–protein interaction motifs, such as KELCH or meprin and TRAF (tumor necrosis factor receptor-associated factor) homology (MATH) motifs, indicating involvement in various biological processes [5,10–13].

It has recently been demonstrated for animals and plants that members of the BTB/POZ family use their BTB/POZ domain to assemble with CUL3 proteins [2–4,14–16]. Cullins are scaffolding subunits of multimeric E3-ligases that can polyubiquitinate their substrates and thereby target them for degradation via the 26S proteasome [17]. Thus, plant BTB/POZ proteins potentially serve as substrate adaptors for CUL3-based E3-ligases. Furthermore, self-assembly of BTB/POZ proteins has been established for Arabidopsis and rice [2–4]. However, although plants encode for a large number of BTB proteins, a functional role has only been assigned for a few of them, including ETO1 (ethylene biosynthesis [14]), NPH3 (blue light signal transduction [18]), BOP1 (leaf development [19]), ARIA (abscisic acid signaling [20]), NPY1 (auxin signaling [21]) and NPR1 (salicylic acid signaling [22]).

Some BTB proteins from plants and animals contain a secondary MATH domain which comprises around 150 amino acids forming eight β-sheets [23]. The motif was noted on the basis of homology with the C-terminal region of meprins A and B and the TRAF-C domain, and, like the BTB domain, facilitates protein–protein interaction [24]. Meprins are tissue-specific metalloendopeptidases implicated in developmental and pathological processes in animals by hydrolyzing a variety of peptides and proteins [25–27]. In mammals, TRAFs regulate cell growth signaling and apoptosis by interacting with membrane-bound receptors through their TRAF-C domains [28,29]. Although TRAFs and meprins have not been described in plants, a variety of plant proteins functionally unrelated to meprins and TRAFs contain MATH domains [30], and proteins carrying both BTB and MATH motifs are common in plants. Arabidopsis, for example, expresses six members of this BTB subfamily [referred to as the BPM (BTB/POZ-MATH) family] [2,16], whereas, in rice, 74 members are annotated [5]. Although it has been established that the BTB domain is employed to facilitate assembly with CUL3 and other BTB proteins [2,16], it remains unclear what kind of interactions are mediated by the MATH domain in plants.

In this article, we show that the MATH domain of BPM proteins is used to assemble with members of the ethylene response factor/Apetala2 (ERF/AP2) transcription factor family. We also provide a detailed description of the Arabidopsis thaliana BPM family expression and subcellular localization profile, including promoter:β-glucuronidase (promoter:GUS) and green fluorescent protein (GFP) fusion protein studies for all six members. Overall, the work demonstrates a novel role for BPMs as potential regulators that affect transcriptional activities of ERF/AP2 proteins in higher plants.

Results

BPM proteins assemble with members of the ERF/AP2 transcription factor family

Because it has been shown previously that Arabidopsis BPM proteins use their BTB/POZ domain to interact with the cullins CUL3A and CUL3B [3,4,16], we investigated what kind of protein–protein interactions were facilitated by their MATH domains by performing two
yeast two-hybrid (Y2H) screens on a root-specific cDNA library. One screen was performed with a full-length BPM3 (At2g39760), whereas, for the other, we used a BPM1 (At5g19000) fragment that lacked the BTB/POZ domain [denoted as BPM1(1–189); Fig. 1B]. As both the MATH and BTB domains mediate assembly with other proteins, we speculated that this dual approach would not only identify specific interactors for the MATH domain, but would also provide information on to what extent the two different MATH domains of BPM1 and BPM3 target the same group of proteins (see Table S1A,B for identity/similarity comparisons of BPM proteins and their MATH domains, respectively).

In total, 250 yeast clones were analyzed as primary positives and, consistent with earlier studies [2], BPM4 was found using BPM3 as bait (data not shown). However, predominantly, we identified RAP2.4 (At1g78080; related to Apetala2.4), which was found 15 times with BPM3 and 18 times with the BPM1(1–189) fragment (Fig. 1A). RAP2.4 belongs to the ERF/AP2 family of transcription factors and contains a single AP2 domain. The protein has been described previously in context with abiotic stress tolerance, red light response and ethylene signaling [31]. We also identified once At1g22190 in the BPM3 screen, which is the closest relative of RAP2.4 [32]. It should be noted that both RAP2.4 and At1g22190 were isolated as partial clones lacking the first 60 amino acids (Fig. 1B), demonstrating that this region is not essential for assembly with BPM proteins. As 12 RAP2 proteins have been annotated previously [33], we retained this nomenclature and denoted At1g22190 as RAP2.13.

To further confirm the interaction of RAP2.4 and RAP2.13 with BPM proteins, we cloned the corresponding full-length cDNAs for both genes, generated GST fusion proteins and tested these in pulldown assays with BPM1(1–189). As shown in Fig. 1C, BPM1(1–189) coprecipitated with both GST:RAP2 proteins, but not with GST alone, further corroborating the Y2H data. Because RAP2.4 and RAP2.13 are closely related to each other, we mainly focused on RAP2.4 as a representative example in subsequent experiments. Here, RAP2.4 also interacted with a full-length BPM1 (using a GST:BPM1 protein) and with itself (using GST:RAP2.4) (Fig. 1D). Additional pull-down assays positively confirmed binding to BPM2, BPM4, BPM5 and BPM6 (Fig. 1E). To exclude non-specific assembly with BPM proteins, we decided to test At1g65050. This protein has no BTB motif, but contains a MATH domain that is most closely related to those from BPMs [30]. In these experiments,
RAP2.4 did not interact with At1g65050 (Fig. 1E), which is a critical finding as it suggests that RAP2.4–BPM assembly is specific.

BPM1–RAP2.4 interaction requires a complete MATH domain and the N-terminal region of RAP2.4

The use of a truncated version of BPM1 in the Y2H screens demonstrated that the BTB domain is not involved in the assembly with RAP2.4. However, BPM1(1–189) still contains nearly 80 amino acids that are not part of the MATH domain and which could represent possible interaction sites for RAP2.4. To further confirm that a full-length MATH domain is sufficient for binding to RAP2.4, we generated a new truncated BPM1 version of 151 amino acids [BPM1(1–151)] comprising the first 38 amino acids of BPM1 followed by the complete MATH domain. As shown in Fig. 2A, BPM1(1–151) is entirely capable of binding to GST:RAP2.4, making it highly probable that only the MATH domain is required for RAP2.4–BPM1 assembly.

Likewise, we were interested in the RAP2.4 region that mediates the assembly with BPM1 proteins. Its AP2 domain stretches from amino acid residue 150 to 214. To test whether a functional AP2 domain is critical for assembly with BPMs, we took advantage of an earlier description of ap2-1 and ap2-5 mutants, in which mutation of a glycine residue in the AP2 domain disrupts the protein’s DNA-binding affinity [33,34]. This glycine is highly conserved and can also be found in RAP2.4 at position 179 [35]. However, the introduction of a point mutation that changed the glycine residue to serine [RAP2.4(G179S)] did not affect assembly with GST:BPM1, indicating that a functional AP2 domain is not required for this type of interac-

Fig. 2. Mapping of the interactive sites in BPM1 and RAP2.4. (A) In vitro-translated BPM1(1–151) can interact with GST:RAP2.4. (B) Schematic drawing of BPM1. The triangle indicates the fragment used for the Y2H screen and for pulldowns in (A). (C) In vitro-translated RAP2.4(1–251) is able to interact with GST:BPM1. (D) GST:BPM1 can assemble with in vitro-translated RAP2.4(G179S), RAP2.4(116–END) and RAP2.4(125–END), but not with RAP2.4(135–END). (E) GST:RAP2.4 can interact with in vitro-translated RAP2.4(125–END), but not with RAP2.4(135–END). (F) Schematic drawing of RAP2.4. Triangle indicates the fragment found in the Y2H screen.
tion. Next, we generated several truncated versions of RAP2.4 that were translated in vitro and tested for interaction with GST:BPM1. As we originally found truncated versions of RAP2.4 and RAP2.13 that were missing the first 60 amino acids in the Y2H screens, we started out with further reduced versions that lacked the first 116, 125 and 134 amino acids. Although complete deletion of the first 116 and 125 amino acids [RAP2.4(125–END)] did not affect coprecipitation with GST:BPM1, we could not detect interaction with a truncated version that lacked the first 134 amino acids [RAP2.4(134–END)] (Fig. 2D). In addition, deletion of amino acid residues C-terminal from the AP2 domain [RAP2.4(1–251)] did not affect the interaction with GST:BPM1 (Fig. 2C). We therefore conclude that a critical region for assembly with BPM proteins is located within amino acid residues 125–251 of RAP2.4.

**Detailed expression analysis of BPM and RAP2.4 shows distinct patterns for the different genes**

Although the interaction studies presented demonstrate that all BPM proteins can assemble with RAP2.4, and even provide strong evidence for the assembly of the transcription factor in planta, it is still unclear whether BPM and RAP2.4 genes are expressed in the same tissues. Consequently, we analyzed the tissue-specific expression patterns of all BPM genes and RAP2.4 via semiquantitative RT-PCR, and further described their expression in greater detail using promoter:GUS lines (referred to as proBPM:GUS and proPRAP2.4:GUS, respectively).

The results from RT-PCR showed that BPM2 and BPM5 were strongly expressed in all tested tissues (roots, rosette and cauline leaves, stems and flowers) (Fig. 3A). Although BPM6 was also strongly expressed, its expression level was weaker overall in comparison with BPM2 and BPM5. For BPM1 and BPM3, we could hardly detect expression in the different tissues, and had to load double the amount of RT-PCR products on the gels to visualize any PCR products (Fig. 3A). BPM1 showed only slightly higher expression levels in root and flower, and BPM3 expression levels showed little variation between the different tissues (Fig. 3A). BPM4 also showed little variation, and expression was lower than that of the other BPM genes. In this case, we had to load triple the amount of RT-PCR product relative to that used for BPM2 and BPM5. Finally, RAP2.4 was expressed strongly in roots, rosette and cauline leaves, and flowers, with slightly weaker expression levels in the stem (Fig. 3A).

![Fig. 3. Expression profiles of BPMs and RAP2.4 genes in Arabidopsis thaliana analyzed by semiquantitative RT-PCR. (A) Total RNA (100 ng), extracted from roots, rosette and cauline leaves, sections of stems and open flowers of mature plants grown in soil, was used for RT-PCR. The expression of all tested genes was detected in all tested tissues, but with considerable differences in expression strength/intensity: For BPM1 and BPM3 twofold, and for BPM4 threefold, the amount of RT-PCR product was loaded (compared with actin2 control reaction). (B) RT-PCR analysis showing BPM1, BPM2, BPM5 and RAP2.4 up-regulated by salt (200 mM NaCl for 6 h) and osmotic stress (200 mM sorbitol for 6 h). Sorbitol treatment also induced BPM3 and BPM4. (C) On drought treatment (drying for 6 h on a laboratory bench), only BPM1 and BPM4 showed up-regulation in expression. Numbers in parentheses indicate the fold amount of RT-PCR loaded in comparison with actin2. Asterisks indicate correct RT-PCR products.](image-url)
Because RAP2.4 has been described previously to play a role in abiotic stress tolerance, we tested whether expression of the different BPM genes was regulated by salt (NaCl), osmotic (sorbitol) and drought stress. Treatment of Col0 wild-type plants with 200 mM NaCl for 6 h resulted in a clear up-regulation of BPM1 and BPM5 expression (Fig. 3B). We also observed an up-regulation of BPM1 and BPM5 after treatment with sorbitol for 6 h, together with increased BPM4 levels (Fig. 3B). RAP2.4 also responded to both treatments with enhanced expression, which is in agreement with earlier findings from Lin et al. [31] (Fig. 3B). Drought stress only induced the expression of BPM1 and BPM4; all other BPM genes and RAP2.4 remained unchanged (Fig. 3B).

Overall, these data indicate that BPM1, BPM4 and BPM5 are involved in the abiotic stress response.

The analysis of transgenic plants carrying the different promoter:GUS constructs showed, for proBPM1:GUS lines, GUS expression in pollen, but also in stipules and leaf hyathodes (Fig. 4A). Rosette leaves showed staining within the vascular tissue at the end of the leaf blade, whereas the basal parts close to the petiole remained almost unstained. We observed clear GUS expression in the primary root of 7-day-old seedlings, strongest at the base of emerging lateral roots, but no expression at detectable levels in the tips of primary and budding lateral roots. proBPM2:GUS lines (Fig. 4B) showed strong expression in the vascular tissue of cotyledons and rosette leaves, and in most parts of the flower. Similar to proBPM1:GUS, strong staining was detectable in the stipules, pollen and at the base of siliques. Expression in roots was detectable along the primary but not lateral roots, with strongest staining present at the budding lateral root primordia. proBPM3:GUS lines showed clear GUS expression in the root tips, but also in the stipules, anthers and in the central veins and petioles of rosette leaves (Fig. 4C). proBPM4:GUS showed GUS staining very similar to that of proBPM3:GUS in the stipules, the central veins of rosette leaves and in the anthers of differentiated flowers. We also detected meagre expression along the root, with most obvious staining present at the lateral root primordia and the base of the lateral roots, and also in the columella (Fig. 4D).

Like proBPM2:GUS, proBPM5:GUS plants showed a wide range of expression patterns in all tested organs (Fig. 4E). Both cauline and rosette leaves showed strong GUS expression, as did the primary root tips and the stem, whereas, in the flower, expression was detectable in the petals, stamen and stigmata. In proBPM6:GUS lines, we saw GUS expression in the vascular tissue of cotyledons and mature leaves, whereas, in the flowers, the anthers, connectives and filaments and the base and tip of the stigmata were stained. Similar to BPM2, BPM3 and BPM5 promoter:GUS lines, the root tips were strongly stained, with the exception of columella cells which remained nearly white (Fig. 4F). Finally, proPRAP2.4:GUS lines showed blue staining in cotyledons of 3-day-old seedlings, but not in parts of the hypocotyls (Fig. 4G). In rosette leaves, we observed expression in the vascular tissue of the leaf blade, whereas, interestingly, in older parts of the mid-rib, no GUS expression was detectable. This was different from cauline leaves, in which all vascular tissue was stained. In the flower, we detected GUS expression exclusively in the pollen. Siliques were stained at the base and at the tip, with overall very weak staining of the fused carpels. In the root, the central cylinder was stained, whereas the primary root tips and tips of emerging lateral roots showed no blue staining (Fig. 4G, part f, marked by arrows).

Subcellular localization analysis of BPM proteins and their interactors

Using a GFP:RAP2.4 fusion protein, it has recently been established that RAP2.4 is primarily located in the nucleus [31]. Accordingly, one would expect that this organelle would be the most likely location for the assembly of BPM proteins with RAP2.4. We generated expression constructs for all BPM genes; however, only for BPM4 were we able to obtain GFP:BPM4 overexpressing plants. As an alternative approach to investigate the subcellular localization of the different BPM proteins, we transiently expressed them in tobacco leaves. We also included GFP:RAP2.4 and GFP:CUL3a in these experiments to compare their localization with that of BPM proteins.

Transient expression of GFP:BPM1 and GFP:BPM2 revealed that both proteins, like GFP:RAP2.4, were primarily localized to the nucleus (Fig. 5 and Fig. S2). The predominantly nuclear localization of BPM1 and BPM2 GFP fusion proteins contrasted with all other BPMs, as GFP:BPM3, GFP:BPM5 and GFP:BPM6 were found inside as well as outside the nucleus. Remarkably, GFP:BPM4 was the only BPM protein that was excluded from the nucleus, suggesting that BPM4 and RAP2.4 are not present in the same cellular compartments. We observed this in transient expression assays, but also in Arabidopsis plants that stably expressed GFP:BPM4 (Fig. 5 and Fig. S3). Also noteworthy was the observation that GFP:CUL3a showed a subcellular localization pattern similar to GFP:BPM3, GFP:BPM5 and GFP:BPM6. Overall, these analyses revealed a very distinct and different
Fig. 4. Expression profile of proBPM:GUS and proRAP2.4:GUS in Arabidopsis thaliana. (A) proBPM1:GUS. Hydathodes and stipules of 5-day-old seedlings showed staining (a–c), as did fully developed siliques (d; base and stigma region) and vascular tissue of rosette leaves (f). In flowers, expression was restricted to pollen and anthers (e). The primary root (g) was stained throughout, but the strongest expression was detectable at the points of emerging lateral roots, indicated by the arrows. (B) proBPM2:GUS showed the strongest expression of all promoter:GUS lines, detected in all tested tissues. Although cotyledons, hypocotyls and rosette leaves were strongly stained (a–c), GUS expression in cauline leaves was restricted to the base and apex of the lamina (c). Siliques showed staining at their bases and tips (stigma region) (d). In flowers, the petals, stamens, receptacle and upper pistil were stained (e). Close-up of stigma with strong staining of the stigma’s papillae (f). proBPM2:GUS plants showed GUS expression in the primary root (g, i) and lateral root primordia (h), but not in developed lateral roots (g). (C) proBPM3:GUS lines showed altogether very weak expression. In seedlings, staining was only detectable in the stipules (a). Rosette and cauline leaves showed good GUS expression in the central vein and petioles (b, c), as well as the anthers in flowers (d). proBPM3:GUS plants also showed expression in the root tips (e–g). (D) GUS expression under the BPM4 promoter was also weak, but with clear expression in the stipules (a), midrib of rosette leaves (b), mature anthers and stigmata (c, d). In roots, faint expression was detected along the primary root and its tip (g, h), whereas the lateral root primordia and base of the developed lateral roots showed stronger staining (e–g). (E) GUS staining for proBPM5:GUS lines was strong in the vascular tissue of the cotyledons (a) and in mature leaves (b, rosette; c, cauline), but also in the hypocotyl (a), young siliques (d) and flowers (g). (F) For BPM6, strong expression was observed in 3-day-old seedlings (a), as well as in the entire lamina and petiole of rosette leaves, including vascular veins (b). In flowers, GUS expression started in the early stages of the receptacles and stigmata (c) in older flowers, mature anthers and, later, filaments were also stained. In roots, GUS was expressed only in the tips of primary roots (d), and at the base of differentiated siliques (e). (G) proRAP2.4:GUS lines showed GUS expression in 3-day-old seedlings in cotyledons and in the central cylinder of the root, but not in the lower parts of the hypocotyl (a). Rosette leaves were stained in the vascular tissue of the leaf blade, whereas the petiole and older parts of the midrib remained unstained (b). However, the cauline leaf blade was stained very evenly (c). In flowers, staining was only detectable in the pollen (d). Expression levels in siliques were very low, with staining mainly present at the base and at the tip (e). A close-up of a root section showed that the central cylinder was stained (f), whereas the lateral root primordia (marked by arrows) did not show any GUS expression.
localization for the different BPM proteins, which might reflect their diverse biological roles in the cell. In addition, they indicate that, except for BPM4, all other BPM proteins are potentially able to interact with RAP2.4 in planta, and also that CUL3a can assemble with other proteins either in the nucleus or the cytoplasm.

Discussion

This work provides novel information, including the first description linking ERF/AP2 proteins to the BPM family, but also a detailed report of BPM expression and subcellular localization.

Our intensive interaction studies have identified the domains required for RAP2.4/BPM assembly to the BPM MATH domain and a region of RAP2.4 that stretches from amino acid residues 125 to 251, which encompasses the AP2 domain. Although the point mutation G179S in the AP2 domain of RAP2.4 does not affect assembly with BPMs, this does not rule out a possible role of the domain for this type of interaction. However, to date, there is no evidence that the AP2 domain mediates protein–protein interaction and, because of this, we consider it probable that it is also not involved in assembly with the BPM proteins.

Both RAP2.4 and RAP2.13 belong to a small subgroup within the ERF/AP2 superfamily that comprises eight members and is called the A-6 subfamily [32,36]. We tested more members of this subgroup, At1g36060, At4g39780 and At4g13620, in pulldown assays with GST:BPM1. Although At4g39780 and At4g13620 were both able to assemble with GST:BPM1, At1g36060 was not (Fig. S1), indicating that assembly with ERF/AP2 proteins is restricted to the A-6 subgroup and, in this case, even to a subset of eight members. The finding that, within the A-6 subgroup, not all of its members interact with BPM1 indicates that BPM proteins assemble only with a very limited set of ERF/AP2 proteins, which potentially does not extend beyond the A-6 subgroup. However, we experienced a high degree of redundancy from the BPM site, as all BPMs were able to interact with RAP2.4. It will be of interest to determine whether this redundancy is also present for all other ERF/AP2 proteins that bind to BPM1.

Studies on gene expression showed that BPMs have a widely overlapping pattern of expression. This was further corroborated by the promoter:GUS lines, localization for the different BPM proteins, which might reflect their diverse biological roles in the cell. In addition, they indicate that, except for BPM4, all other BPM proteins are potentially able to interact with RAP2.4 in planta, and also that CUL3a can assemble with other proteins either in the nucleus or the cytoplasm.

Discussion

This work provides novel information, including the first description linking ERF/AP2 proteins to the BPM family, but also a detailed report of BPM expression and subcellular localization.

Our intensive interaction studies have identified the domains required for RAP2.4/BPM assembly to the BPM MATH domain and a region of RAP2.4 that stretches from amino acid residues 125 to 251, which encompasses the AP2 domain. Although the point mutation G179S in the AP2 domain of RAP2.4 does not affect assembly with BPMs, this does not rule out a possible role of the domain for this type of interaction. However, to date, there is no evidence that the AP2 domain mediates protein–protein interaction and, because of this, we consider it probable that it is also not involved in assembly with the BPM proteins.

Both RAP2.4 and RAP2.13 belong to a small subgroup within the ERF/AP2 superfamily that comprises eight members and is called the A-6 subfamily [32,36]. We tested more members of this subgroup, At1g36060, At4g39780 and At4g13620, in pulldown assays with GST:BPM1. Although At4g39780 and At4g13620 were both able to assemble with GST:BPM1, At1g36060 was not (Fig. S1), indicating that assembly with ERF/AP2 proteins is restricted to the A-6 subgroup and, in this case, even to a subset of eight members. The finding that, within the A-6 subgroup, not all of its members interact with BPM1 indicates that BPM proteins assemble only with a very limited set of ERF/AP2 proteins, which potentially does not extend beyond the A-6 subgroup. However, we experienced a high degree of redundancy from the BPM site, as all BPMs were able to interact with RAP2.4. It will be of interest to determine whether this redundancy is also present for all other ERF/AP2 proteins that bind to BPM1.

Studies on gene expression showed that BPMs have a widely overlapping pattern of expression. This was further corroborated by the promoter:GUS lines,
which showed that most BPMs were expressed in anthers, root tips and rosette leaves. Although some patterns were highly specific, such as, for example, expression of BPM1 and BPM4 at the junction of primary to lateral roots, or BPM3 expression specifically in root tips, overall our results indicated that BPM proteins were functionally redundant. Consequently, one would expect no obvious developmental defects in plants affected in single BPM genes, which is the case for available T-DNA insertion mutants (H. Weber and H. Hellmann, unpublished work). However, on the basis of the expression patterns described, it is predictable that mutants affected in multiple BPMs will show aberrant flower, leaf and root development. Likewise, the inducible expression of BPM1, BPM4 and BPM5 on abiotic stress treatment suggests that corresponding single or multiple mutants will display an altered tolerance when exposed to these stressors.

The widely overlapping expression patterns of BPMs with RAP2.4 also suggest that the transcription factor can potentially assemble with most BPM proteins. This was further supported by our subcellular localization studies, in which all of the BPMs, except BPM4, were present in the nucleus. However, the specific nuclear localization of BPM1 and BPM2 currently makes both proteins the most favorable candidates for in planta assembly with the transcription factor RAP2.4. As BPM3, BPM5 and BPM6 were also present in the cytosol, it is probable that they assemble with additional, yet unknown proteins in this cellular compartment, and this is especially likely for BPM4, which was never found in the nucleus. In this case, it is of interest that CUL3a was also localized to the nucleus and the cytosol. Because a proposed role of BPMs is to function as substrate adaptors to a CUL3-based E3-ligase, the current findings suggest that such an assembly can occur within the nucleus and the cytoplasm and that, in both compartments, proteins can be ubiquitinated and potentially marked for degradation via the 26S proteasome.

**Conclusion**

In future work, it will be critical to verify our findings on BPM–ERF/AP2 assembly in planta and to identify the motif in RAP2.4 that mediates this type of interaction, as this has the potential to predict possible protein binding to the BPMs. It will also be of importance to define the functional impact of the BPM family on the activity of ERF/AP2 transcription factors. For example, do they affect the stability of these proteins and does this require CUL3s? In this case, it is noteworthy that we observed the instability of in planta-expressed myc-tagged RAP2.4 in a 26S proteasome-dependent manner (Fig. S4). We currently favor a working model in which they bind to ERF/AP2s, potentially interfering with their DNA-binding ability, but which ultimately results in the degradation of ERF/AP2 proteins (Fig. 6). Independent of this hypothetical role, the work described here opens up a novel and important connection between two plant protein families, and provides a forecast on a new regulatory mechanism controlling ERF/AP2 transcription factor activities.

**Materials and methods**

**Plant growth conditions and transformation**

*Arabidopsis thaliana* (ecotype Col0) and tobacco (*Nicotiana benthamiana*) plants were grown under standard conditions with 16 h : 8 h light : dark cycles in either soil or sterile culture, using ATS medium [37] without supplemented sucrose. *Arabidopsis* floral dip transformation was performed as described in [38].

---

*Fig. 6.* Schematic model for assembly and functional impact of BPM–ERF/AP2 assembly. BPM proteins function as substrate adaptors to CUL3-based E3-ligases. They also assemble with ERF/AP2 transcription factors, and this interaction serves to bring bound ERF/AP2 proteins to the core E3-ligase. Docking of the BPM–ERF/AP2 complex to the E3-ligase results in ubiquitination and subsequent degradation of bound transcription factor.
Molecular cloning and mutagenesis

Full-length cDNAs of BPM genes were amplified from a seedling-specific cDNA library [39]. The promoters of the different BPM genes and RAP2.4 (for sizes, see Table S1), as well as AP2/ERF transcription factors, were amplified directly from Col0 genomic DNA. In all cases, Pfu polymerase (Promega, Mannheim, Germany) was used and the PCR products were controlled for correct sequences. The Klenow fragment (Promega, Mannheim, Germany) was used and the PCR products were controlled for correct sequences. The cDNAs obtained were subcloned into pDONR221 (Invitrogen, Carlsbad, CA, USA) and shuffled into Agrobacterium vectors using GATEWAY technology (Invitrogen): pACT2 and pBTV116 [2] for Y2H studies, pDEST15 (Invitrogen) for Escherichia coli expression and the binary vector pK7FWG2 [40] for subcellular localization analysis. The amplified promoters were subcloned into pCR2.1 by TOPO TA reaction (Invitrogen). Afterwards, using the BamHI and XhoI restriction sites, the promoters were fused to the GUS gene in the binary vector pCB308 [41]. The primers used in this and other sections are given in Table S2. Mutagenesis was performed using a mutagenesis kit from Stratagene (La Jolla, CA, USA) as described previously [2].

Expression analysis

Expression was studied by RT-PCR with gene-specific primers, and histochemically using promoter-GUS fusions. RT-PCR was performed on 100 ng of total RNA isolated from different tissues of mature Arabidopsis ecotype Col0 plants grown on soil, or on total RNA extracted from 7-day-old seedlings grown on plates, respectively. For histochemical analysis, promoters in the binary vector pCB308 were introduced into Arabidopsis plants. Transgenic plants were selected by BASTA herbicide (Aventis Crop Science, Leverkusen, Germany). GUS staining was carried out by vacuum infiltration of plant material with staining solution [2] and subsequent incubation at room temperature for up to 24 h. For stress treatment, 7-day-old sterile-grown seedlings were transferred for 6 h into liquid ATS medium supplemented with either 200 mM NaCl or sorbitol. To impose drought stress on 7-day-old seedlings, the lids from culture dishes were removed for 6 h before the samples were harvested.

Y2H assay

Screening for BPM-interacting clones was performed using a root-specific suspension cell cDNA library in the prey vector pACT2-GW [42]. The MATH domain of BPM1 and full-length BPM3 were cloned into the bait vector pBTV116-D9-GW [42]. Yeast transformation and testing for interaction were performed as described in [2]. Clones were transformed into yeast with an efficiency of 1.5 million clones per transformation. All BPM-interacting clones were tested for auto-activation and sequenced for the correct open reading frame in pACT2.

Subcellular localization analysis

Fluorescent fusion proteins of the six BPM proteins, CUL3a and RAP2.4 were transiently expressed in tobacco epidermal cells using the method of Agrobacterium infiltration as described in [43]. The bacterial attenuation (D) at 600 nm was 0.01–0.03 for all constructs. In addition, BPM4 localization was also analyzed in stable transgenic Arabidopsis plants expressing GFP:BPM4 fusion protein. In all cases, binary GFP expression vectors obtained from [40] were used. Transfected leaf sections were imaged using a Zeiss (Jena, Germany) LSM 510 Meta confocal microscope.

In vitro transcription/translation assays

For interaction studies, full-length BPM proteins, fragments of BPM1 and selected ERF/AP2 proteins were expressed in the TNT-reticulocyte lysate system (Promega) as described previously [2]. In vitro-translated proteins were labeled with either [35S]methionine (Amersham, Chalfont St Giles, UK) or [3H]Lysine (Promega).

Acknowledgements

We thank Sutton Mooney for critical reading of the manuscript. Financial support for this project was provided by the Deutsche Forschungsgemeinschaft (DFG) grants HE3224/5-1 and 5-2 and Washington State University to HH.

Accession numbers

BPM1 (At5g19000/Q8L765); BPM2 (At3g06190/Q9-M8J9); BPM3 (At2g39760/Q9SRV1); BPM5 (At5g21010/Q1EBV6); BPM6 (At3g43700/A1L4W5); RAP2.4 (At1g78080/Q8H1E4); RAP2.13 (At1g22190/Q9LM15).

References


**Supporting information**

The following supplementary material is available:

Fig. S1. *In vitro*-translated A-6 ERF/AP2 proteins At4g39780 and At4g13620 coprecipitate with GST:BPM1, but not At1g36060.

Fig. S2. Verification of nuclear localization for GFP:BPM1 and GFP:RAP2.4 in transient expression experiments using *Nicotiana benthamiana* leaves.

Fig. S3. Generation of stable transgenic *Arabidopsis* P35S:GFP:BPM4 plants.

Fig. S4. Instability of RAP2.4:myc in plants overexpressing myc-epitope-tagged RAP2.4 under the control of a 35S promoter (pro35S:RAP2.4:myc).

Table S1. Comparison of whole BPM proteins (A) and the BPM MATH domains only (B). Similarities and identities between the amino acid sequences were determined by MATGAT 2.02 software using the default settings (BLOSUM62, first gap 12, extending gap 2) [44].

Table S2. Overview of primers used in this work and expected PCR products.

This supplementary material can be found in the online version of this article article.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.