Arabidopsis Mutants Resistant to S(+)-β-Methyl-α, β-Diaminopropionic Acid, a Cycad-Derived Glutamate Receptor Agonist[1]

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Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that are the predominant neuroreceptors in the mammalian brain. Genes with high sequence similarity to animal iGluRs have been identified in Arabidopsis. To understand the role of Arabidopsis glutamate receptor-like (AtGLR) genes in plants, we have taken a pharmacological approach by examining the effects of BMAA [S(+)-β-methyl-α, β-diaminopropionic acid], a cycad-derived iGluR agonist, on Arabidopsis morphogenesis. When applied to Arabidopsis seedlings, BMAA caused a 2- to 3-fold increase in hypocotyl elongation and inhibited cotyledon opening during early seedling development. The effect of BMAA on hypocotyl elongation is light specific. Furthermore, BMAA effects on early morphogenesis of Arabidopsis can be reversed by the simultaneous application of glutamate, the native iGluR agonist in animals. To determine the targets of BMAA action in Arabidopsis, a genetic screen was devised to isolate Arabidopsis mutants with a BMAA insensitive morphology (*bim*). When grown in the light on BMAA, *bim* mutants exhibited short hypocotyls compared with wild type. *bim* mutants were grouped into three classes based on their morphology when grown in the dark in the absence of BMAA. Class-I *bim* mutants have a normal, etiolated morphology, similar to wild-type plants. Class-II *bim* mutants have shorter hypocotyls and closed cotyledons when grown in the dark. Class-III *bim* mutants have short hypocotyls and open cotyledons when grown in the dark, resembling the previously characterized constitutively photomorphogenic mutants (*cop, det, fus,* and *shy*). Further analysis of the *bim* mutants should help define whether plant-derived iGluR agonists target glutamate receptor signaling pathways in plants.

Glu is the predominant neurotransmitter in the brain. As a neurotransmitter, it activates Glu receptors at the post-synaptic membrane, which are involved in sensing environmental cues and in memory function (Nowak et al., 1984; Isquierdo and Medina, 1995; Tsien et al., 1996). Improper ionotropic Glu receptor (iGluR) function has been implicated in a variety of human diseases including Alzheimers and Parkinsons dementia (Ikonomidou and Turski, 1996; Forsythe and Barnes-Davies, 1997). One subgroup of Glu receptors is comprised of the iGluRs, which function as Glu-gated ion channels that convey rapid synaptic transmission. iGluRs are pharmacologically classified into subgroups based on agonist response. The two main iGluR subfamilies in animals are *N*-methyl-D-Asp (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)/kainate (KA) (non-NMDA) activated iGluRs (Ikonomidou and Turski, 1996; Forsythe and Barnes-Davies, 1997).

In plants, it appears that Glu may also act as a signaling molecule. Glu supplied to plant growth media has been shown to alter the expression of genes encoding enzymes involved in amino acid metabolism (Lam et al., 1994, 1998b; Oliveira and Coruzzi, 1999). Despite the evidence that amino acids may act as signals in higher plants, the mechanism of amino acid sensing and signaling is poorly understood. Genes for putative amino acid sensors have been uncovered in Arabidopsis that have high sequence similarity to ionotropic Glu receptors of animals (Lam et al., 1998a; Chiu et al., 1999). Arabidopsis GLRs have all the signature features of animal iGLRs, including a plasma membrane signaling peptide, two putative ligand-binding domains, and a "three-plus-one" transmembrane region (Lam et al., 1998a; Chiu et al., 1999).

To assess the function of putative Glu receptor genes in plants, Arabidopsis seedlings were treated with the iGluR antagonist 6,7 dinotropuinoxaline 2,3(1H, 4H) dione (DNQX), known to block AMPA/KA iGLRs in animals (Muller et al., 1989). It was shown that DNQX inhibits two key aspects of seedling photomorphogenesis in Arabidopsis: light-induced hypocotyl shortening and light-induced greening (Lam et al., 1998a). To further explore the targets of AtGLR function in plants, we tested whether other compounds known to block iGluR function in animals could also block aspects of Ara-

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bidopsis growth and development. Several of these iGluR agonists are plant-derived products: kainate (KA; Monaghan et al., 1989; Bettler and Mulle, 1995) made by seaweed (*Digenea simplex*); β-*N*-oxalylamino-L-alanine (Ross et al., 1989) made by chickpeas (*Lathyrus* sp.), and BMAA (Copani et al., 1991) made by cycads. BMAA [S(+)-β-methyl-α, β-diaminopropionic acid] has been detected in members of the family Cycadaceae or "cycads" (Pan et al., 1997). Cycads are believed to be the most primitive of gymnosperms, whose remnant surviving members descended from the Mesozoic and the Paleozoic when cycads predominated the vegetation (Chamberlain, 1919). BMAA was first isolated as the suspected cause of Parkinsonians dementia complex and amyotrophic lateral sclerosis in Guam's Chamorro human population, where consumption of *Cycas circinalis* L., a local food source, was prevalent (Whiting, 1963; Spencer et al., 1987). Subsequent to its detection and purification, BMAA has been shown to cause neural degeneration in primates when supplemented in their food (Spencer et al., 1987). Because BMAA is a natural plant product that blocks iGluR function in animals, we decided to test whether it would have any effect on plant GLRs using Arabidopsis as a model. In this study, we show that BMAA promotes hypocotyl elongation and inhibits cotyledon opening when applied to light-grown Arabidopsis seedlings. To identify the targets of BMAA action in plants, we used BMAA as a pharmacological tool to screen for Arabidopsis mutants resistant to BMAA-induced changes in photomorphogenesis. The isolation and preliminary characterization of these mutants is described below.

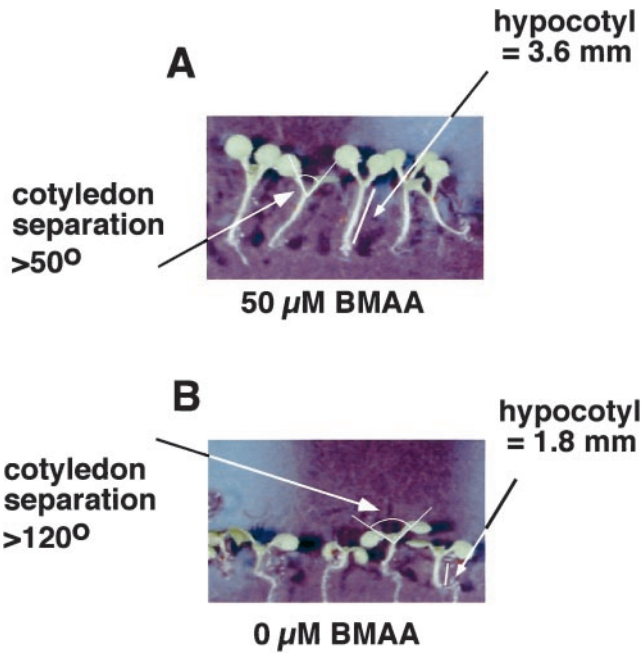

Figure 1. BMAA causes hypocotyl elongation and inhibits cotyledon unfolding in light-grown Arabidopsis. Arabidopsis plants were germinated and cultivated on Murashige and Skoog media and 0.7% (w/v) agar containing 50 μM BMAA (A) or no BMAA (B) for 8 d in the light. The average hypocotyl length for each treatment, as well as the arc-angle of opening for the cotyledons, is shown ($n = 30$).

RESULTS

BMAA, a Cycad-Derived Glu Receptor Agonist, Causes a Long Hypocotyl Phenotype in Arabidopsis

To probe the putative function of AtGLR genes in plants, we sought to determine whether the iGluR agonist, BMAA, caused any observable phenotypic effects on plant growth when supplied to Arabidopsis seedlings. Arabidopsis seedlings were germinated and cultivated on Murashige and Skoog media in the presence or absence of BMAA. BMAA-treated seedlings were evaluated for phenotypic alterations in treated plants compared with untreated control plants. At 8 d post-germination, the hypocotyls of seedlings grown in the light on Murashige and Skoog media containing 50 μM BMAA, displayed elongated hypocotyls (Fig. 1A), compared with control untreated plants (Fig. 1B). The effect of BMAA on hypocotyl elongation was quantified. A dose-dependent response was observed at increasing concentrations of BMAA (Fig. 2A). In light-grown plants, a concentration of 20 μM BMAA caused an increase in length of approximately 30%, and 50 μM BMAA caused approximately a 100% increase in hypocotyl length when compared with untreated plants (Fig. 2A). The effect on hypocotyl elongation is weaker at 100 μM BMAA, and at greater concentrations (200 μM) BMAA becomes inhibitory to growth (data not shown). In contrast, BMAA does not induce hypocotyl elongation in dark-grown plants (Fig. 2B). Instead, BMAA has an increasingly negative effect on hypocotyl length in dark-grown plants at concentrations of 50 μM or greater (Fig. 2B). BMAA was also inhibitory to root growth (Fig. 1A). BMAA also inhibits cotyledon opening in the light (Fig. 1A). The arc of cotyledon opening is reduced to 50° in BMAA-treated plants (Fig. 1A) when compared with 120° in control plants (Fig. 1B).

To determine whether the effects of BMAA (a Glu analog) could be reversed by Glu (the native agonist of iGluRs in animals), plants treated with 25 μM BMAA were grown on media containing increasing amounts of L-Glu (Fig. 3). The BMAA-induced effects on hypocotyl elongation and inhibition of cotyledon opening can be partially reversed by the simultaneous addition of L-Glu to the growth media (Fig. 3, A and B). The L-Glu reversal of the BMAA effects occurs in a dose-dependent manner. BMAA-induced hypocotyl elongation is reversed by approximately 50% with 1 mM L-Glu and by approximately 100% with 10 mM L-Glu (Fig. 3A). BMAA-induced inhibition of cotyledon opening is reversed by 20% with 1

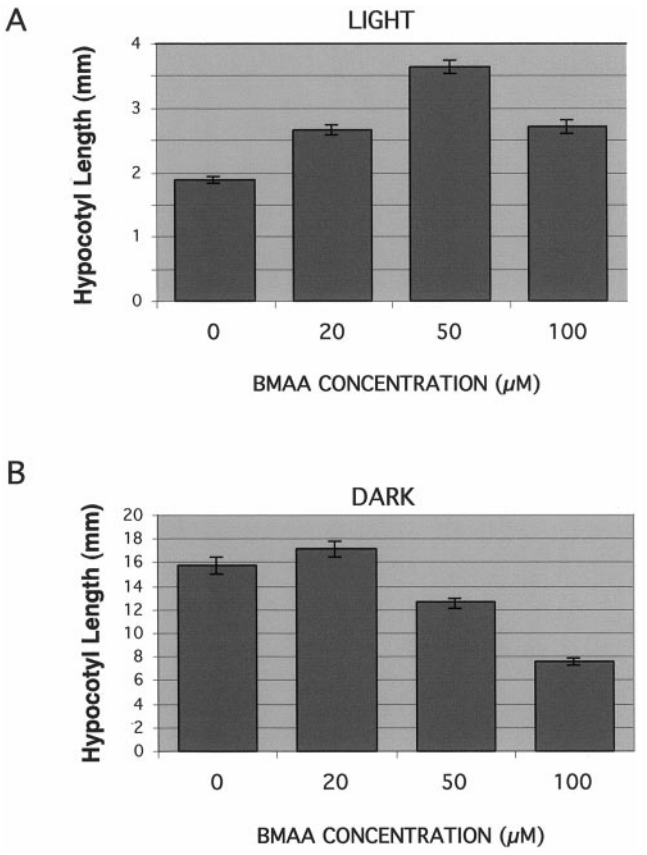

Figure 2. Quantification of BMAA-induced hypocotyl elongation in light-grown and etiolated plants. Arabidopsis plants were grown on increasing concentrations of BMAA (0–100 μM), in the light (A) or in continuous dark (B) for 5 d. The average hypocotyl length for each treatment is shown. Error bars show the SE of the mean ($n = 30$).

mM L-Glu, and by nearly 50% with 10 mM L-Glu (Fig. 3B). To determine whether the reversal of BMAA effects on morphogenesis was specific to the amino acid L-Glu, we tested whether two other amino acids could similarly counteract the effects of BMAA. L-Asp could not reverse the effects of BMAA, however L-Gln could also reverse the effects of BMAA (data not shown).

Selection of Arabidopsis Mutants Resistant to the Effects of BMAA on Morphogenesis

To determine the targets and mode of action of BMAA in Arabidopsis, a screen was devised to isolate mutants insensitive to the BMAA-induced effects on seedling morphology. For this screen, mutagenized (M2) Arabidopsis seeds were plated and grown in the light on Murashige and Skoog media containing 50 μM BMAA (Fig. 4). On this BMAA-containing media, wild-type plants exhibit elongated hypocotyls and partially closed cotyledons. M2 plants that exhibited short hypocotyls and open cotyledons when grown on BMAA were identified as "BMAA insensitive morphology" (*bim*) mutants (Fig. 4A). A total of 18,000 ethyl methanesulfonate (EMS) M2 seedlings were screened in the light on 50 μM BMAA, and 10 *bim* mutants were isolated (*bim 18*, *26*, *40*, *50*, *59*, *77*, *131*, *136*, *167*, and *175*). A representative *bim* mutant seedling, *bim 26*, identified in the M2 screen is shown in Figure 4B.

M3 progeny from M2 *bim* plants were tested for genetic inheritance of resistance to the effects of BMAA. M3 progeny of two *bim* mutants are shown in Figure 5. When treated with 50 μM BMAA and grown in the light, wild-type plants have elongated hypocotyls (Fig. 5A). In contrast, two representative mutants, *bim 131* and *bim 26*, each have visibly shorter hypocotyls when grown in the light on 50 μM BMAA (Fig. 5A). When grown in the light minus BMAA, *bim 131* and *bim 26* are indistinguishable from wild type as young seedlings (Fig. 5B). BMAA treatment impairs root growth in wild-type plants, and in the majority of *bim* mutants. However, one mutant, *bim 50*, showed partial resistance to BMAA-mediated inhibition of root growth when compared with wild type (data not shown).

The effect of BMAA on hypocotyl length of light-grown plants was quantified for all *bim* mutants and wild-type plants (Fig. 6A) and was compared with untreated plants (Fig. 6B). When grown in the light plus 50 μM BMAA, wild type has a 2- to 3-fold increase in hypocotyl length, compared with *bim* mutants (Fig. 6A). In contrast, when grown in the light minus BMAA, the majority of *bim* mutants are indistinguishable from wild type with regard to hypocotyl length (Fig. 6B). Only *bim 131* and *bim 167* have obviously shorter hypocotyls than wild type, when grown in the absence of BMAA (Fig. 6B).

A Subset of *bim* Mutants Exhibit Constitutive Photomorphogenesis in the Dark

The *bim* mutants have been grouped into three classes based on their dark morphology when grown in the absence of BMAA (Fig. 7). Hypocotyl length of the etiolated plants is quantified in Figure 8. Class I *bim* mutants have a normal etiolated morphology (elongated hypocotyls and closed cotyledons) when cultivated in the dark in the absence of BMAA (Fig. 7A). This is shown for a representative *bim* mutant (*bim 131*) (Figs. 7A and 8A). Class-II and class-III *bim* mutants each have short hypocotyls when grown in the dark minus BMAA (Figs. 7, B and C, and 8A). Class-II *bim* mutants (*bim 18*, *40*, *77*, *136*, *59*, *167*) have short hypocotyls in the dark, but their cotyledons remain closed (Fig. 7B). Class-III *bim* mutants (*bim 26* and *50*) have short hypocotyls but also display open cotyledons in the dark (Figs. 7C and 8A), similar to the *cop/det/fus* mutants. The effects of BMAA on a representative *cop* mutant (*cop1-6*), is shown in Figure 6 and 8. BMAA has no effect on hypocotyl elongation of the *cop 6-1* mutation in the light (Fig. 6B versus 6A) or in the dark (Fig. 8B versus 8A). By contrast, BMAA

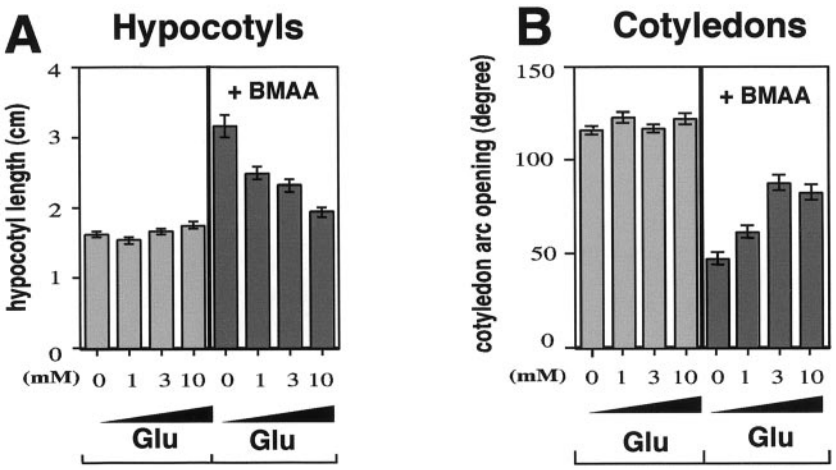

Figure 3. BMAA-induced hypocotyl elongation, as well as inhibition of cotyledon opening is reversed in a dose-dependent manner by Glu. Arabidospsis seedlings were cultivated in the light on Murashige and Skoog media containing 1, 3, or 10 mM Glu in the absence (left, A and B) or presence (right, A and B) of 25 μM BMAA. A, The average hypocotyl length for each treatment. B, The arc of cotyledon opening. Error bars show the SE of the mean (n = 30)

causes slight elongation of *bim* mutants grown in the light (Fig. 6A). Thus, it appears that the mutation conferring BMAA-resistance in the *bim* mutants affects aspects of skotomorphogenesis.

DISCUSSION

We have shown that BMAA, a plant-derived agonist that blocks Glu receptor function in animals, appears to alter early morphogenesis of light-grown Arabidopsis seedlings. BMAA promotes hypocotyl elongation and inhibits cotyledon opening in the light. As such, BMAA-induced effects on Arabidopsis seedlings phenocopy the long hypocotyl or "*hy*" mutants, defective in perceiving light and/or transmitting light signals in Arabidopsis (von Arnim and Deng, 1996; Fankhauser and Chory, 1997).

We reported previously that DNQX, an antagonist of AMPA/KA receptors in animals, also causes a "*hy*"-like phenotype when supplemented in the culture media of Arabidopsis seedlings (Lam et al., 1998a). The fact that two different iGluR interacting compounds (DNQX and BMAA) each induce hypocotyl elongation in light-grown seedlings provides support for the hypothesis that endogenous AtGLR genes in plants may be involved in photomorphogenic development in Arabidopsis. DNQX (an iGluR antagonist) could potentially antagonize Arabidopsis GLRs, which may be involved in light-mediated inhibition of hypocotyl growth. BMAA (an iGluR agonist) might inhibit AtGLR function but via a different mechanism. In animal systems, non-native agonists such as BMAA, can impair iGluR function because often iGluRs remain sensitized to these non-native

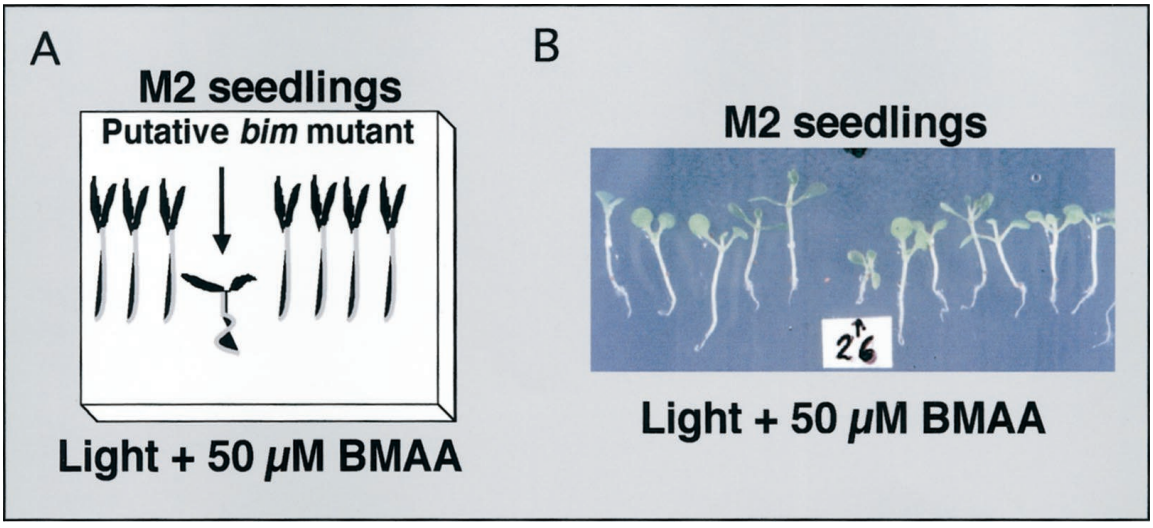

Figure 4. A genetic screen to isolate BMAA insensitive morphology (*bim*) mutants. A, Strategy to isolate Arabidopsis mutants resistant to the effects of BMAA is shown. Individual EMS mutagenized M2 seedlings are grown for up to 2 weeks on Murashige and Skoog media containing 50 μM BMAA in the light. M2 individuals with short hypocotyls, compared with neighboring plants, are recovered from the BMAA-containing media and allowed to produce seed for analysis in the M3 generation. B, Representative M2 *bim* plant (*bim26)* is shown as detected in the primary screen.

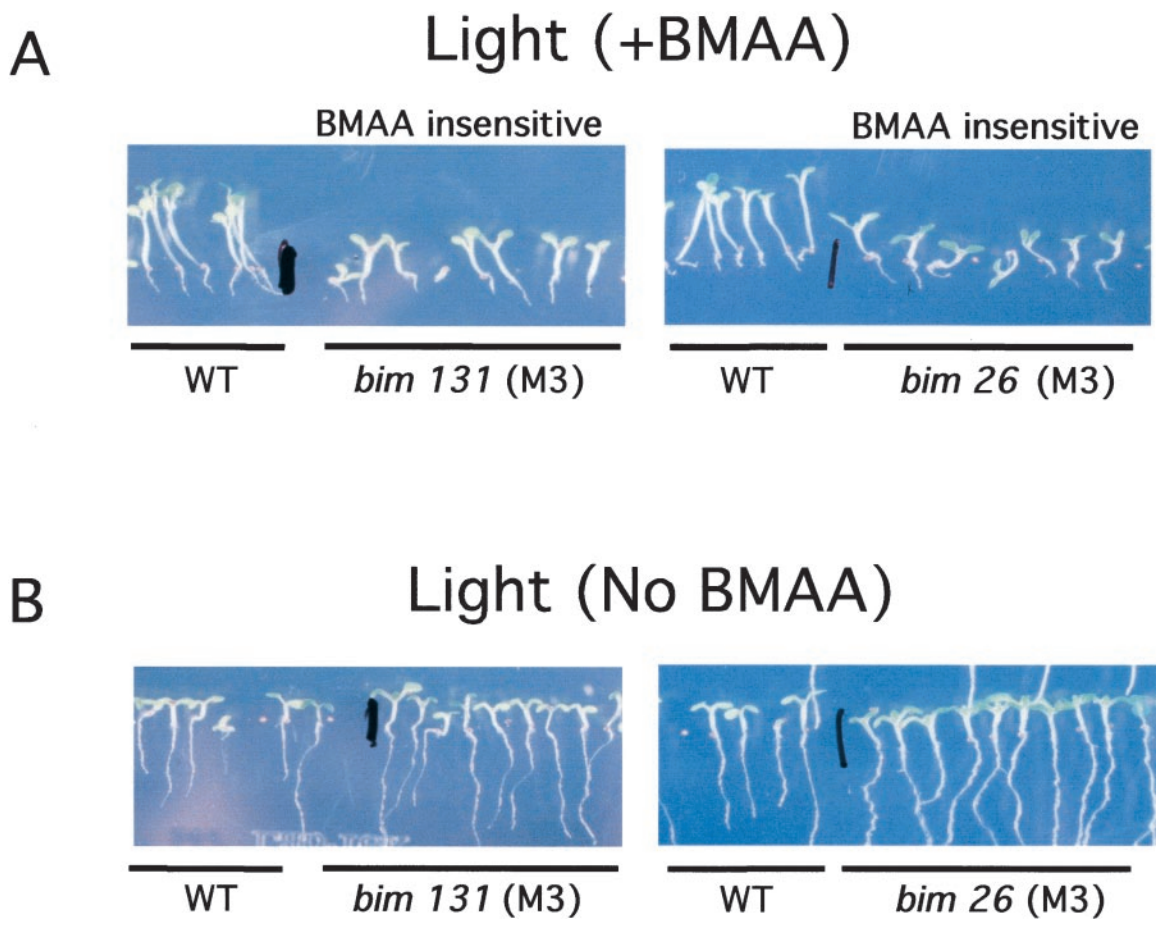

Figure 5. *bim* mutants are insensitive to BMAA-induced hypocotyl elongation. Two representative M3 *bim* mutants (*bim 131* and *bim 26)* were cultivated for 5 d in the light on Murashige and Skoog media in the presence of 50 μM BMAA (A) or in the absence of BMAA (B). A, Both *bim 131* and *bim 26* have a short hypocotyl phenotype (right) when compared with wild type (left), which has an elongated hypocotyl in the presence of BMAA. B, Plants grown in the absence of BMAA where *bim 131* and *bim 26* appear similar to wild type at the early seedling stage.

ligands (Ross et al., 1989). In contrast, animal iGluRs become desensitized to the native agonist, Glu. Desensitization is necessary for ion channel closure and proper iGluR function (Geoffroy et al., 1991; Sprengel and Seeburg, 1995). The fact that the BMAA-induced effects on Arabidopsis morphogenesis are reversed by the addition of Glu (the native iGluR ligand), is consistent with the hypothesis that BMAA may act by blocking plant AtGLR signaling in Arabidopsis. In this scenario, increasing levels of Glu would compete with BMAA at the ligand-binding site and restore normal AtGLR desensitization and function. Alternatively, BMAA could act as an agonist to activate and open iGluR channels in plants, potentially regulating ion flow necessary for hypocotyl elongation. Hypocotyl expansion is largely due to increases in cell size, since most cells in the hypocotyl are formed during embryogenesis (Gendreau et al., 1997). Previous work has already detected the activation of chloride channels during hypocotyl elongation (Cho and Spalding, 1996). In this scenario, we hypothesize that BMAA-induced hypocotyl elongation may be caused by activation of Arabidopsis GLRs important for cell expansion during hypocotyl elongation.

Among other amino acids tested, we have found that Gln could also reverse the effects of BMAA on Arabidopsis growth, whereas Asp could not. Thus, Gln may also act similar to Glu as a potential agonist at a BMAA responsive site. Gln and Glu both trigger ion transport of Glu receptors from the cyanobacteria, *Synechocystis* (Chen et al., 1999), when expressed in a heterologous system. Glu receptors from cyanobacteria show the strongest sequence similarity to Arabidopsis Glu receptors. Another possibility is that exogenously supplied Gln is assimilated and metabolized to Glu, which is then able to reverse the effects of BMAA on Arabidopsis. In fact, HPLC analysis has shown that exogenously added Gln leads to significantly higher levels of endogenous Glu in Arabidopsis (Oliveira and Coruzzi, 1999).

To test these different hypotheses and to determine the targets of BMAA action in plants, we have undertaken a mutant screen in Arabidopsis using BMAA as a pharmacological tool. This molecular-

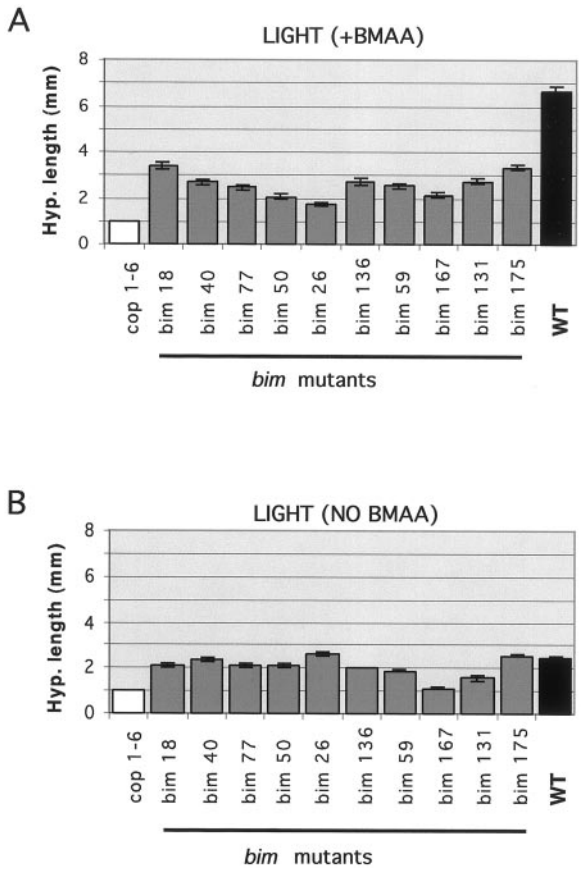

Figure 6. Quantification of hypocotyl lengths of *bim* mutants grown in the light in the presence or absence of BMAA. *bim 18, 40, 77, 50, 26, 136, 59, 131, 167,* and *175* were grown for 5 d in the light in the presence of 50 μM BMAA (A) or with no BMAA (B). Hypocotyl lengths of *bim* seedlings and wild type were measured and quantified. Wild type is indicated with a black bar on the far right of each graph. *cop1-6* mutant is indicated with a white bar on the far left of each graph. The average hypocotyl length for each treatment is shown (n = 30). Error bars show the SE of the mean.

genetic approach should enable us to understand how BMAA might induce hypocotyl elongation and block cotyledon separation in light-grown Arabidopsis. We have isolated Arabidopsis mutants insensitive to the effects of BMAA on early morphogenesis in the light. BMAA insensitive morphology (*bim*) mutants have short hypocotyls when grown in the light in the presence of BMAA (Figs. 4–6). In contrast, wild-type plants display elongated hypocotyls under these conditions. The *bim* mutants were further separated into three classes based on their morphology in the dark (Fig. 7). The first class of *bim* mutants has a normal etiolated morphology in the dark. We have identified two *bim* mutants in this class (*bim 131* and *bim 175)*. The second class of *bim* mutant (*bim 18*, *40*, *59*, *77*, *136*, *167*), has short hypocotyls in the dark and closed cotyledons. This phenotype is similar to the *proscute* (Desnos et al., 1996) and *korrigan* (Nicol et al., 1998) mutations, which affect cell wall formation during development. This phenotype is also similar to a number of hormone mutants deficient in growth including *gai* (Gendreau et al., 1999) and *ctr1* (Kieber et al., 1993). The third class of *bim* mutants (*bim 26* and *50)* has short hypocotyls and open cotyledons. These two characteristics are analogous to the photomorphogenic mutants *cop* (Hou et al., 1993), *det* (Chory et al., 1989, 1991b), *fus* (Miséra et al., 1994; Kwok et al., 1996), and *shy* (Reed et al., 1994; Tian and Reed, 1999) mutants, which share these phenotypes. We tested the effects of BMAA on one constitutively

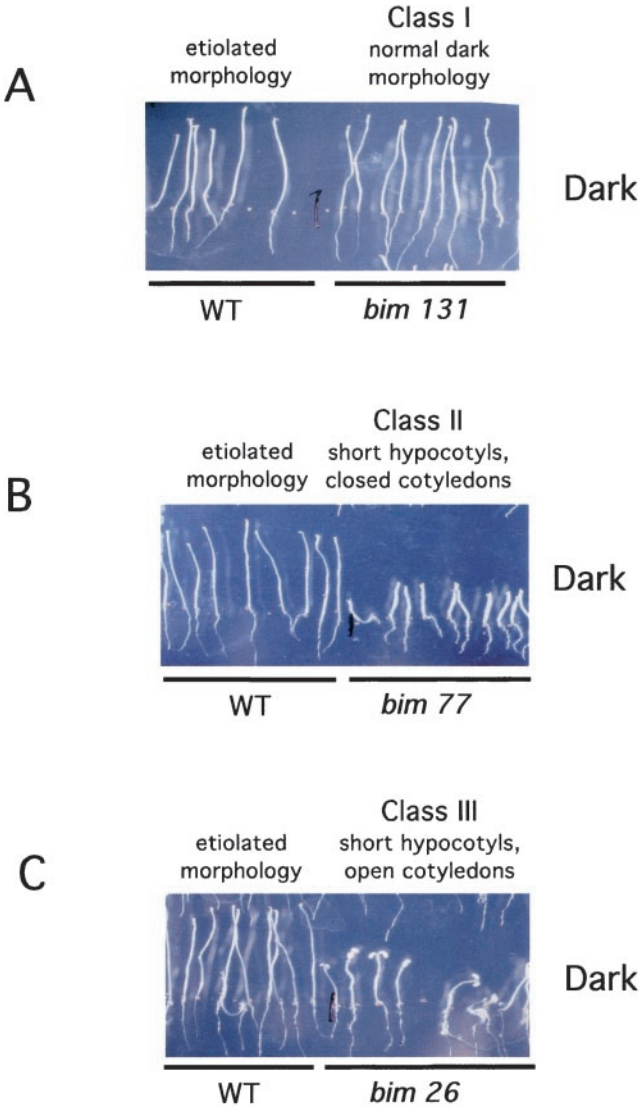

Figure 7. *bim* mutants are subgrouped into three separate classes based on their dark-grown morphology. *bim* mutants were cultivated on Murashige and Skoog media in the absence of BMAA and grown in the dark for 5 d. Class-I *bim* mutants exhibit a normal etiolated phenotype (Fig. 7A, right) when grown in the dark compared with wild type (Fig. 7A, left). Class-II *bim* mutants have a short hypocotyl and closed cotyledons (Fig. 7B, right) when compared with wild type (Fig. 7B, left). Class-III *bim* mutants have a short hypocotyl and open cotyledons (Fig. 7C, left) when compared with wild type (Fig. 7C, right).

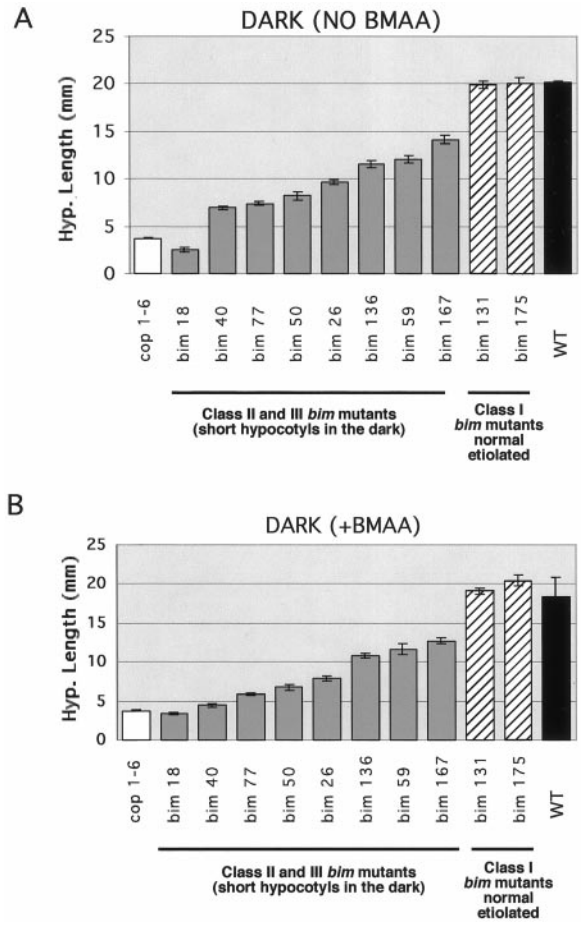

Figure 8. Quantification of hypocotyl lengths of *bim* mutants grown in the dark in the absence or presence of BMAA. *bim 18, 40, 77, 50, 26, 136, 59, 131, 167,* and *175* and wild type were grown for 5 d in the dark in the absence (Fig. 8A) or presence of 50 μM BMAA (Fig. 8B). Hypocotyl lengths were quantified. Wild type is indicated with black bars. Class-I *bim* mutants (*bim 131* and *bim 175*) have wild-type length hypocotyls and are indicated with hatched bars. Class-II and -III *bim* mutants, which have short hypocotyls in the dark, are marked with gray shaded bars. *cop1-6* is shown in the far left side of the graph (white bar). The average hypocotyl length for each treatment is shown (n = 30). Error bars show the SE of the mean.

photomorphogenic mutant, *cop1-6* (Kendrick and Nagatani, 1991; Deng and Quail, 1992) (Figs. 6 and 8). BMAA does not induce elongation of the *cop1-6* hypocotyl. However, interpretation of these results must await molecular analysis of lesions in the *bim* mutants. It is important to test whether class II and III *bim* mutants are allelic to these previously characterized *cop*, *det*, *fus*, and *shy* mutants or whether they represent new loci. It is also important to test for allelism between the different *bim* mutants and to map the *bim* mutants to determine whether they are genetically linked to any AtGLR genes in Arabidopsis.

An important aspect of development in seedlings involves the complicated interplay of light and various phytohormones. Auxin (Jensen et al., 1998; Kim et al., 1998), gibberellin (Jacobsen and Olszewski, 1993; Steber et al., 1998), and brassinolide (Fujioka et al., 1997; Azpiroz et al., 1998) all act as positive regulators of hypocotyl elongation in Arabidopsis. Ethylene (Kieber et al., 1993) and cytokinins (Chory et al., 1991a), conversely, are believed to act as inhibitors of hypocotyl elongation. Thus, the BMAA-mediated effects on hypocotyl length in light-grown plants may also involve the interaction of one or more of these phytohormones. It is also possible that BMAA blocks transduction of light signals, which inhibit hypocotyl elongation. Blue light (Liscum and Hangarter, 1991; Ahmad and Cashmore, 1993; Lasceve, et al., 1999), red light (Somers et al., 1991; Nagatani et al., 1993), and far-red light (Dehesh et al., 1993) are the major, incident wavelengths perceived by plants that repress hypocotyl elongation in Arabidopsis. In future studies, it will be important to determine whether BMAA effects are specific to one or more of these wavelengths in Arabidopsis.

One interesting result from our studies is that BMAA induces hypocotyl elongation in Arabidopsis specifically in the light at low concentrations (20–50 μM BMAA) (Fig. 2A). Because BMAA exerts its effects at low (μM) concentrations, this suggests that BMAA could act as a signaling compound in plants. In species of the Cycadaceae, BMAA is detected at high levels (milligram BMAA/gram of tissue) (Vega and Bell, 1967; Duncan et al., 1989; Pan et al., 1997). The presence of high levels of BMAA in such tissues has led some researchers to suggest that BMAA may act as a toxin against predators (Ladd et al., 1993). This theory of herbivore deterrence may explain why neurotoxins, such as BMAA are synthesized at high levels in plants. Our phylogenetic studies on GLR genes in plants and animals suggests that iGluRs are derived from a primitive signaling mechanism that existed before plants and animals diverged (Chen et al., 1999; Chiu et al., 1999). Those studies, plus the ones described herein, suggest the intriguing possibility that iGluR agonists made by plants may serve not only as herbivore deterrents, but may also act as signaling molecules affecting developmental processes in plants. We postulate, for example that BMAA, which appears to affect photomorphogenesis in Arabidopsis, may also alter light signaling in cycads. Whether BMAA plays a signaling role in cycads, or is even present at low levels in other species of higher plants are open questions that remain to be answered. Using the Arabidopsis *bim* mutants to understand the mode of action of a cycad-derived iGluR agonist in plants may help to address these questions. Furthermore, using *bim* mutants to understand how BMAA mediates its effects in Arabidopsis could potentially lead to new therapeutic treatments of iGluR-related neurological disorders in humans.

MATERIALS AND METHODS

Culture of Arabidopsis Plants

Arabidopsis ecotype Columbia seeds were plated on Murashige and Skoog media (Murashige and Skoog, 1962), 0.1% (w/v) MES [2-(*N*-morpholino)ethanesulfonic acid] (Sigma M-2933) containing 0.5% (w/v) Suc and 0.7% (w/v) agar. Arabidopsis seeds were placed for 2 d at 4°C on the growth media. Plants were grown in square (100 × 15 mm) plates in a vertical position. Light grown plants were grown at 22°C during a cycle of 16-h light/8-h dark under cool-white fluorescent bulbs (General Electric, Fairfield, CT). Plants received a fluence level ranging from 40 to 60 μE. For dark grown seedlings, plants were incubated for an initial 4 h in the light to stimulate germination. After the light pretreatment, dark-grown plants were wrapped in two layers of foil and grown in the dark for 5 d at 22°C.

BMAA (L-BMAA hydrochloride) was purchased from RBI. L-Glu (Sigma G-1501), L-Asp (Sigma A-6558), and L-Gln (Sigma G-3126) stocks were dissolved in water and the pH was adjusted to 5.7.

cop 6-1 seeds used as a control were a gift from Dr. Kameda, Hokkaido University (Hokkaido, Japan).

Measurement of Hypocotyl Length and Cotyledon Opening

Plants were grown in the light for 5 to 8 d. Hypocotyl length was measured under the view of a dissecting scope. The top of the hypocotyl was defined as the point where the petioles of the cotyledons are attached to the axis. The bottom of the hypocotyl was determined as the root-shoot junction (with the root being defined as the point where root hairs are initially apparent). Cotyledon separation was measured by projecting the slide image of the seedlings onto a screen. Two lines were then drawn along the petioles over the image of the cotyledons and the angle was measured where these two lines intersected (at the shoot apex of the seedling).

Screen for *bim* Mutants in EMS Mutagenized M2 Lines

EMS mutagenized Columbia Co-3, glabrous seedlings (Lehle Seeds, Round Rock, TX) were cultivated as described above on Murashige and Skoog media (with 0.1% [w/v] MES, pH 5.7; 0.5% [w/v] Suc) plates containing 50 μM BMAA. Mutants with a *BMAA insensitive morphology* (*bim*) were screened after 8 to 12 d of growth, at which time they were transferred to Murashige and Skoog media lacking BMAA. After 1 to 2 weeks, the plants were transplanted to soil and allowed to set seed.

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