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Regular Article

Quantitative evaluation of the biological activity of various brassinosteroids using spiral root induction in rice seeds

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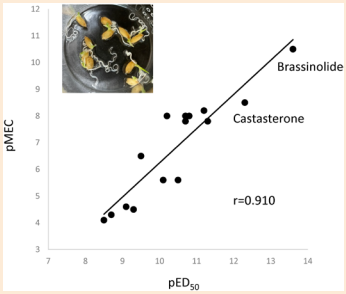
Supplementary material

Spiral roots are induced in germinated rice seeds through treatment with nanomolar brassinosteroids (BRs) but not with other plant hormones. Here, we determined the minimum effective concentration (MEC) of various BRs to induce spiral roots in germinated rice seeds. The reciprocal logarithm of MEC, pMEC, was used as the BL-like activity index, which was linearly correlated with the reciprocal logarithm of a 50% effective dose (pED_{50}) as evaluated in the rice lamina inclination assay. Furthermore, a ligand-receptor docking simulation was performed against the BL receptor complex, *Arabidopsis thaliana* BRI1/SERK1, and the binding free energy (ΔG_{bind}) was calculated for the tested BRs. The ΔG_{bind} calculation was performed using the molecular mechanics/generalized Born surface area method on an ensemble of uncorrelated snapshots collected *via* molecular dynamics to predict biological activity.



Keywords: brassinosteroids, spiral root induction, docking simulation, MM/PBSA, MM/GBSA.

Introduction

Brassinolide (BL, **1**; Fig. 1) was discovered as a plant growth promoter in 1979 by a USDA group. BL has a steroidal skeleton constructed from four fused rings (A/B/C/D) and hydroxyalkyl chains at the C17 position.[1)] The B-ring of BL is an ε-caprolactone structure (seven-membered ring), but a few years later, castasterone (CS, **2**; Fig. 1) with a six-membered cyclohexanone B-ring was isolated from chestnut insect gall.[2)] Various BL and CS analogs have been identified in nature and/or chemically synthesized, which are collectively called brassinosteroids (BRs).[3)] In previous structure–activity studies, the BL-specific bioassay systems described below were necessary to distinguish BRs from other plant hormones. To search for BL-like compounds, an *in vitro* rice lamina inclination assay (RLIA) was established,[4)] and later, an *in vivo* RLIA was developed.[5)] Previously, we synthesized various BL and CS analogs and quantitatively measured their BL-like activity using an *in vivo* RLIA.[6–8)] Other bioassay methods have been developed to measure the BL-like activity specifically, but many of them were not as simple.[9)]

Whether BL is a genuine plant steroid hormone was disputed after the first characterization of BL from *Brassica napus* pollen, because many steroidal compounds are found in plants as secondary metabolites. Two decades later, however, BL was confirmed as a 6th plant hormone by the discovery of a BL-deficient mutant.[10)] Later, BR insensitive receptor kinase 1 (BRI1) was identified as the BL receptor,[11)] and the binding site of BL to BRI1 was confirmed by Kinoshita *et al.* in 2005.[12)] In 2011, the three-dimensional structures of BRI1 were worked out by two groups,[13,14)] and two years later, the crystal structure of BRI1-BL complexed with a kinase, somatic embryogenesis receptor-like kinase 1 (SERK1), was elucidated.[15)] The establishment of

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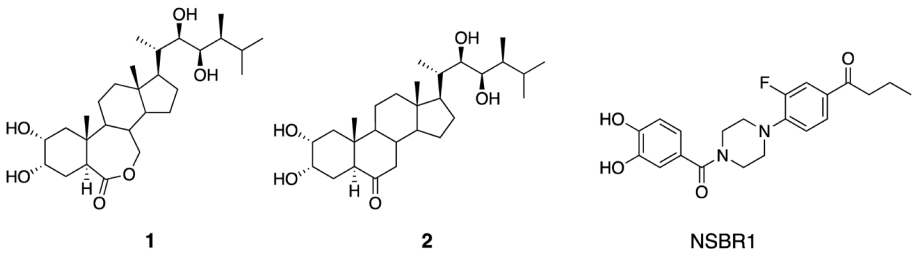


Fig. 1. Structures of brassinolide (**1**), castasterone (**2**), and a non-steroidal BL agonist, NSBR1.

3-D structures of the BL receptor opened the door for *in silico* screening of the BL-like compounds. We found three BL antagonists to the rice plant[16] and two BL antagonists to Arabidopsis[17] as shown in Fig. 2, using LigandScout[18] in our *in silico* approach.

Sugiura *et al.* modified the structure of the antagonists (**B1** and **B2**; Fig. 2) successfully to get an agonist toward Arabidopsis, which was named NSBR1 (Fig. 1).[17,19] However, the activity of NSBR1 was 30,000 times less potent than BL in terms of the 50% effective dose [ED_{50} (mol/plant)] in the RLIA. Watanabe *et al.* calculated the binding free energy (ΔG_{bind}) of BL (**1**), CS (**2**), and their analogs to the BL receptor of Arabidopsis using molecular dynamics (MD) and the molecular mechanics/Poisson Boltzman surface area (MM/PBSA)[20] and found the linear correlation between pED_{50} and ΔG_{bind}.[17] Sugiura *et al.* also executed a docking simulation for NSBR1 to calculate ΔG_{bind}, which was predictable from the correlation between the ΔG_{bind} and pED_{50} derived for BRs.[17]

Even though the RLIA is a good bioassay system to determine the BL-like activity quantitatively, several processes are required to obtain reproducible data. Particularly, it is not easy to evaluate the activity of weak compounds, because they easily crystallize on the surface of the rice plant from the applied droplet of highly concentrated MeOH or EtOH solutions. Dimethyl sulfoxide (DMSO) is often used in bioassays to relieve the drawback of the property of alcohols, but DMSO cannot be used in the RLIA due to its toxic effect on the rice plant (personal communication). Therefore, the RLIA is difficult to use as a bioassay system to find leads and/or hits in a drug discovery study when the biological activity is generally very weak. To overcome the drawback of the RLIA, we developed a bioassay system named the spiral root induction assay (SRIA).[21] The SRIA, regarding the spiral root induction as an index of BL-like activity is very simple and user friendly.

The aim of the present study is to measure the biological activity of various BRs quantitatively using the SRIA and discuss the structure–activity relationship of BRs. The minimum effective concentrations (MECs) required to induce the spiral root in the germinated rice seeds were determined for various BRs, and their reciprocal logarithm values (pMECs) were compared with corresponding pED_{50} values determined in the RLIA. Furthermore, the ΔG_{bind} of ligands to the receptor is calculated using the molecular mechanics generalized Born surface area (MM/GBSA) method to examine the relationship with biological activity. While two popular methods, MM/PBSA and MM/GBSA, have been used to estimate the ΔG_{bind} of small ligands for biological macromolecules,[22–24] we previously employed MM/PBSA to calculate the ΔG_{bind} of ecdysteroids[25] and brassinosteroids[8] and found good correlation between ΔG_{bind} and biological activity. In this study, however, we used MM/GBSA, since MM/GBSA calculation is accurate and relatively simple, providing stable and reproducible results that are computationally effective for the *in silico* study.[23]

Materials and methods

1. Observation of the root grown in rice seeds

The bioassay method is basically the same as that previously reported by our group.[21] Rice seeds of *Oryza sativa* Koshihikari cultivated in Shiga Prefecture were soaked in running tap water for a few days to germinate. Bioassay solutions were prepared by

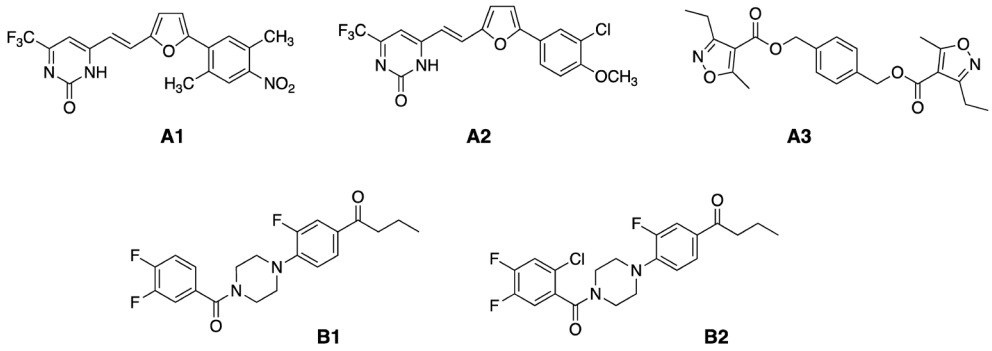


Fig. 2. Chemical structures of BL antagonists found through *in silico* screening against the rice plant (**A1**–**A3**) and Arabidopsis (**B1** and **B2**).

Table 1. The BL-like activity of various BRs and the ΔG_{bind} to the BL receptor protein

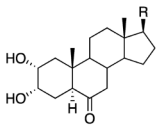
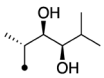
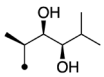
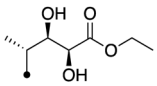
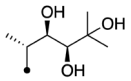
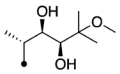
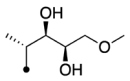
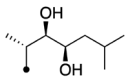
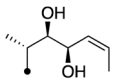
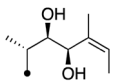
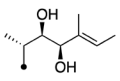
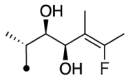
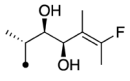
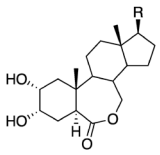
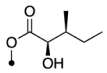
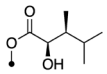
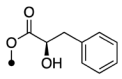
		BL-like activity		ΔG_{bind}[c)]	
No.	Compounds	pMEC[a)]	pED_{50}[b)]	*A. thaliana*	*O. sativa*
1	BL	10.5±0.23 (4)	13.6	−78.7±5.0	−79.2±5.8
2	CS	8.5±0 (3)	12.3	−75.1±5.3	−90.1±5.3
					
	R				
3		8.0±0 (3)	10.7	−71.3±2.8	−87.7±1.0
4		4.1±0.17 (3)	8.5	−67.3±3.7	−80.9±3.7
5		4.3±0 (3)	8.7	−64.9±5.0	−74.9±8.5
6		6.1±0 (3)	<10.0 (10%)	−72.4±3.1	−75.2±10.8
7		6.4±0.17 (3)	<9.0 (39%)	−69.2±3.8	−77.6±1.3
8		6.0±0.17 (3)	<9.2 (0.1%)	−71.8±2.1	−84.1±2.0
9		5.9±0.17 (3)	<9.3 (11%)	−67.5±3.3	−85.3±4.0
10		8.0±0.17 (3)	10.2	−72.6±3.9	−87.4±6.4
11		6.4±0.17 (3)	9.5	−70.0±6.7	−82.2±9.0
12		7.8±0 (3)	10.7	−75.0±6.1	−80.8±1.4
13		7.8±0 (3)	11.3	−69.4±6.0	−79.4±4.5
14		8.0±0.17 (3)	10.8	−73.0±5.0	−86.7±6.6
15		8.2±0.17 (3)	11.2	−68.4±2.2	−83.5±0.4

Table 1. Continued

No.	Compounds	BL-like activity		ΔG_{bind}[c)]	
		pMEC[a)]	pED_{50}[b)]	*A. thaliana*	*O. sativa*
					
	R				
16		4.15±0.21 (2)	<8.0 (13%)	−57.3±2.5	−75.3±3.2
17		4.0±0.17 (3)	<8.0 (41%)	−66.1±1.6	−78.6±1.6
18		<4.3 (2)	<8.0 (48%)	−68.8±0.9	−79.6±0.9
19		<4.3 (2)	<8.0 (44%)	−61.8±5.6	−71.4±2.8
20		4.5±0.17 (3)	9.3	−60.6±4.9	−72.8±4.9
21		5.6±0 (3)	10.5	−69.6 ±2.7	−82.5±6.6
22		5.6±0 (3)	10.1	−64.5±3.1	−83.7±6.5
23		5.1±0 (3)	<8.7 (16%)	−67.8±4.8	−72.8±4.4
24		4.6±0 (3)	9.1	−62.1±5.5	−81.4±2.5

[a)] Mean±standard deviation. The numbers in parentheses are the replication numbers. [b)] From Ref. 6–8. Measured by RLIA. The numbers in parentheses are the promotion of the lamina inclination at the corresponding dose. [c)] Mean±standard deviation for three calculations.

adding an aliquot of DMSO or EtOH solution to 2 mL of water in a 50 mL beaker (Fig. S1). The 10 germinated seeds were put into beakers containing bioassay solutions. Germinated seeds were cultured for two days at 25°C, and the grown root was observed.

The EtOH and DMSO stock solutions for test compounds were prepared by roughly threefold dilutions (*i.e.*, 10 mM, 3.33 mM, 1 mM, 0.33 mM, *etc.*), and 10 μL of each stock solution was added to the water (2 mL) to prepare the bioassay solutions. In some cases, the addition of an aliquot volume of stock solutions was changed to prepare the bioassay solutions, as shown in Fig. S2 and Fig. S3. In some cases, the volume of the additional aliquot of stock solutions was changed. Changing the stock solution aliquot volume is a convenient way to prepare the various bioassay solutions. To determine the MEC of BL precisely, the volume of the added aliquot of EtOH stock solutions (200 nM and 2 nM) was changed. As shown in Fig. S2, spiral roots were induced in all concentrations prepared from a 200 nM stock solution. However, when the diluted stock solution (2 nM) was used, the spiral roots were observed in the bioassay solution prepared by adding 30 μL of a 2 nM stock solution. The MEC of BL required to induce the spiral root in more than 50% of the treated seeds was determined to be 3×10^{-11} (M). We examined another concentration-response as shown in Fig. S3. The reciprocal logarithm of MEC, pMEC, was used as the activity index. We repeated this experiment three times and took the average of three pMEC values (10.7, 10.5, and 10.3) as 10.5±0.2 (n=3) for BL. The pMEC values of other BRs were obtained in the similar way, and they are listed in Table 1.

2. MD and the binding free energy calculation

We previously conducted a simulation of BR docking to the

crystal structure of BRI1/SERK1 (PDB: 4LSX) using the FRED of OEDocking (OpenEye), then the calculated ΔG_{bind} value using the MD-MM/PBSA of AMBER14 (http://ambermd/org/).[8,17,25] In this study we used AMBER20. MD simulation was performed for 150 ps and 150 trajectories at 1.0 ps intervals were obtained. Energy calculation was performed using the MM/GBSA, because the MM/GBSA produces better performance in ranking the binding affinities for systems without metals and has a much lower computer-resource demand than the MM/PBSA method.[22–24]

Results and discussion

1. BL-like activity of various BRs

We previously reported that BL induced the spiral root in germinated rice seeds at an extremely low concentration (5 nM) in the SRIA.[21] We also reported that a BL antagonist unwound the spiral root induced by BL treatment, and this can be used as a bioassay method to detect both BL-like compounds and their antagonists. Since other plant hormones, such as gibberellin and auxin, did not induce the spiral root even at high concentrations (50 μM), the SRIA is a good bioassay method to find BL-like compounds. Some of the assay water solutions were prepared by changing the addition of stock solution. As shown in the supplementary figure (Fig. S1), the addition of 100 μL of EtOH to 2 mL of water did not affect the root growth, but 100 μL of DMSO significantly inhibited the root growth. The addition of 40 and 50 μL of EtOH and DMSO seemed to be nontoxic to rice seeds, but the maximum volume of the aliquot of stock solutions in 2 mL of water was kept below 30 μL (<1.5%) to avoid the toxic effect of the solvent.

In this study, the MECs required to induce the spiral root were determined for 14 BL and 10 CS analogs, and their pMEC values are listed in Table 1. Among 24 tested BRs, only two compounds (**18** and **19**) were inactive in the SRIA. As shown in Table 1, the pMEC of BL (**1**) was determined to be 10.5, 100 times more potent than CS (**2**; pMEC=8.5). This is comparable to the result evaluated by in the RLIA (pED_{50}: 13.6 *vs.* 12.3). Even though the pED_{50} values of CS analogs (**6–9**)[8] and BL analogs (**16–19**, and **23**)[6] could not be obtained using an RLIA, their pMEC values were determined to be 4.0–6.4 in the SRIA. MEC values were not determined for compounds **18** and **19**, but MEC values of **16** and **17** were determined at the lower concentrations than MECs of compounds **18** and **19**. This is probably due to the difference of solubility among compounds. We compared the pMEC and pED_{50} values for 15 compounds to derive a good correlation (r=0.910), as shown in Fig. 3.

Figure 3 indicates that the SRIA can be used as a suitable bioassay method to quantitatively measure BL-like activity against rice plants. The SRIA is also advantageous in that the person who is not familiar with the bioassay of BRs can perform it without training and quantitatively determine the BL-like activity of BRs within a few days. Moreover, germinated seeds for the SRIA can be easily prepared, whereas it takes a week or more to prepare the biological materials, such as a rice shoot with a second leaf, in an RLIA. Furthermore, DMSO can be used as a carrier solvent for the test compound in an SRIA, which is not the case in an RLIA.

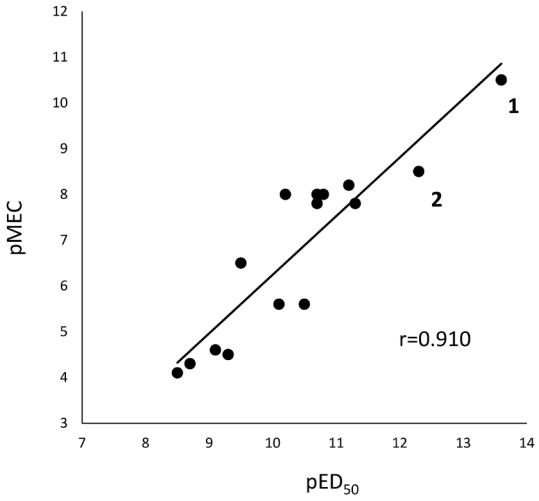


Fig. 3. Relationship between pED_{50} (RLIA) and pMEC (SRIA).

2. In silico *study*

As shown in Table 1, we could quantitatively determine the biological activity of various BRs in an SRIA in terms of the pMEC. Here, we conducted MD for all 24 BRs and calculated their ΔG_{bind} using the MM/GBSA. As stated above, the MM/GBSA was used in this study to calculate the ΔG_{bind}, instead of the MM/PBSA, because the calculation cost of the MM/GBSA method is low for obtaining results comparable with those using an MM/PBSA. As shown in Fig. 4, the MM/GBSA worked well to calculate the ΔG_{bind} to the Arabidopsis BL receptor to show a linear correlation with the pMEC. At present, MD simulation using the MM/PBSA or MM/GBSA is the most important tool for evaluating the affinity of ligand molecules to a receptor by calculating the free energy change in the binding process.

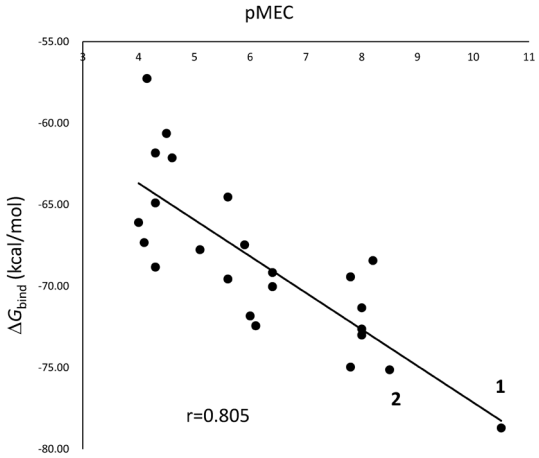


Fig. 4. Relationship between the pMEC and the ΔG_{bind} to Arabidopsis receptor.

Yamamuro *et al.* isolated the rice *BRI1* homologous gene, *O. sativa BRI1* (*OsBRI1*), which was very similar to the *Arabidopsis thaliana BRI1* (*AtBRI1*) gene.[26] Since the bioassay was conducted using rice materials, we built a 3-D structure of OsBRI1 to calculate the ΔG_{bind} to the rice receptor. The 3-D model of OsBRI1 is well superposed on the crystal structure of AtBRI1 (PDB: 4LSX). The ΔG_{bind} values calculated using the MM/GBSA (Table 1) were linearly correlated with the pMEC, but the correlation (r=0.553) was not as good as that shown in Fig. 4. This is probably due to the poor prediction of the side chains of amino acid residues surrounding ligands in the 3-D model constructed in this study. It is very likely that a good correlation will be observed when the experimentally solved 3-D structure of OsBRI1 is available or the modelling system is further improved.

In our previous study, the ΔG_{bind} to the Arabidopsis receptor was calculated for BRs using the MD-MM/PBSA method and linearly correlated with the pED_{50} values measured in the RLIA.[8] Sugiura *et al.* also calculated the ΔG_{bind} of NSBR1, which is predicted well by the relationship between ΔG_{bind} and pED_{50} for BRs. However, some of the ΔG_{bind} values of weak compounds, including NSBR1, were positive, which is not adequate to express the specific ligand-receptor binding. In the MM/PBSA, the exterior dielectric constant is set as 78.5, but the interior dielectric constant (ε_{in}) is changeable from 1 to 4, and 1 was used in the previous calculation.[8,17] According to Wang,[24] the higher solute dielectric constant (ε_{in}=2 or 4) is preferable to improve the rescoring accuracy of the MM/PBSA. A value of 2 was assigned for the dielectric constant of non-polar residues, a value of 3 for polar residues, and a value of 4 charged residues. Matsuo recalculated ΔG_{bind} using 2 in the MM/PBSA and obtained negative ΔG_{bind} for all active compounds (unpublished). The correlation between the pED_{50} and ΔG_{bind} calculated using ε_{in}=2 was slightly better than the previous one (Fig. S4). The ΔG_{bind} values of all BL-like compounds calculated in the MM/GBSA were negative. MM/PBSA calculations are very time consuming, but the MM/GBSA requires far fewer computer resources than the MM/PBSA method. It is said that the accuracy of the calculated energy using the MM/GBSA is compromised at the expense of computational speed.[24] The correlation shown in Fig. 4 means that the MM/GBSA calculation is sufficient for practical use, such as *in silico* drug design.

Conclusion

The SRIA is a simple method to detect BL-like activity. The MEC of BL was determined to be 3×10^{-11} M (pMEC=10.5). The BL-like activity in terms of the pMEC was linearly correlated with the pED_{50} measured in the RLIA. DMSO can constitute up to 1.5% of the assay solution in the SRIA. Therefore, the concentration of test compounds can be enhanced in the SRIA. This constitutes an advantage over the RLIA, in which DMSO cannot be used. The docking and MD simulations were performed for BRs against the crystal structure of the BRI1/SERK1 complex of Arabidopsis and the modelled receptor structure of the rice plant. The ΔG_{bind} calculated in the MD-MM/GBSA was linearly correlated with the pMEC determined in the SRIA. This study contributes to the search for and rational design of BL-like compounds.

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Electronic supplementary materials

The online version of this article contains supplementary materials (Fig. S1–S4) which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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