The selectivity of 6-nor-ABA and 7’-nor-ABA for abscisic acid receptor subtypes

The selectivity of 6-nor-ABA and 7′-nor-ABA for abscisic acid receptor subtypes

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Abstract

Abscisic acid (ABA), a plant hormone, is involved in many plant development processes and environmental stress responses that are regulated by a Pyrabactin Resistant 1 (PYR)/Pyrabactin Resistant-Like (PYL)/Regulatory Component of ABA Receptor (RCAR) receptor protein-mediated signal transduction pathway. In *Arabidopsis thaliana*, PYL proteins constitute a 14-member family comprising two distinct subclasses: dimeric receptors (PYR1 and PYL1–PYL3) and monomeric receptors (PYL4–PYL13). The individual contributions of PYL subclasses/subtypes with specific physiological actions are still poorly understood; consequently, the development of PYL subclass/subtype-selective agonists should be useful to reveal the different functions of these receptors. In this study, we focused on the ABA analogs 6-nor-ABA and 7′-nor-ABA, which were expected to function as monomeric receptor-selective agonists on the basis of crystal structures of PYL-ABA complexes and sequence alignments of PYL subtypes. In a protein phosphatase 2C (PP2C) assay, the agonist activities of both analogs were lower than those of ABA towards all tested PYL proteins, regardless of subclass/subtype. Nevertheless, we found that 6-nor-ABA acts as a selective agonist at the physiological level: it induced stomatal closure but did not inhibit seed germination and root growth. On the basis of observed inhibitory activity against PP2C among different PYL subtypes, this biological effect of 6-nor-ABA may be attributed to the activity of that agonist on PYL5 and/or PYL6.

Keywords

Abscisic acid, PYR/PYL/RCAR receptor, Agonist

The plant hormone abscisic acid (ABA, compound **1**) plays a key role in many physiological processes, such as seed dormancy, root growth, stomatal closure and abiotic stress response [1,2]. The physiological actions of ABA are controlled by a signal transduction process involving the interaction of two types of proteins: a PYR/PYL/RCAR (PYL) receptor and group-A protein phosphatases 2C (PP2Cs)—including HAB1, ABI1 and ABI2—that act as negative regulators of ABA signaling. By binding to PYL proteins, ABA induces a conformational change associated with a mobile loop (gate) closure that enables the receptor to bind and inhibit PP2Cs [3–6]. Arabidopsis PYL proteins, which constitute a 14-member family [7,8], are divided into two distinct subclasses according to their oligomeric state: dimeric receptors (PYR1 and PYL1–PYL3) and monomeric receptors (PYL4–PYL13) [9–11]. Among these proteins, the receptor property of PYL13 remains controversial. Several studies have indicated that PYL13, which lacks the lysine residue crucial for ABA binding, does not bind ABA and inhibits specific PP2Cs independently of ABA [11,12]. In contrast, a recent report has claimed that PYL13 inhibits these PP2Cs in an ABA-dependent manner[13]. Further study is needed to resolve this discrepancy.

Both dimeric and monomeric receptors are involved in ABA-induced physiological responses, with each receptor contributing additively to regulation of ABA responses [14,15]. These receptors differ substantially in function, as evidenced by the contrasting expression patterns of the genes encoding various PYL-subtype members. Although PYL8 is known to play a nonredundant role in root sensitivity to ABA [15], characterization of the individual PYL subtypes is generally difficult because of the functional redundancy of the receptors. The details of these functional differences are thus largely unknown. A chemical compound capable of selectively activating PYL subclasses/subtypes would be a valuable tool for evaluation of the effect of the specific receptors on the various roles of ABA. Although some selective agonists of dimeric receptors, such as pyrabactin and quinabactin, have been described [16,17], no reports have appeared of agonists that preferentially activate monomeric receptors. In the present study, we explored the possibility that a structural analog of ABA could function as a monomeric receptor-selective agonist, a role suggested by an examination of the crystal structures of several PYL-ABA complexes [18,19] and sequence alignments of PYL subclasses/subtypes. Although

numerous ABA analogs have been designed and synthesized [20], few have been evaluated from this perspective.

In PYL-ABA complexes, ABA establishes a hydrophobic network with amino acid residues at the α3 helix and the gate loop to stabilize the gate-closed form of PYL proteins. A comparison of PYL subtypes reveals only one residue differing between dimeric and monomeric receptors, namely, a valine (Val) present in dimeric receptors that is replaced by leucine (Leu) (PYL7-10) or isoleucine (Ile) (PYL4-6, PYL11 and PYL12) in monomeric receptors (Supplementary Fig. S1). Because of its location in the α3 helix, this residue should be involved in the hydrophobic interaction with the C6 and/or C7′ methyl group of the ABA molecule. A consideration of steric bulkiness suggests that hydrophobic interactions should be induced most easily by Leu, followed by Ile and then Val. We therefore predicted that monomeric receptors can adopt the gate-closed form; this should be true even in ABA analogs lacking the C6 or C7′ methyl group (6-nor-ABA, compound **2**, and 7′-nor-ABA, compound **3**, respectively) because of their bulky residues, which likely compensate for the weakening of the hydrophobic interaction due to elimination of the C6 or C7′ methyl group. These two compounds have been reported to be weak ABA agonists [21], but their activities towards PYL proteins have not been investigated. We therefore investigated whether 6-nor-ABA and 7′-nor-ABA can function as monomeric receptor-selective agonists.

(±)-6-Nor-ABA was prepared according to the method of Ueno et al. (2005), while the synthesis of (±)-7′-nor-ABA was carried out following a modification of the route of Nanzyo et al. (1977), as shown in Scheme 1. In particular, the carbonyl group of compound **4** (prepared as reported previously)[23] was protected by treating with ethylene glycol in the presence of pyridinium *p*-toluenesulfonate to afford the ketal **5** [24]. Allylic oxidation with manganese (III) acetate and *tert*-butyl hydroperoxide afforded ketone **6** [25]. The side chain was introduced by direct addition of (*Z*)-3-methyl-2-penten-4-yn-1-ol using *n*-butyllithium, generating the alcohol **7**. Reduction (generating compound **8**), oxidation (generating compound **9**) and esterification of **7** resulted in the formation of the ester **10**, followed by acidic and basic hydrolysis of the ester to give (±)-7′-nor-ABA (**11**). (±)-6-Nor-ABA and (±)-7′-nor-ABA were optically resolved

by HPLC on a chiral column to obtain the corresponding (+)-isomers with the same exciton chirality as that of *S*-(+)-ABA [21]. Since the unnatural type of ABA analogs showed weaker activities than the natural type of them (Supplementary Fig. S2), we used analogs with the same exciton chirality of the natural type of ABA for all assays.

To examine the agonist activities of 6-nor-ABA and 7′-nor-ABA towards different subclasses/subtypes of PYL proteins, we assessed receptor-mediated PP2C inhibition using nine Arabidopsis PYL proteins (dimeric subclass: PYR1 and PYL1–PYL3; monomeric subclass: PYL4–PYL6, PYL8 and PYL10) and the PP2C HAB1. 6-Nor-ABA induced PYL3, PYL5 and PYL6 to inhibit HAB1, while 7′-nor-ABA caused activation of PYL2, PYL3, PYL5, PYL6 and PYL10. The activities of both analogs were weaker than those of ABA (Fig. 2a). Dose-response analysis revealed that the IC_{50} values of 6-nor-ABA and 7′-nor-ABA for PYL5, the PYL subtype most sensitive to both compounds, were 510 and 360 nM, respectively (Fig. 2b). These values were approximately 10-fold higher than that of ABA (50 nM). Additionally, isothermal titration calorimetry (ITC) was used to measure the binding affinity of 6-nor-ABA and 7′-nor-ABA for PYL5 (Fig. 3). The K_d values of 6-nor-ABA and 7′-nor-ABA were 16.7 and 4.7 μM, respectively, which were 5- to 20-fold higher than that of ABA (0.88 μM) [26]. Thus, 6-nor-ABA and 7′-nor-ABA were less effective than ABA towards all PYL proteins tested, implying that the C6 and C7′ methyl groups of ABA play an important role in receptor binding regardless of PYL subclass/subtype. These results suggest that the PYL-subtype selectivity of ABA analogs is not solely determined by the amino acid residues involved in the gate closure.

To analyze the plant physiological effects of ABA and its analogs, we focused on 6-nor-ABA, a more narrow-spectrum agonist for PYL subtypes (PYL3, PYL5 and PYL6). As PYL3 expression is extremely low throughout plant development [14], 6-nor-ABA may act as a weak PYL5- and PYL6-selective agonist in plants. With respect to its potency, the PP2C assay and the ITC experiment revealed that 6-nor-ABA was able to activate PYL5 as strongly as ABA when present in a 20-fold excess over ABA. This result suggested that 6-nor-ABA could be used to examine the effect of PYL5 and PYL6 on the various physiological actions of ABA. To investigate the relationship between the activation of PYL5/PYL6 and ABA-induced

physiological responses, we tested the effect of 6-nor-ABA in three different physiological assays. In an Arabidopsis seed germination assay, the inhibitory activity of 6-nor-ABA was at least 100-fold lower than that of ABA (Fig. 4). A similar trend was observed in root growth inhibition of 5-day-old seedlings (Fig. 5). In contrast, 6-nor-ABA showed relatively potent agonistic activity in the induction of stomatal closure and drought tolerance. Because transpiration lowers leaf temperatures through evaporative cooling [27], we tested the effect of 6-nor-ABA on stomatal apertures using thermal-imaging methods. We observed that ABA-treated seedlings showed increased leaf surface temperatures by reducing transpiration. Treatment with 6-nor-ABA induced stomatal closure (Fig. 6a) and drought tolerance (Fig. 6b) as effectively as did ABA when the former was present in 10-fold excess over ABA; this result is consistent with the observations of agonist potency towards PYL5. Additionally, the profile of this ligand was similar to that of 1′-*O*-methyl-ABA, which has been found to selectively activate PYL5 in a PP2C assay as well[28]. These results suggest that PYL5 plays an important role in the induction of stomatal closure, which is in agreement with the phenotype of the PYL5 over-expression[6]. The selectivity of 6-nor-ABA for PYL subtypes in plants should be investigated in detail at the genetic levels; however, this selectivity has potential use, both as a chemical probe and as a practical application to induce drought tolerance in plants without inhibiting growth.

References and notes

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Figure legends

Graphical abstract 6-Nor-ABA, a weak selective agonist for PYL3, PYL5 and PYL6, showed ABA-like activity in the induction of stomatal closure but not in the inhibition of seed germination and root growth.

Figure 1. Structures of abscisic acid (ABA) and its analogs.

Figure 2. The comparative effects of 6-nor-ABA, 7′-nor-ABA and ABA on inhibition of HAB1 by ABA receptors. Chemical inhibition of HAB1 by (a) various ABA receptors in the presence of 10 μM of each test compound or (b) PYL5 in the presence of various concentrations (0.05, 0.1, 0.5, 1, 5 and 10 μM) of 6-nor-ABA or 7′-nor-ABA.

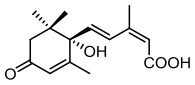
Figure 3. Isothermal titration calorimetric analysis of the binding of 6-nor-ABA and 7′-nor-ABA to PYL5.

Figure 4. The comparative effects of 6-nor-ABA and ABA on Arabidopsis seed germination. The seed germination rate in response to ABA or 6-nor-ABA was measured at 48 h after stratification (n = 3; error bars represent s.d.).

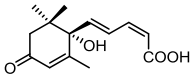
Figure 5. The comparative effects of 6-nor-ABA and ABA on Arabidopsis seedling root growth.

Figure 6. The comparative effects of 6-nor-ABA and ABA on Arabidopsis (a) leaf surface temperature and (b) drought tolerance.

Scheme 1. Synthesis of (±)-7′-nor-ABA. Reagents: (i) ethylene glycol, PPTS; (ii) $Mn_3O(OAc)_9$, tBuO$_2$H, O_2, EtOAc; (iii) (*Z*)-3-methylpent-2-en-4-yn-1-ol, n-BuLi, THF; (iv) SMEAH, THF; (v) MnO_2, acetone; (vi) MnO_2, NaCN, AcOH, MeOH; (vii) 1 M HCl, acetone; (viii) 1 M NaOH, MeOH.



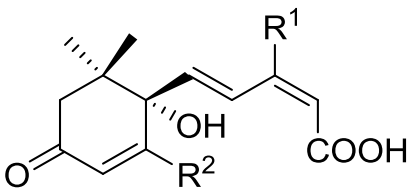
ABA



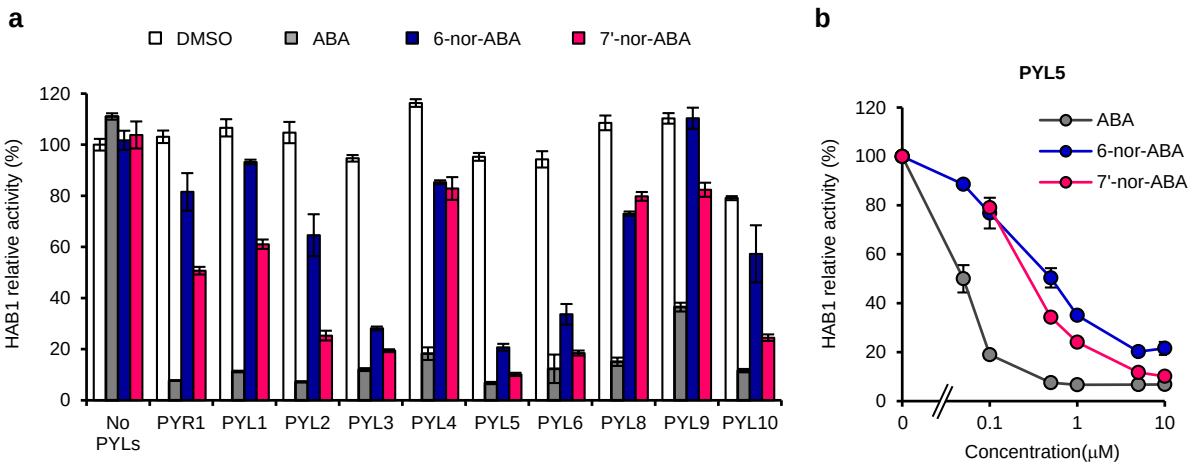
6-nor-ABA

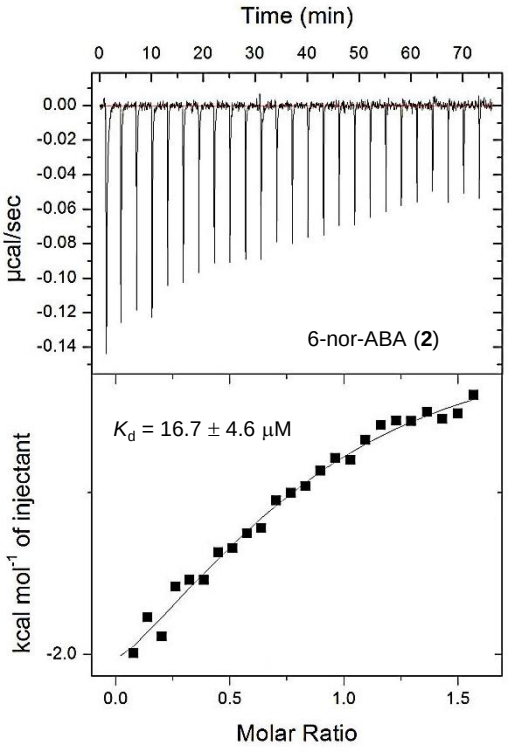
Activated PYL subtypes	PYR1, PYL1-12	PYL3, PYL5, PYL6
Drought tolerance	++	+
Inhibition of seed germination	++	–
Inhibition of root growth	++	–

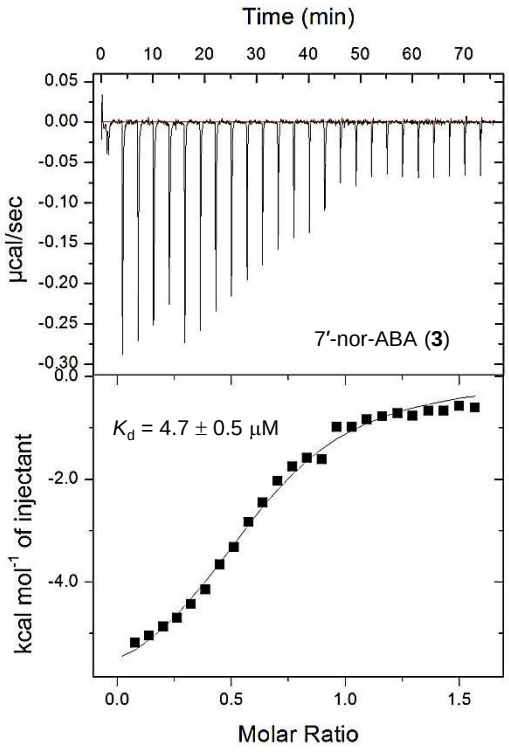
++, 1×; +, 1/10; −, <1/100

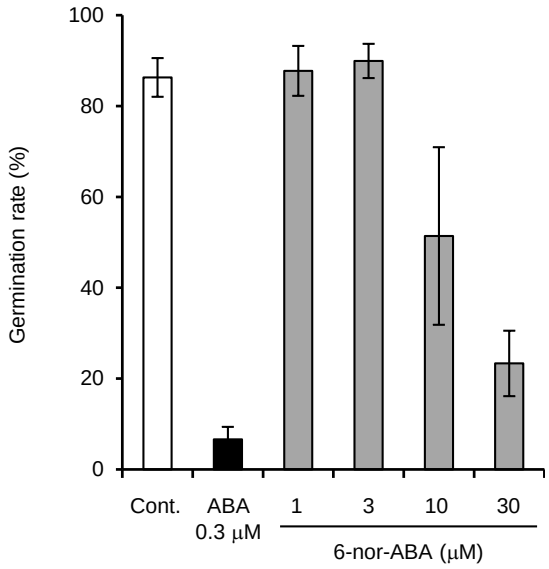


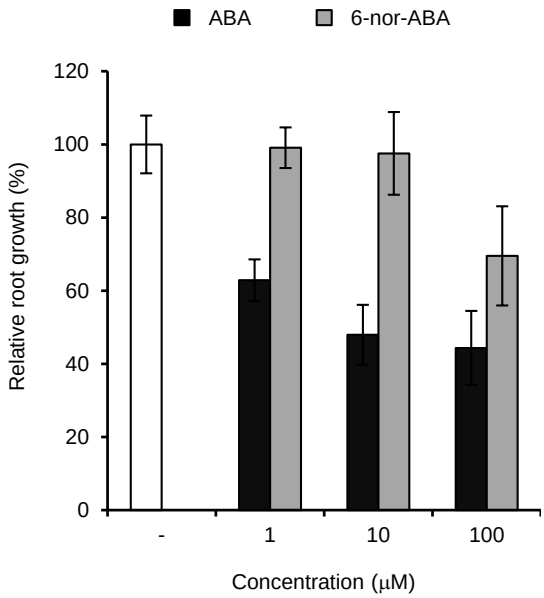
ABA (**1**): R^1 = Me, R^2 = Me
6-nor-ABA (**2**): R^1 = H, R^2 = Me
7'-nor-ABA (**3**): R^1 = Me, R^2 = H

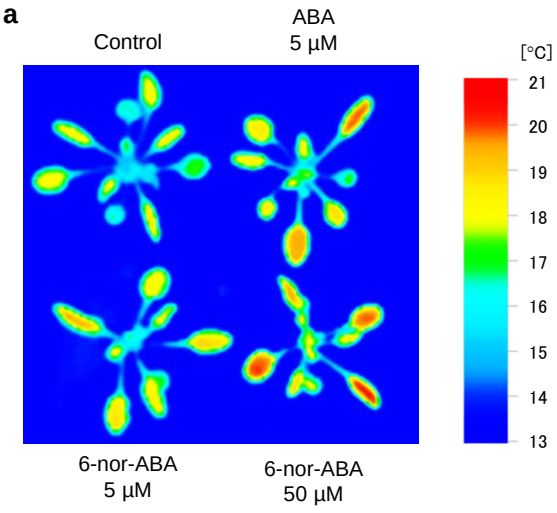

a
DMSO
ABA
6-nor-ABA
7'-nor-ABA
HAB1 relative activity (%)
120
100
80
60
40
20
0
No PYLs
PYR1
PYL1
PYL2
PYL3
PYL4
PYL5
PYL6
PYL8
PYL9
PYL10
b
PYL5
ABA
6-nor-ABA
7'-nor-ABA
HAB1 relative activity (%)
120
100
80
60
40
20
0
0
0.1
1
10
Concentration(μM)

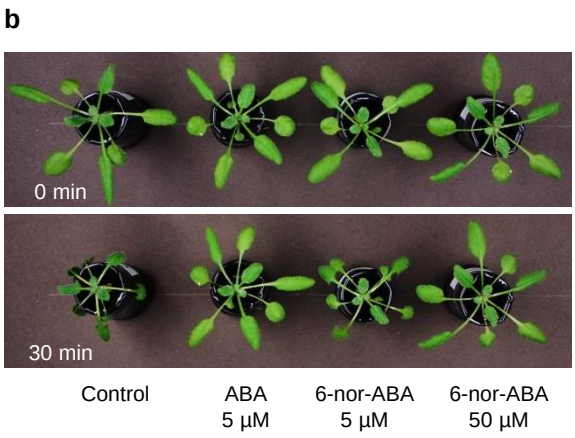

Time (min)
0 10 20 30 40 50 60 70
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
-0.14
µcal/sec
6-nor-ABA (2)
K_d = 16.7 ± 4.6 µM
kcal mol^{-1} of injectant
-2.0
0.0 0.5 1.0 1.5
Molar Ratio

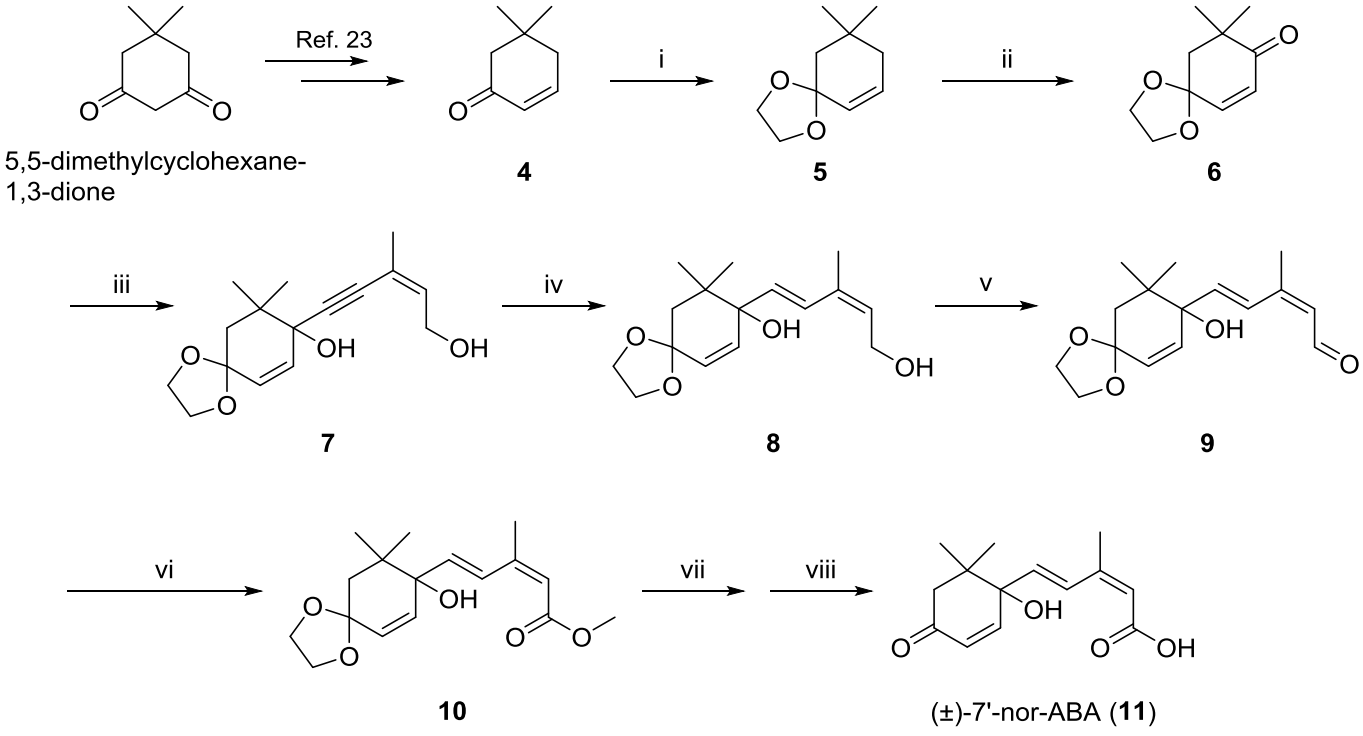

Time (min)
0 10 20 30 40 50 60 70
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
µcal/sec
7′-nor-ABA (3)
K_d = 4.7 ± 0.5 µM
kcal mol^{-1} of injectant
0.0
-2.0
-4.0
0.0 0.5 1.0 1.5
Molar Ratio


Germination rate (%)
100
80
60
40
20
0
Cont.
ABA
0.3 μM
1
3
10
30
6-nor-ABA (μM)


ABA
6-nor-ABA
120
100
80
60
40
20
0
Relative root growth (%)
-
1
10
100
Concentration (μM)


a
Control
ABA
5 µM
[°C]
21
20
19
18
17
16
15
14
13
6-nor-ABA
5 µM
6-nor-ABA
50 µM


b
0 min
30 min
Control
ABA
5 µM
6-nor-ABA
5 µM
6-nor-ABA
50 µM



5,5-dimethylcyclohexane-1,3-dione

4 **5** **6**

7 **8** **9**

10

(±)-7'-nor-ABA (**11**)

The selectivity of 6-nor-ABA and 7′-nor-ABA for abscisic acid receptor subtypes

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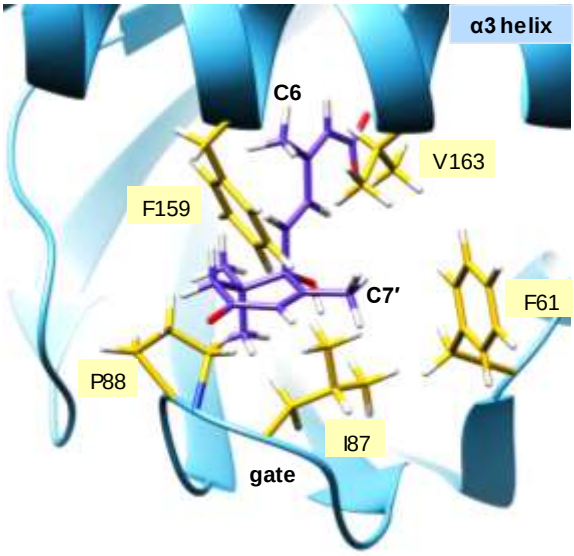
Supplementary data

1) Supplementary Figures

2) The procedures for preparation of (±)-7′-nor-ABA, ITC experiments and biological assays

1. Supplementary Figures

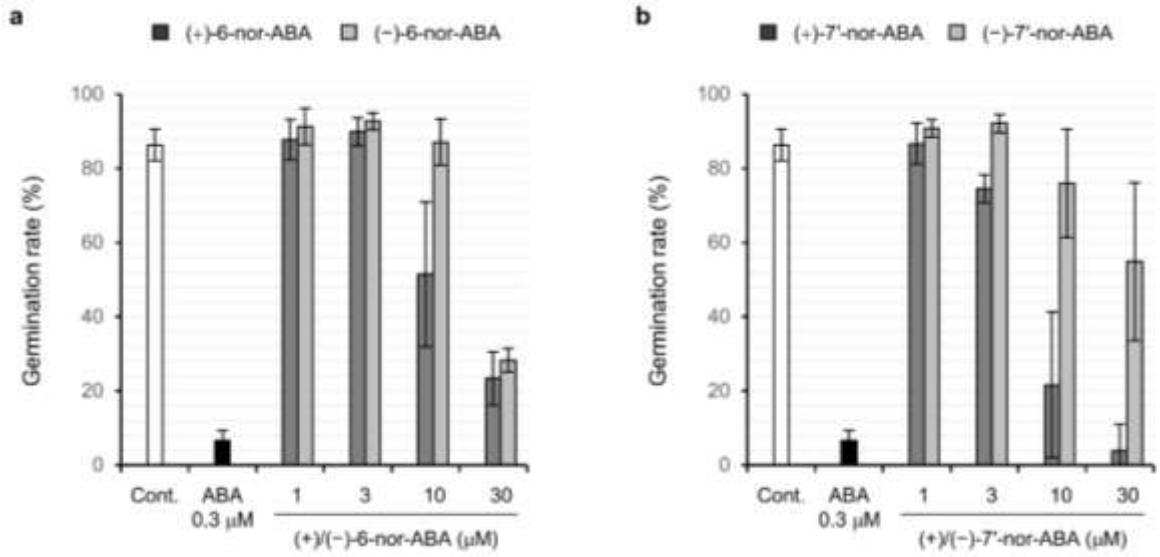
a



b

		Amino acid (PYR1 #)				
		61	87	88	159	**163**
Dimeric	PYR1	F	L	P	F	**V**
	PYL1	F	L	P	F	**V**
	PYL2	F	L	P	F	**V**
	PYL3	F	L	P	F	**V**
Monomeric	PYL4	F	L	P	F	**I**
	PYL5	F	L	P	F	**I**
	PYL6	F	L	P	F	**I**
	PYL7	F	L	P	F	**L**
	PYL8	F	L	P	F	**L**
	PYL9	F	L	P	F	**L**
	PYL10	F	L	P	F	**L**
	PYL11	F	L	P	F	**I**
	PYL12	F	L	P	F	**I**
	PYL13	F	F	P	F	**I**

Supplementary Figure S1. Comparison of dimeric- and monomeric-receptor amino acid residues involved in gate closure via hydrophobic interactions with the ABA molecule. (a) The crystal structure of the PYR1-ABA complex (PDB code 3K3K). (b) Sequence alignment of amino acid residues located at the entrance of the ligand binding pocket of PYL subtypes.



Supplementary Figure S2. Comparison of the activity of (+)-isomers of ABA analogs and their (−)-isomers. Arabidopsis seed germination rate in response to 6-nor-ABA (a) and 7′-nor-ABA (b) at 48 h after stratification (n = 3; error bars represent s.d.).

2. Preparation of (±)-7′-nor-ABA

2-1. 9,9-dimethyl-1,4-dioxaspiro[4.5]dec-6-en-8-one (6)

To a solution of **4** (2.02 g, 16.2 mmol), ethyleneglycol (1.36 mL, 24.4 mmol) and *p*-toluenesulfonate pyridinium (20.4 mg, 0.08 mmol) in anhydrous benzene (45 mL) was heated for 10.5 h under reflux in a Dean-Stark unit. After cooling the reaction mixture at room temperature, it was quenched with sat. $NaHCO_3$ solution (5.5 mL) and then diluted with diethyl ether (80 mL). The organic layer was separated, washed with brine and dried over Na_2SO_4, and then concentrated *in vacuo*. The residual oil was purified by silica gel chromatography with 3% EtOAc in hexane to obtain **5** (405 mg) as a mixture of the undesired regioisomer (1:1, according to ^{1}H NMR), inseparable by chromatography. To a mixture of **5** and its regioisomer (400 mg, 2.4 mmol) in EtOAc (20 mL) was added *tert*-butyl hydroperoxide in decane (2.4 mL, 12 mmol). The mixture was stirred at room temperature for 30 min under an atmosphere of Ar. Manganese (III) acetate dihydrate (64 mg, 0.24 mmol) was added. The reaction mixture was filled with oxygen (balloon), and stirred for 3 d. The reaction was filtered through a pad of Celite® and concentrated *in vacuo*. The crude products were purified by silica gel chromatography with 10% EtOAc in hexane to obtain **6** (82.2 mg, 3%) as a colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$): δ_H 1.21 (6H, s, 2 × CH_3), 2.09 (2H, d, *J*=1.0 Hz, H_2-5), 4.03 (4H, br s, -O*C*H_2*C*H_2O-), 5.95 (1H, d, *J*=10.2 Hz, H-2), 6.51 (1H, dt, *J*=10.2 and 1.0 Hz, H-3). HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{10}H_{14}O_3Na$, 205.0841; found, 205.0844.

2-2. (*Z*)-8-(5-hydroxy-3-methylpent-3-en-1-yn-1-yl)-9,9-dimethyl-1,4-dioxaspiro[4.5]dec-6-en-8-ol (7)

(*Z*)-3-Mehylpent-2-en-4-yn-1-ol (side chain) (68 mg, 0.70 mmol) in dry THF (3.5 mL) was cooled to −80 °C under an atmosphere of Ar. *n*-Butyllithium (0.90 mL, 1.58 M) was then added slowly. After being stirred for 30 min at −80 °C, a solution of the **6** (64 mg, 0.35 mmol) in dry THF (0.5 mL) was added to the stirred mixture. The reaction mixture was stirred for a further 15 min at −80 °C and then the ice bath was removed. The reaction mixture was stirred at room temperature for 90 min. After quenching with sat. NH_4Cl solution (100 mL), it was extracted with EtOAc (15 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residual oil was purified by silica gel chromatography with 55% EtOAc in hexane to obtain **7** (58 mg, 59%) as a yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$): δ_H 1.12 (3H, s, H_3-8′ or 9′), 1.17 (3H, s, H_3-8′ or 9′), 1.88 (3H, d, *J*=1.0, H_3-6), 1.94 (2H, s, H_2-5′), 2.08 (1H, s, -O*H*), 3.96 (4H, br s, -O*C*H_2*C*H_2O-), 4.29 (2H, d, *J*=5.3 Hz, H_2-1), 5.60 (1H, d, *J*=9.9 Hz, H-2′ or 3′), 5.87 (1H, td, *J*=5.3 and 1.0 Hz, H-2), 5.89 (1H, d, *J*=9.9 Hz, H-2′ or 3′); ^{13}C NMR (68 MHz, $CDCl_3$): δ_C 22.6, 23.0, 25.6, 39.2, 43.7, 61.6, 64.3, 64.5, 72.0, 77.2, 84.1, 104.7, 120.5, 127.0, 134.0, 136.1. HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{16}H_{22}O_4Na$, 301.1416; found, 301.1415.

2-3. 8-((1*E*,3*Z*)-5-hydroxy-3-methylpenta-1,3-dien-1-yl)-9,9-dimethyl-1,4-dioxaspiro[4.5]dec-6-en-8-ol (8)

To a stirred solution of **7** (55 mg, 0.20 mmol) in dry THF (2 mL) was cooled to −18 °C and added sodium bis (2-methoxyethoxy) aluminum hydride in toluene 65 % w/w (SMEAH) (0.21 mL, 0.69 mmol) under an atmosphere of Ar. The mixture was stirred for 60 min at room temperature. After quenching with sat. NH_4Cl solution (5 mL), it was extracted with EtOAc (10 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residual oil was purified by silica gel chromatography with CH_2Cl_2-EtOAc stepwise to obtain **8** (18.7 mg, 34%) as a yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$): δ_H 0.93 (3H, s, H_3-8′ or 9′), 1.10 (3H, s, H_3-8′ or 9′), 1.39 (1H, br s, -O*H*), 1.69 (1H, s, -O*H*), 1.79 (1H, d, *J*=15.2 Hz, H-5′), 1.87 (1H, d, *J*=15.2 Hz, H-5′), 1.87 (3H, s, H_3-6), 3.96 (4H, m, -O*C*H_2*C*H_2O-), 4.31 (2H, d, *J*=6.6 Hz, H_2-1), 5.59 (1H, t, *J*=6.6 Hz, H-2), 5.61 (1H, d, *J*=9.9 Hz, H-2′ or 3′), 5.66 (1H, d, *J*=9.9 Hz, H-2′ or 3′), 5.81 (1H, d, *J*=15.8 Hz, H-5), 6.65 (1H, d, *J*=15.8 Hz, H-4); ^{13}C NMR (68 MHz, $CDCl_3$): δ_C 20.6, 23.3, 24.9, 38.8, 44.4, 58.2, 64.2, 64.6, 77.2, 104.9, 126.2, 127.2, 128.7, 132.6, 134.7, 135.5. HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{16}H_{24}O_4Na$, 303.1572; found, 303.1570.

2-4. Methyl (2*Z*,4*E*)-5-(8-hydroxy-9,9-dimethyl-1,4-dioxaspiro[4.5]dec-6-en-8-yl)-3-methylpenta-2,4-dienoate (10)

To a stirred solution of **8** (18 mg, 0.06 mmol) in dry acetone (2 mL) was added MnO_2 (131 mg, 1.28 mmol) at room temperature. After string at room temperature for 30 min, all the starting material had disappeared. The reaction mixture was then filtered through a pad of Celite® and concentrated *in vacuo*. The crude material (22.5 mg) was carried through to the next stage without further purification. The crude aldehyde **9** (22.5 mg) was dissolved in methanol (2 mL) and stirred with MnO_2 (131 mg, 1.28 mmol), NaCN (9.4 mg, 0.19 mmol) and AcOH (4 µL, 0.06 mmol) at room temperature. After 2 h, the reaction mixture was filtered through a pad of Celite® and concentrated *in vacuo*. The residual oil was purified by silica gel chromatography with 30% EtOAC in hexane to obtain **10** (16.5 mg, 83%) as a yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$): δ_H 0.94 (3H, s, H_3-8′ or 9′), 1.11 (3H, s, H_3-8′ or 9′), 1.82 (1H, s, -O*H*), 1.86 (2H, s, H_2-5′), 2.00 (3H, d, *J*=1.0 Hz, H_3-6), 3.71 (3H, s, -O*C*H_3), 3.99 (4H, m, -O*C*H_2*C*H_2O-), 5.65 (1H, d, *J*=10.6 Hz, H-2′ or H-3′), 5.68 (1H, d, *J*=10.6 Hz, H-2′ or H-3′), 5.70 (1H, br s, H-2), 6.16 (1H, d, *J*=16.2 Hz, H-5), 7.76 (1H, d, *J*=16.2 Hz, H-4); ^{13}C NMR (68 MHz, $CDCl_3$): δ_C 21.2, 23.4, 25.0, 38.8, 44.6, 51.1, 64.2, 64.6, 77.2, 104.8, 117.5, 127.0, 127.4, 135.2, 138.7, 150.2, 166.7. HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{17}H_{24}O_5Na$, 331.1512; found, 331.1516.

2-5. (2*Z*,4*E*)-5-(1-hydroxy-6,6-dimethyl-4-oxocyclohex-2-en-1-yl)-3-methylpenta-2,4-dienoic acid, (±)-7′-nor-ABA (11)

A solution of 1 M HCl (0.4 mL) was added to a solution of **10** (13 mg, 0.04 mmol) in acetone (2 mL), and the resulting mixture was stirred for 60 min at room temperature. After quenching with sat. $NaHCO_3$ (3 mL), it was diluted with H_2O (5 mL) and extracted with EtOAc (8 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude material (16.8 mg) was carried through to the next stage without further purification. A solution of 1 M NaOH (2 mL) was added to a solution of crude methyl ester (16.8 mg) in MeOH (2 mL), and reaction mixture was stirred for 3 h at room temperature. The pH of the reaction mixture was adjusted to 2 using 1 M HCl, it was extracted with EtOAc (10 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residual oil was purified by silica gel chromatography with 50% EtOAc in hexane containing 0.2% AcOH to obtain **(±)-7′-nor-ABA** (8.4 mg, 80%) as a white solid. ^{1}H NMR (270 MHz, CD_3OD): δ_H 1.00 (3H, s, H_3-8′ or 9′), 1.08 (3H, s, H_3-8′ or 9′), 2.04 (3H, d, *J*=1.0 Hz, H_3-6), 2.28 (1H, d, *J*=16.5 Hz, H-5′), 2.49 (1H, d, *J*=16.5 Hz, H-5′), 5.74 (1H, br s, H-2), 5.98 (1H, d, *J*=10.2 Hz, H-3′), 6.31 (1H, d, *J*=16.2 Hz, H-5), 6.62 (1H, d, *J*=10.2 Hz, H-2′), 7.80 (1H, d, *J*=16.2 Hz, H-4); ^{13}C NMR (68 MHz, CD_3OD): δ_C 21.2, 23.7, 24.8, 42.7, 50.3, 77.6, 119.6, 128.4, 129.3, 138.3, 151.1, 154.3, 169.4, 201.8. HRMS (*m/z*): $[M+Na]^+$ calcd. for $C_{14}H_{18}O_4Na$, 273.1094; found, 273.1103.

A Chiralcel OD HPLC column (250 × 10.0 mm i.d., Daicel; solvent, 12% 2-propanal in hexane containing 0.1% AcOH; flow rate, 4.7 ml/min; detection, 254 nm) was injected with (±)-7′-nor-ABA. The material at t_R 10.8 and 15.2 min were collected to give (−)-7′-nor-ABA (2.3 mg) and the (+)-enantiomer (2.3 mg) with an optical purity of 99.9% and 99.7%, respectively. (+)-7′-nor-ABA: $[\alpha]_D^{29}$ +458.8 (MeOH; *c* 0.115); CD λ_{ext} (MeOH) nm (Δε): 264.0 (34.7), 225.0 (−29.2). (−)-7′-nor-ABA: $[\alpha]_D^{29}$ −448.9 (MeOH; *c* 0.115); CD λ_{ext} (MeOH) nm (Δε): 264.0 (−35.0), 225.0 (26.7).

3. ITC experiment

The ITC experiments were performed with an iTC_{200} calorimeter (Microcal, GE Healthcare Bio-Sciences AB) as described previously (Takeuchi, J.; Okamoto, M.; Akiyama, T.; Muto, T.; Yajima, S.; Sue, M.; Seo, M.; Kanno, Y.; Kamo, T.; Endo, A.; Nambara, E.; Hirai, N.; Ohnishi, T.; Cutler, S. R.; Todoroki, Y. *Nat. Chem. Biol.* **2014**, *10*, 477–82). His_6-tagged PYL5 was assayed at a concentration of 50 μM with (+)-6-nor-ABA and (+)-7′-nor-ABA stock solutions in the injection syringe at concentration of 500 μM. All of the titrations were carried out via a series of 25 injections of 1.25 μL each. The data were corrected by subtracting the mixing enthalpies for the (+)-6-nor-ABA or (+)-7′-nor-ABA solutions into protein-free solutions and fitted by Origin for ITC (GE Healthcare Bio-Sciences AB) with a 1/1 binding model.

4. Arabidopsis seed germination assay

Twenty to thirty-five seeds (Columbia accession) were sterilized successively with soaking in 70% aqueous ethanol (EtOH, v/v) for 30 min and reagent-grade EtOH for 1 min. They were then soaked in distilled water and incubated in darkness at 5 °C for 3 d. The stratified seeds then soaked in 100 μL of a test medium liquid agar in 96-well plates and allowed to germinate under continuous illumination at 22 °C. All of the assays were conducted at least three times.

5. Root growth assay

Seedlings were grown on 1/2 MS plates for 5 days afterward, 12 plants were transferred to new 1/2 MS plates lacking or supplemented with the indicated concentrations of 6-nor-ABA or ABA. The plates were scanned on a flatbed scanner after 7 days to measure the root growth using the Image software ImageJ version 1.48. Data are averages from three independent experiments (n = 12 each; error bars represent s.d.).

6. Thermal imaging

Four-week-old *Arabidopsis* plants were treated by aerosol spray with 1 mL containing 5 or 50 μM compound and 0.1% spreader, and were incubated under continuous illumination at 22 °C for 1 h. Thermal images were obtained using Testo 881-2 thermography (Testo, Inc., Sparta, NJ, USA).

7. Drought tolerance assay

Four-week-old *Arabidopsis* plants were treated by aerosol spray with 1 mL containing 5 and 50 μM compound and 0.1% spreader, and were incubated under continuous illumination at 22 °C for 2 h. The plants were transferred to empty tubes and exposed to drought stress under same conditions for 30 min.