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Screening and characterization of an inhibitory chemical specific to Arabidopsis gibberellin 2-oxidases

Masato Otani, Jung-Min Yoon, Seung-Hyun Park, Tadao Asami, Masatoshi Nakajima *

Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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ABSTRACT

The hydroxylation of gibberellin (GA) at the 2-position is known as the major cause of inactivation of GAs, whose reaction is catalyzed by 2-oxoglutarate dependent dioxygenases, also termed GA 2-oxidases (GA2oxs). To block GA catabolism in plants, a few chemicals can be used. To obtain novel inhibitors specific to GA2oxs, we performed in vitro random screenings by using ^{3}H-16,17-dihydro-GA$_4$ and recombinant Arabidopsis GA2ox2. As a result, one candidate, methyl 6-chloro-3*H*-1,2,3-benzodithiazole-4-carboxylate 2-oxide (CBTC), was selected from the screening, and was subjected to in-planta evaluations. CBTC promoted both the germination and elongation of Arabidopsis seedlings. This strongly suggests that CBTC inhibits GA2oxs in Arabidopsis with high specificity.

Gibberellin (GA) is a plant hormone that regulates various developmental processes in plants like seed germination, stem elongation, flowering, and fruit ripening.[1,2] For those phenomena, the fluctuation pattern of GAs and their related gene expression are precisely regulated. The biosynthetic pathway of GA in plants is generally illustrated in Fig. 1. GA$_4$, which shows the highest affinity to GA receptors, is thought to be the most active form of GA.[3] The endogenous amount of biologically active GA depends on the balance of its metabolism and catabolism. For the catabolism of GA, hydroxylation at the 2-position by GA 2-oxidase (GA2ox) was initially reported.[4] Besides the GA2oxs, other enzymes which catalyze the methylation of the 6-carboxy group,[5] or the epoxydation of exo-methylene at C-16 and 17,[6] have also been identified (Fig. 1).

The analysis of multiple loss-of-function mutants revealed that GA2oxs are the major enzymes for GA inactivation in the growth of Arabidopsis.[7] The orthologous genes for GA2ox were cloned from various plants.[4,8] GA2ox is classified as a *2-oxoglutarate dependent dioxygenase* (2ODD). Other 2ODDs, GA 3-oxidase (GA3ox) and GA 20-oxidase (GA20ox) function in GA biosynthesis (Fig. 1).[1,9] Consequently, the two 2ODDs and GA2ox are homologous to each other in their amino acid sequences.[4]

Prohexadione (PHX, **1** shown in Fig. 2) is a universal inhibitor of 2ODDs carrying a cyclohexadione moiety.[10,11] The target of PHX is the binding site of 2-oxoglutarate,[10,12] and therefore the chemical is effective for other 2ODDs, for example, biosynthetic enzymes for flavonoids.[12] So GA20ox, GA3ox, and GA2ox are all inhibited by PHX.[10] This implies that the application of PHX to plants leads to a starvation of endogenous GAs caused by the inhibition of the GA-biosynthetic enzymes, GA20ox and GA3ox (Fig. 1). This broad characteristic of PHX is advantageous for the application to various kinds of crops to reduce the endogenous GAs, although its low specificity may be a weak point to the fine regulation of GAs. In this report, by using a recombinant GA2ox from Arabidopsis and its radio-labeled substrate ^{3}H-16,17-dihydro-GA$_4$, we tried to screen new inhibitors for GA2ox having more specificity than PHX.

Firstly, we selected one Arabidopsis GA2ox as a tool.[13] Seven genes (*GA2ox1* through *GA2ox8*, excluding *GA2ox5*) encoding GA2ox exist in the Arabidopsis genome.[1] After the biochemical characterization of those gene products, they were divided into two groups; the first shows a preference to biologically active GAs (GA$_4$/GA$_1$) or to their direct precursors (GA$_9$/GA$_{20}$),[4] while the second shows a preference to precursors distant from active GAs (GA$_{12/53}$) (Fig. 1).[14] We focused on GA2ox2 which belongs to the first group and has a major function in Arabidopsis seed germination.[15] Recombinant GA2ox2 fused with thioredoxin- and His-tags was prepared and confirmed by an SDS–PAGE profile (60 kDa, data not shown).

To validate the enzymatic activity of GA2ox2, GA$_4$ or 16,17-dihydro-GA$_4$ were incubated with the affinity-purified

Abbreviations: GA, gibberellin; GA2ox, gibberellin 2-oxidase; 2ODD, 2-oxoglutarate dependent dioxygenase; PHX, prohexadione.

* Corresponding author. Tel.: +81 3 5841 5192; fax: +81 3 5841 8025.
E-mail address: nkjm@pgr1.ch.a.u-tokyo.ac.jp (M. Nakajima).

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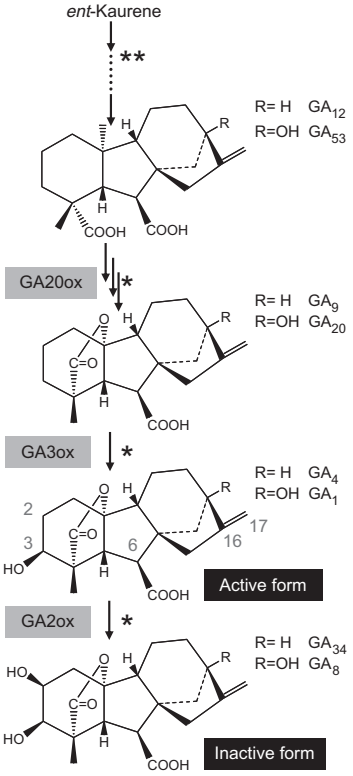

Figure 1. Biosynthesis of gibberellin in higher plants. The inhibition sites of prohexadione (PHX, **1**) are indicated with an asterisk (*). The inhibition site of paclobutrazol is indicated with double asterisks (**). GA20ox, gibberellin 20-oxidase; GA3ox, gibberellin 3-oxidase, and GA2ox, gibberellin 2-oxidase.

recombinant GA2ox2 and other required adjuncts. Each metabolite was then identified by using GC/MS on the basis of Kovats' retention index (KRI) and the full-scan mass spectra of each methyl ester trimethylsilyl ether derivative.[16] As a result, the 2 beta-hydroxylated metabolite for each examined GA was identified; GA_{34} (as for the substrate GA_4), KRI 2721, 506 (100), 459 (15), 416 (12), 387 (18), 372 (18), 356 (11), 313 (15), 288 (13), 272 (17), 241 (20), 223 (38), 217 (36); 16,17-dihydro-GA_{34} (as for the substrate 16,17-dihydro-GA_4), KRI 2769, 508 (100), 461 (9), 420 (15), 374 (13), 345 (9), 315 (9), 291 (24), 263 (15), 235 (17), 181 (14), 129 (18), 103 (45). As a negative control, no 2 beta-hydroxylated metabolite was detected in the reaction mixture when the affinity-purified negative control fraction (vector only) was used instead of the GA2ox2 fraction. Hereby we confirmed that the recombinant GA2ox2 has enzymatic activity, just as other GA2oxs.

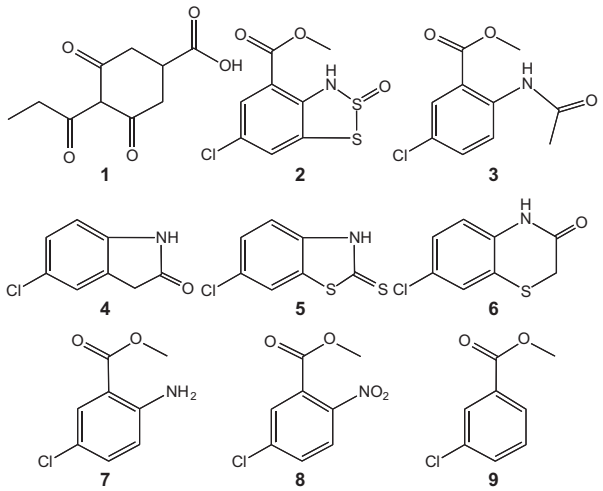

Figure 2. Chemical structures appearing in this Letter. (**1**) Prohexadione (PHX); (**2**) methyl 6-chloro-3*H*-1,2,3-benzodithiazole-4-carboxylate 2-oxide (CBTC); (**3**) 2-acetamido-5-chlorobenzoic acid methylester; (**4**) 5-chlorooxindole; (**5**) 6-chloro-2-mercaptobenzothiazole; (**6**) 7-chloro-2*H*-1,4-benzothiazin-3(4*H*)-one; (**7**) methyl 5-chloroanthranilate; (**8**) methyl 5-chloro-2-nitrobenzoate; and (**9**) methyl 3-chlorobenzoate.

The enzymatic activity of GA2ox2 is also visible by using radiolabeled substrate ^{3}H-16,17-dihydro-GA_4 and an imaging analyzer.[8] To evaluate the concentration of inhibitors, different amounts (0, 1–3000 μM) of PHX were added to the reaction mixture as a positive control assigning an inhibitory effect to GA2ox. As shown in Figure 3a, PHX surely inhibited the production of the metabolite from ^{3}H-16,17-dihydro-GA_4 in a dose-dependent manner.[17] In this system, the IC_{50} of PHX was estimated to be ca. 30 μM.

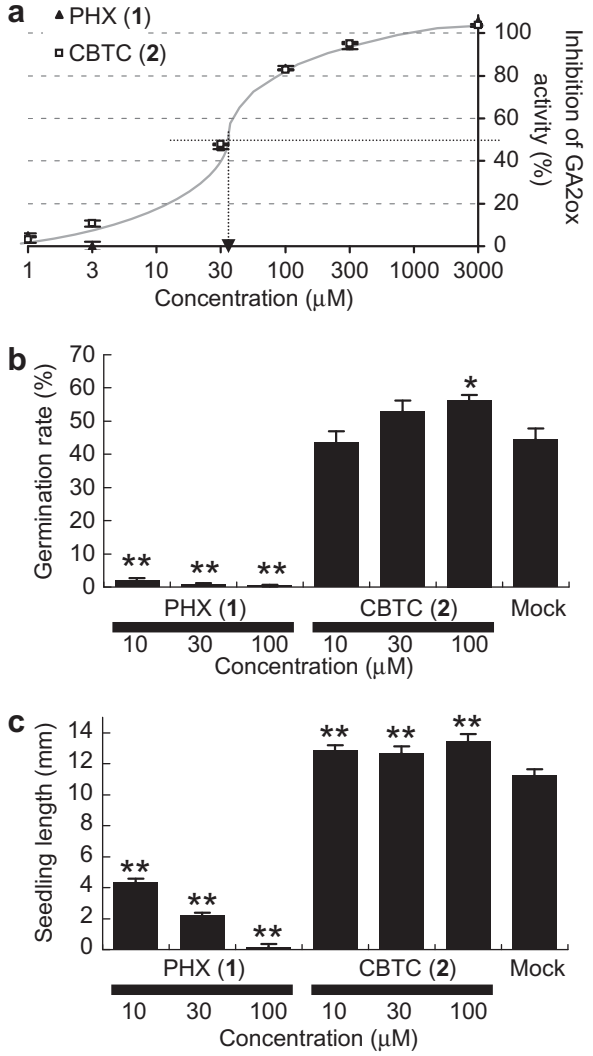

Figure 3. A comparison of the in vitro and in-planta effects caused by CBTC and PHX. (a) Dose responses of the inhibition to GA2ox2 activity in vitro. The values represent the mean of three independent experiments and SE. (b) Effect on Arabidopsis seed germination. In imbibition, seeds were treated with a solution of paclobutrazol (10 μM) to reduce the endogenous GAs moderately. The values (n = ca. 60) represent the mean of six independent experiments and SE. (*) value means p <0.05, and (**) value means p <0.01 compared with mock treatment, as determined with the Z-test. (c) Effect on the growth of Arabidopsis seedlings. We measured the aerial part of each seedling. The values (n = 20) represent the mean of three independent experiments and SE. (**) value means p <0.01 compared with mock treatment, as determined with the student's t-test.

Table 1
Inhibitory activities of CBTC-related chemicals to GA2ox2 in vitro

	IC_{50}[a] (M)	Relative activity[b]
1 (PHX)	3×10^{-5}	100
2 (CBTC)	3×10^{-5}	100
3	$>3 \times 10^{-3}$	<1
4	$>3 \times 10^{-3}$	<1
5	6×10^{-4}	5
6	$>3 \times 10^{-3}$	<1
7	9×10^{-4}	3
8	$>3 \times 10^{-3}$	<1
9	3×10^{-3}	1

[a] Experiment was repeated twice, and very similar values were obtained. Values from one experiment are listed as a representative.
[b] Each relative activity was calculated based on the value for CBTC (**2**).

Next, we decided to use this system for random screening of the GA2ox inhibitors from a commercially-available chemical library. Each chemical (0.01 mg, 0.3 mM in average) was added to the reaction mixture. In this screening, we found that one chemical, methyl 6-chloro-3*H*-1,2,3-benzodithiazole-4-carboxylate 2-oxide (**2** shown in Fig. 2), showed inhibition against recombinant GA2ox enzyme activity. We designate this chemical CBTC. The IC_{50} of CBTC was comparable (ca. 30 μM) to that of PHX (Fig. 3a). Structural variations in CBTC are commercially available (**3**–**9** shown in Fig. 2) and, to assess the structure–activity relation, we evaluated their IC_{50} values in the GA2ox2 reaction. The values are listed in Table 1. CBTC showed the highest activity in them. 5-chlorooxindole (**5**) and 7-chloro-2*H*-1,4-benzothiazin-3(4*H*)-one (**7**) had 1/20–1/30 of the activity of CBTC, although other chemicals examined showed very low activities.

Hereafter, we focus on CBTC. To evaluate CBTC in-planta,[18] we studied its effect on the growth of Arabidopsis. It is known that the *GA2ox2* gene expresses during Arabidopsis seed germination, and the gene product has a decisive function to regulate the amount of endogenous GAs among GA2oxs.[15] Firstly, we examined the effect of CBTC on Arabidopsis (Columbia) seed germination. CBTC (10–100 μM) was absorbed by seeds after imbibition with a known GA-biosynthetic inhibitor, paclobutrazol (10 μM, Fig. 1) and cold treatment (2 d, 4 °C). The germination rate was calculated after 45 h-incubation at 23 °C under continuous light. As shown in Figure 3b, CBTC promoted seed germination at 100 μM, whereas PHX strongly inhibited it in the range of 10–100 μM. In this experiment, paclobutrazol was used to reduce the amount of endogenous GAs moderately, which treatment was effective to evaluate the promotive effect of CBTC because all seeds germinate if we use distilled water for their imbibition.

We also examined the effect of CBTC on the growth of Arabidopsis seedlings in a similar fashion to the above-mentioned experiment for germination. Unlike the germination test, we did not use paclobutrazol in this experiment. As expected (Fig. 3c), CBTC clear promoted the growth of seedlings, even at 10 μM whereas PHX inhibitory growth, just as in the germination test (Fig. 3b). Taking both in-planta experiments into consideration, the results strongly suggest that CBTC inhibits only GA2oxs but not GA3oxs or GA20oxs, whereas we have not yet confirmed that in vitro.

As an additional point, we examined whether CBTC may also have agonist activity to GA receptors, because, in principle, CBTC should show promotive activity in-planta if it were to bind to any GA receptor like GA. We have already established a monitoring system for the GA-binding activity of GA receptors in vitro.[3] After the application of CBTC to the system, we confirmed that CBTC had no effect on the perception of the receptors to GA (data not shown). We concluded that CBTC is not an agonist of GA.

In general, we succeeded in finding one chemical, CBTC, which was able to fulfill our initial aim, that is, to promote both Arabidopsis seed germination and seedling growth, which strongly suggests that CBTC has a inhibitory effect only on GA2oxs with a high specificity, but not on other 2ODDs. We can expect that the selective inhibition of GA2oxs' activity will lead to the delay of GA catabolism in plants, and therefore the life of endogenous GA will be prolonged unlike the case where CBTC is not applied.

Acknowledgments

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13. A coding region for a full-length *GA2ox2* gene (Clone Name: U20502) was purchased from ABRC, and amplified by polymerase chain reaction with specific primers to its full-length cDNA sequences and suitable restriction enzyme sites (*Sac*I and *Xho*I) at both ends of each primer. After a confirmation by sequence analyses, the cDNA fragment of *GA2ox2* gene was ligated to pET32a vector (Novagen/Merck Biosciences, Madison, WI, USA). The preparation of recombinant GA2ox2 was confirmed on a SDS–PAGE profile, compared with a profile from negative control fraction (vector only). According to the manufacturer's procedure, affinity purification of GA2ox2 was performed by using its His-tag (Clontech TARON metal affinity resin, Takara Bio, Shiga, Japan).
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16. Purified GA2ox2 (ca. 20 μg) was dissolved in 100 mM Tris–HCl (pH 7.5) containing 1 mM 2-oxoglutarate, 1 mM $FeSO_4$, and 4 mM ascorbate. After the addition of a substrate, the assay solution (100 μl) was incubated for 2 h at 30 °C, and then the enzymatic reaction was terminated by adding 5 μl acetic acid. The reaction mixture was applied to a C_{18} Sep-Pak™ cartridge (1 ml, Waters, Milford, MA, USA), and GAs were eluted with 2.5 ml of MeOH after washing with 1.2 ml of distilled water. The MeOH eluate was dried in vacuo. The concentrate was methylated with CH_2N_2 and then trimethylsilylated in *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide. An aliquot of the reaction mixture was subjected to GC/MS [Hitachi M-4100 MS connected with a HP-5890 ser. II (Hitachi High-Technologies Co., Tokyo, Japan) under the following conditions; column: DB-1 (0.25 mm id × 15 m, 0.25 μm thick; J&W Scientific, Foster City,

CA, USA), temperature program: 120 °C (2 min), 120–216 °C linear gradient at 16 °C min^{-1}, 216–280 °C linear gradient at 8 °C min^{-1}, He flow: 1.0 ml/min, ion source temperature: 280 °C, ionization: EI (70 eV)]. Each metabolite was identified by comparing its MS fragment pattern and Kovats' retention index (KRI) to those of authentic specimen.

17. A radio-labeled substrate for GA2ox2, [1,2,16,17-$^3H_4$]-16,17-dihydro-GA_4 (4.55 TBq/mmol) was prepared according to a previous report.[8] To screen, each examined chemical (0.01 mg, 0.3 mM on average) from the commercially-available library (Maybridge, Cornwall, UK) was added to the reaction mixture (total 100 μl); GA2ox2 (20 μg), ^{3}H-16,17-dihydro-GA_4 (0.59 kBq), Tris–HCl buffer (100 mM, pH 7.5) containing 1 mM 2-oxoglutarate, 1 mM $FeSO_4$, and 4 mM ascorbate. After 15 min incubation at 30 °C, the enzymatic reaction was terminated by adding 10 μl acetic acid. Metabolites were extracted twice with 100 μl EtOAc, concentrated in vacuo, and subjected to thin-layer chromatography developed in a solvent mixture of $CHCl_3$/EtOAc/AcOH (20:20:1, v/v/v). Radioactivity was visualized with an imaging plate (BAS-TR2040, Fuji Photo Film, Tokyo, Japan) and analyzer (FLA-5000, Fuji Photo Film). Quantification of metabolites and substrate that remained were estimated as pixels by scanning the imaging spots with CS Analyzer (Version 3.00.1010, ATTO, Tokyo).
18. For the germination test, wild type seeds (Columbia) of Arabidopsis were placed on filter paper (No.2, ADVANTEC Toyo, Tokyo) absorbing each examined chemical after imbibition with a solution of paclobutrazol (10 μM) and cold treatment (2 d, 4 °C). Germination was checked at 45 h-cultivation (23 °C, continuous light, 32 μmol m^{-2} s^{-1}) under an optical microscope (SZX12, Olympus, Tokyo). For the seedling growth test, germinating seeds without any chemical were placed on half Murashige and Skoog medium[19] (pH 5.8) containing each examined chemical, and the length of their seedlings was measured after 2 d-cultivation (23 °C, continuous light, 15 μmol m^{-2} s^{-1}).
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