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Increased benzoxazinoid (Bx) levels in wheat seedlings via jasmonic acid treatment and etiolation and their effects on *Bx* genes including *Bx6*

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ABSTRACT

Wheat accumulates benzoxazinoid (Bx) as a defensive compound. While Bx occurs at high concentrations, particularly in the early growth stages, its mechanism of regulation remains unclear. In the present study, we first examined the effects of several plant hormones on Bx concentrations in wheat seedlings. Among the compounds tested, jasmonate (JA) elevated the concentrations of DIMBOA-Glc (2-β-D-glucoside of 2,4-dihydroxy-7-methoy-1,4-benzoxazin-3-one), the primary Bx species in intact wheat seedlings, without a significant increase in HDMBOA-Glc (4-*O*-methyl-DIMBOA-Glc), which is known to be upregulated by stresses. In addition, growing the plants in the dark increased DIMBOA-Glc levels. Quantification of the Bx-biosynthetic genes showed that *TaBx8* (UDP-Glc:Bx glucosyltrasferase) was influenced by neither JA nor etiolation, indicating that *TaBx8* is under the regulation mechanism distinct from the mechanisms influencing the others. In addition, none of the other gene expression patterns exhibited considerable correlation with DIMBOA-Glc accumulation. Since there was no correlation between transcript levels of the genes involved in Bx biosynthesis and Bx accumulation, other factors may control the levels of Bx in wheat. In the course of gene analyses, we isolated *TaBx6*, one of the last two genes that had not been identified in wheat in the DIMBOA-Glc biosynthetic pathway. All the four *TaBx6* genes cloned in the present study were expressed in *Escherichia coli* and characterized their activity.

1. Introduction

Benzoxazinoids (Bx) represent one of the most predominant families of defensive secondary metabolites in wheat (*Triticum aestivum*), maize (*Zea mays*), and rye (*Secale cereale*). The structural variations of Bx and their physiological functions have been explored extensively in several reviews [1–3]. The major Bx species vary across plants, tissues, and growth stages. In rye, for example, most of the all Bx species in the shoots is DIBOA-Glc (2-*O*-glucoside of 2,4-dihydroxy-1,4-benzoxazin-3-one), while DIMBOA-Glc (7-methoxy-DIBOA-Glc) and DIBOA-Glc exist at comparable concentrations in the roots [4]. In wheat and maize, DIMBOA-Glc is the predominant Bx species both in the aerial and underground plant parts [5]. Generally, the concentrations of Bx are higher in young plants (a few days after germination) and gradually decrease as plants grow, suggesting that Bx are involved in defense systems particularly in the early growth stages.

Enhancing the Bx production capacity of plants could minimize the amount of pesticides applied in the field. However, the mechanisms that regulate Bx-biosynthesis and accumulation have not yet been elucidated. The DIBOA biosynthetic pathway was first determined in maize [6], in which ZmBx1–ZmBx5 convert indole-3-glycerolphosphate into DIBOA (Fig. 1). Later, their orthologs in wheat (TaBx1–TaBx5) were isolated [7,8]. Subsequently, DIBOA undergoes glucosylation by UDP-Glc:Bx glucosyltransferase (Bx8) [9–11], followed by two steps including oxidation and methylation by Bx6 and Bx7, respectively, to yield DIMBOA-Glc [12,13]. *Bx6* and *Bx7* have been identified in maize (*ZmBx6* and *ZmBx7*) but not yet in wheat. Recent reports on the isolation of rye Bx-related genes (*ScBx1–ScBx6* and *ScBx8*) [11,14,15] indicate that the pathway is also conserved between maize and rye. More recently, rye *Bx7* (*ScBx7*) was isolated [16] although it has not been characterized enzymatically. *Bx1–Bx8* loci in maize are located close to each other on the short arm of chromosome 4, and are proposed to form a gene cluster [6,9,12,13] that facilitates the coordinated expression of the members. However, this is not the case in wheat and rye, in which the loci are dispersed across several chromosomes [7,11,17].

Some stress factors could influence Bx production and its composition [18–20]. One of the most notable changes reported is increasing HDMBOA-Glc accumulation. HDMBOA, the aglycone of HDMBOA-Glc,

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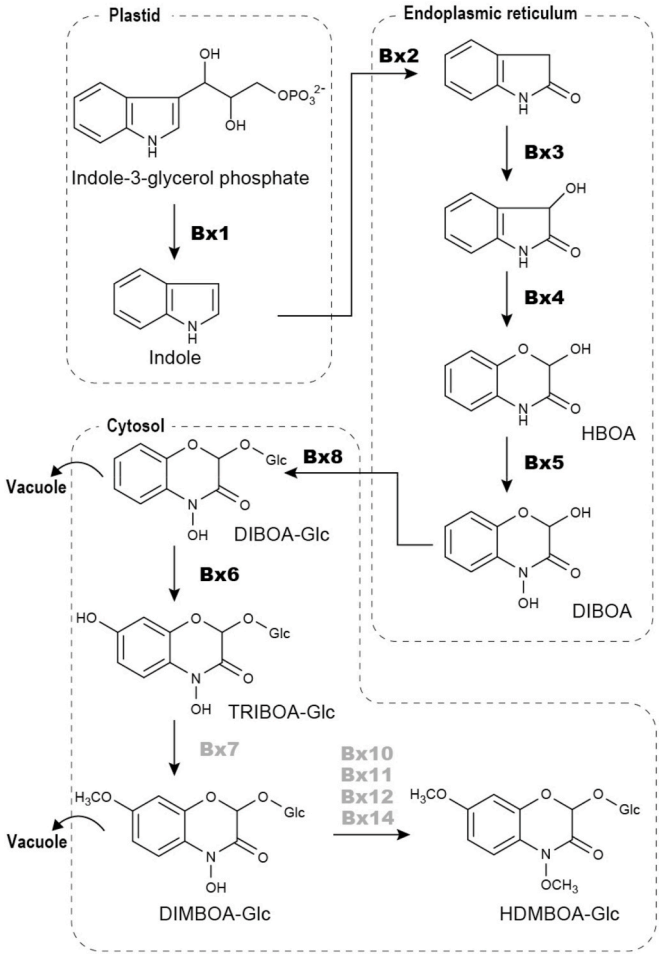

Fig. 1. Biosynthetic pathway of benzoxazinones. The subcellular localization of each enzyme is predicted from its primary structure. Genes yet to be identified are marked in gray. Bx10–Bx14 are based on the enzymes in maize.

is easily degraded into a more toxic methoxy-2-benzoxazolinone (MBOA). Oikawa et al. [21,22] proposed that HDMBOA-Glc was synthesized from the DIMBOA-Glc pool by up-regulation of the methyltransferase that converts DIMBOA-Glc into HDMBOA-Glc to respond to biotic stresses. Since the primary Bx species in young intact plants is DIMBOA-Glc, and HDMBOA-Glc is maintained at lower levels, the transformation of DIMBOA-Glc into HDMBOA-Glc would be under a regulation mechanism distinct from that controlling the DIMBOA-Glc biosynthesis in intact plants.

Exploring the methods that influence Bx production would facilitate the understanding of Bx-regulation mechanisms. The first objective of the present study was to establish growth conditions or treatments that could alter the concentrations of only DIMBOA-Glc and then evaluate the relationships between Bx accumulation and the transcript level of each Bx-related gene to elucidate the mechanisms regulating the Bx accumulation in intact wheat seedlings. Although all the genes involved in DIMBOA-Glc biosynthesis should be analyzed, the *Bx6* and *Bx7* have remained undiscovered in wheat. Therefore, we identified wheat *Bx6* (*TaBx6*) coding a dioxygenase that converts DIBOA-Glc into TRIBOA-Glc (7-hydroxy-DIBOA-Glc), and investigated its expression, in addition to the expression of *TaBx1–TaBx5* and *TaBx8*.

2. Materials and methods

2.1. Plant growth conditions

Wheat (*T. aestivum* cv. Asakazekomugi) seeds were washed in 70% (v/v) ethanol/H_2O for a minute and sterilized further by treatment with sodium hypochlorite solution for 15 min. Subsequently, the seeds were rinsed with distilled water and imbibed in water for 2 h at room temperature. The seeds were placed on 0.8% (w/v) agar containing a plant hormone in a plant culture box (6 seeds/box) and grown at 25 °C under a 12-h photoperiod. Plant hormones (abscisic acid, gibberelline A3, salicylic acid, jasmonic acid, indole acetate, and 1-aminocyclopropane-1-carbonate) were purchased from Sigma-Aldrich Corp. (St. Louis. MO, USA) and Fujifilm-Wako pure chemical corporation (Osaka, Japan) and solved in either ethanol or water.

2.2. Analyses of Bx concentrations and Bx-gene expression levels in wheat shoots

To quantify the Bx concentrations in wheat shoots, each plant was separated into a coleoptile and each leaf, and the tissues were weighed. The tissues were frozen in liquid nitrogen and ground into powder. Thereafter, the Bx was extracted using 10–20 times of volume of methanol. The extracts were diluted by adding equal volume of water and the mixtures were chilled at −25 °C for 20 min. The resultant precipitate was removed by centrifugation, and the Bx in the supernatant was quantified using a HPLC system (LC-10ADvp, Shimadzu, Kyoto, Japan) equipped with SPD-M20A photodiode array detector (Shimadzu). Chromatography was performed on a Kinetex XB-C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA, USA) under the following conditions: a linear gradient from A:B = 91:9 to 85:15 for 15 min (where A = H_2O with 0.1% trifluoroacetic acid and B = acetonitrile) at 40 °C and a flow rate of 1.0 mL/min. The concentrations of Bx were evaluated by monitoring the absorption at a wavelength of 280 nm.

For the analyses of the transcript levels of Bx-related genes (*TaBx1–TaBx6, TaBx8*), total RNA was prepared using RNeasy Plant mini kit (QIAGEN, Germany), and reverse transcribed using PrimeScript RT Master Mix (TaKaRa Bio Inc., Tokyo, Japan) with a mixture of random hexamers and oligo dT as primers. Real-time PCR was carried out using SYBR Green PremixEX Taq II (TaKaRa Bio) and sets of gene-specific primers (Table S1). A two-step PCR protocol was employed for the amplification where denaturation and annealing-elongation were carried out at 98 °C and 60 °C, respectively. Other qPCR conditions were set according to the manufacturer's instructions. The gene expression levels were normalized by RNase inhibitor-like protein (RLI) as a housekeeping gene [23].

2.3. Western blot

Wheat shoots were ground in 5 vol of an extraction buffer (100 mM Tris-HCl [pH 7.5], 25% (w/v) sucrose, 5% (v/v) glycerol, 10 mM EDTA, 5 mM KCl, and 1 mM DTT) and the supernatant was recovered from centrifugation at 15,000 *g* for 15 min. Microsomal fraction was obtained as the pellet of a further centrifugation at 150,000 *g* for 60 min. After resuspension of the pellet in the extraction buffer, the protein was subjected to SDS-PAGE and then transferred to Immobilon-P PVDF membrane (Merck Millipore). TaBx5 was probed using a polyclonal antibody raised against a TaBx5 fragment as a primary antibody and anti-rabbit IgG HRP conjugate (Promega, Madison, WI, USA) as a secondary antibody followed by detection with ECL Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) as a substrate.

2.4. Isolation of wheat Bx6 gene (TaBx6)

To obtain the wheat *Bx6* (*TaBx6*) sequence using 3′-RACE and 5′-RACE, cDNA that could be used for both RACE reactions was synthesized using Gene Racer kit (Thermo Fisher Scientific, Waltham, MA, USA) using total RNA from 72-h-old wheat shoots (cv. Chinese Spring) as a template. To isolate the *TaBx6* fragment, we first designed a set of primers based on the rye *Bx6* (*ScBx6*; GenBank accession number

MG516219) and amplified a part of *TaBx6*. After the sequence verification, 3′-RACE was performed using the gene-specific primers. Then, the primers were designed based on the results and 5′-RACE was performed using a template similar to that for the 3′-RACE, which yielded the DNA fragment corresponding to the 5′-region of *TaBx6*. Finally, the cDNA, including the coding sequence of *TaBx6* (*TaBx6-1*), was amplified using a set of primers, one of which corresponds to the 5′-untranslated region (forward primer) and the other is the adaptor primer for 3′-RACE (reverse primer).

For the isolation of the other *TaBx6*s, the fragments of genomic DNA were first amplified using a set of primers supposed to amplify regions containing introns as well as exons (the forward and reverse primers were similar to those used for 3′-RACE and 5′-RACE, respectively) that was estimated from an alignment of the maize (*ZmBx6*) and *ScBx6*. Several fragments with different lengths were sequenced, and specific forward primers were designed based on intron sequences. A second PCR was performed using genomic DNA (template), the designed forward primers, and reverse primers (designed from exons) to obtain exons (including intron) sequences that were different from *TaBx6-1*. After the isolation of new exon sequences, we designed new specific primers and performed 3′-RACE and 5′-RACE followed by PCR to amplify their full-length coding sequences. During the procedures, pBluescript SK(−) and DH5α were used as cloning vectors and an *E. coli* strain, respectively. All the primers used are listed in Table S1. PrimeStar max DNA polymerase (TaKaRa Bio) was used in all the reactions. The nucleotide sequences of *TaBx6-1*, *TaBx6-2, TaBx6-3*, and *TaBx6-4* have been submitted to the GenBank/EMBL/DDBJ databases with accession numbers LC519324, LC519325, LC519326, and LC519327, respectively.

2.5. Heterologous expression of TaBx6 and validation of its activity

The *TaBx6*s were amplified by PCR using the cloned *TaBx6* as a template to yield fragments flanked by recognition sites of *Nde*I and *Hin*dIII (*TaBx6-1* and *TaBx6-4*) or *Nde*I and *Eco*RI (*TaBx6-2* and *TaBx6-3*). After digestion using the restriction enzymes, the fragment was ligated into the *Nde*I-*Hin*dIII or *Nde*I-*Eco*RI site of pET28. After sequence verification, the resulting plasmid was further transferred into *E. coli*, BL21CodonPlus (DE3)-RIL. Subsequently, the cells were cultured in 150 mL of Luria-Bertani broth containing kanamycin and chloramphenicol at 37 °C till the OD_{600} value reached 0.7, and protein expression was achieved by the addition of IPTG (1 mM) followed by a 16-h culture at 20 °C. The cells were harvested by centrifugation (5000g, 5 min) and resuspended in 5 mL of Tris-HCl (pH 7.2). After cell disruption by sonication (20 s × 6), the soluble enzyme fraction was recovered as the supernatant of centrifugation (15,000 rpm for 15 min). The N-terminal His-tagged TaBx6 was purified by affinity chromatography using TALON metal affinity resin (Clontech, USA). The 2-oxoglutarate-dependent dioxygenase activity of TaBx6 was evaluated by detecting the reaction product, TRIBOA-Glc, using HPLC as described above. The reaction was conducted in 50 mM HEPES (pH 7.2) containing 1 mM $FeSO_4$, 10 mM 2-oxoglutarate, and 10 mM ascorbate with various concentrations of DIBOA-Glc at 30 °C. Kinetic parameters (k_{cat} and K_m) were calculated by fitting the data to Michaelis-Menten equation using SigmaPlot (Systat Software Inc., San Jose, CA, USA).

3. Results and discussions

3.1. Effects of plant hormones on DIMBOA-Glc concentrations in wheat shoots

In the present study, we first intended to determine conditions that can up- or downregulate Bx concentrations without converting DIMBOA-Glc into HDMBOA-Glc. We attempted to evaluate the effects of plant hormones on Bx concentrations in wheat. The time courses of Bx concentrations were recorded from 72 to 144 h after imbibition. Among the hormones examined, abscisic acid inhibited germination or growth extremely at concentrations higher than 1 μM, and lower concentrations (0.1 and 0.01 μM) did not influence Bx concentrations. The other compounds, excluding JA, also had no significant effect on Bx concentrations at concentrations between 0.1 and 100 μM (data not shown).

JA administered at 100 μM elevated DIMBOA-Glc and HDMBOA-Glc concentrations in 72-h-old plants more than 4- and 10-fold, respectively (Fig. 2). However, the treatment reduced the fresh weight of the shoots to about 50% that of the untreated plants. Considering plant growth influences Bx concentrations considerably, the finding was potentially an indirect effect of JA. While there was no significant difference in the plant growth at concentration of JA below 10 μM, the concentration of HDMBOA-Glc was 2.5 times as high as that of the control when JA was administered at 10 μM. For our purposes, it is essential to exclude the influence of growth retardation and to avoid conversion of DIMBOA-Glc into HDMBOA-Glc. Therefore, we conducted further analyses using 5 μM JA, at which the plants retained growth comparable to growth in the untreated individuals, and less conversion of DIMBOA-Glc to HDMBOA-Glc was observed (Fig. 2). The results indicate that JA could be a useful tool for distinguishing between constitutive DIMBOA-Glc production and inducible HDMBOA-Glc production. In the following experiments, the DIMBOA-Glc concentrations in wheat are referred to as Bx concentrations because DIMBOA-Glc accounted for more than 90% of all the Bx species detected.

3.2. DIMBOA-Glc concentrations in JA-treated and etiolated wheat

For further evaluation of the effects of JA, each plant was separated into three parts, i.e., coleoptile, 1st leaf, and 2nd leaf (96 h and after), and the Bx (DIMBOA-Glc) concentrations in each plant part were quantified (Fig. 3). In untreated (control) plants, Bx concentrations peaked 96 h after imbibition and decreased slightly depending on the rate of growth. The concentrations in the coleoptiles were the highest among the three plant parts throughout the experimental period (72–144 h). In addition, the 2nd leaves had higher concentrations than the 1st leaves (about 2-fold at 144 h).

While treatment with JA (5 μM) did not alter Bx concentrations in the coleoptile remarkably, the Bx concentrations in the 1st (except for 96 h) and the 2nd leaves increased about 2-fold. In the 1st leaves, Bx concentrations increased to a level comparable with the levels in the untreated coleoptiles. Notably, in the 2nd leaves, Bx concentrations increased to the highest value in the control and the JA-treated wheat shoots. The insensitivity of the coleoptiles to the JA treatments may be

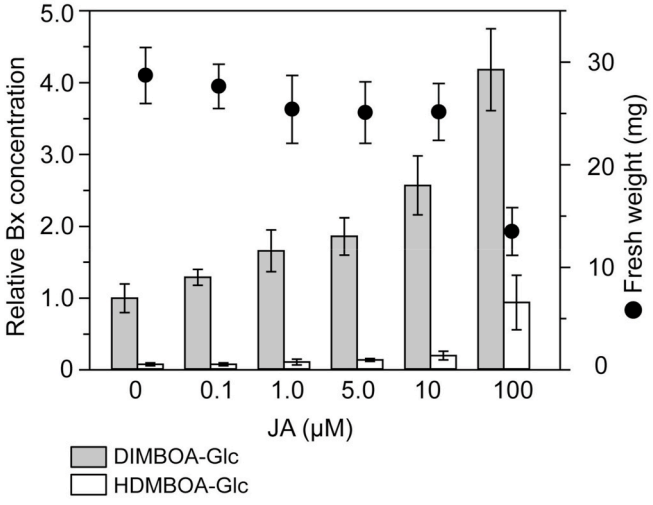

Fig. 2. Effect of jasmonate treatment on Bx accumulation and plant weight. The Bx (DIMBOA-Glc and HDMBOA-Glc) concentrations and fresh weights of each wheat shoot are shown. The concentrations of Bx are described as concentrations relative to the DIMBOA-Glc concentrations in the untreated sample.

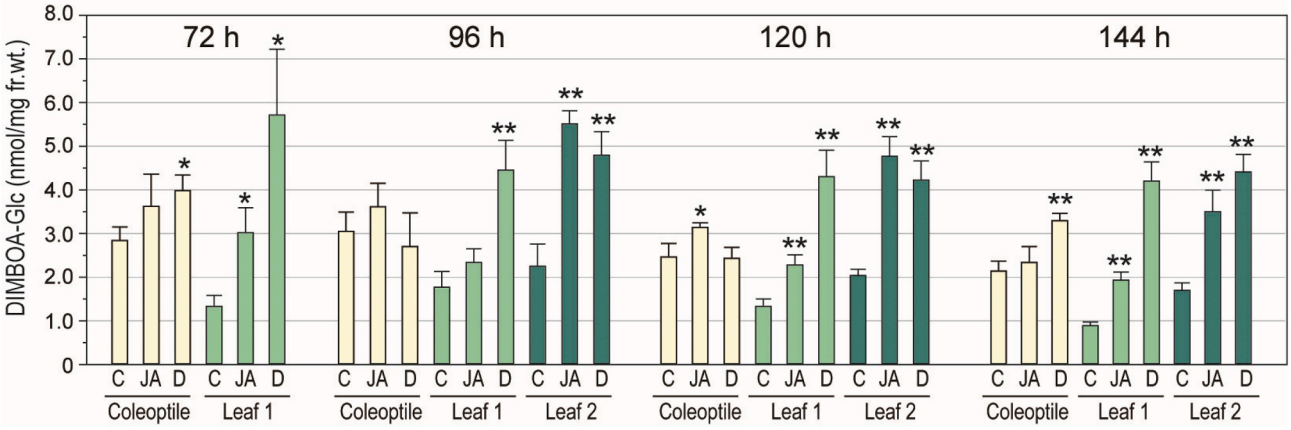

Fig. 3. Time course (72–144 h) of DIMBOA-Glc concentration in jasmonate (JA)-treated and etiolated wheat shoot. A wheat shoot was separated into coleoptile, 1st leaf, and 2nd leaf (after 96 h), and the concentrations of DIMBOA-Glc analyzed using HPLC. JA was administered to agarose at a concentration of 5 μM. Asterisks indicate statistically significant difference from the value of the control (*$p < 0.01$, **$p < 0.001$; Student's *t*-test).

correlated with the minor time-dependent fluctuation in their weight. The fresh weights of coleoptiles decreased slightly in the course of the experiments (control:15.9 to10.7 mg; JA-treated: 15.8 to 10.6 mg), and those of the 1st and 2nd leaves increased about 2.5–3-fold (control 1st leaf: 19.8–50.7 mg; control 2nd leaf: 9.0–22.3 mg; JA-treated 1st leaf: 16.8–37.5 mg; JA-treated 2nd leaf: 5.5–16.2 mg). A potential explanation of the observations is that plant growth and DIMBOA-Glc accumulation in the coleoptiles are already close to peaking at 72 h and exogenous stimulation would not alter the amount of a compound stored.

Subsequently, we investigated the effects of light on Bx concentrations, considering light influences plant metabolism and a considerable part of the 2nd leaf that contained higher Bx concentration than the 1st leaves were shielded from light by being wrapped by a coleoptile or a sheath. When we grew wheat seedlings in the dark, as expected, the coleoptiles became longer and the fresh weights were approximately 2–2.6-fold the fresh weight of the control plants at all growth stages (data not shown). However, the DIMBOA-Glc concentrations in them did not vary considerably and was at most 150% of the control plants (144 h) (Fig. 3). In contrast, the exclusion of light enhanced Bx accumulation in leaves. In particular, the increase in the 1st leaves was marked (2.5–4.7 fold) and the concentration was as high as in the JA-treated 2nd leaves.

3.3. Isolation and heterologous expression of wheat Bx6 (TaBx6)

To elucidate the mechanism by which JA treatment and etiolation enhance Bx accumulation, it is critical to determine the genes that the treatments influence. In the present study, we attempted cloning the *TaBx6* responsible for the conversion of DIBOA-Glc into 7-hydroxy-DIBOA-Glc (TRIBOA-Glc). We first isolated *TaBx6* fragments by 3′-RACE and 5′-RACE using primers designed based on *SxBx6* and then amplified the coding sequences using the specific primers based on the results of RACE. The cloned sequence contained a 1167-bp open reading frame encoding a polypeptide of 388 amino acids (designated as TaBx6-1) whose primary structure shared 94% identity with ScBx6 (Fig. 4). Since wheat is a hexaploid plant consisting of three genomes (2n = 6x = 42), it was assumed that there were at least three homoeologs of *Bx6*. The transcript levels of wheat *Bx* genes identified so far tend to be unbalanced among the homoeologous genes [11,17]. Therefore, we attempted to amplify other *Bx6* fragments using genomic DNA as a template. The PCR isolated three other DNA fragments, which finally yielded three *Bx6* cDNAs (*TaBx6-2* – *TaBx6-4*). *TaBx6-2, TaBx6-3, and TaBx6-4* encode polypeptides consisting of 388, 373, and 373 amino acids, respectively, and they shared more than 93% identity. *TaBx6* genes are located on chromosome 2 as per the analysis using Ensembl-Plants (https://plants.ensembl.org/).

According to the cDNA alignment analyses, all the four *TaBx6*s possess two ATG sequences in their 5′-terminal regions (Fig. 4B). When they are translated from the first ATG, *TaBx6-1* and *TaBx6-2* encode polypeptides with 388 residues, while *TaBx6-3* and *TaBx6-4* encode much shorter polypeptides. Translation from the second ATG yielded polypeptides with 373 residues across all the four *TaBx6*s. The primary structure of the rye orthologue, *ScBx6*, which was found in a database, corresponds to the deduced sequences of TaBx6s translated from the second ATG. We performed 5′-RACE using RNA from rye root to confirm the 5′-UTR sequence of *ScBx6*. According to the results, all the sequences possessed ATG sequences at the position corresponding to the first ATG of *TaBx6s* (Fig. 4B). Starting from the ATG codon, subsequent to translation, ScBx6 should be a polypeptide consisting only of 28 amino acids. Rye possesses an enzyme that catalyzes sequential 7-hydroxylation of DIBOA-Glc, because rye produces DIMBOA-Glc mainly in the roots. In the present study, therefore, we consider the second ATG as the initiation codon for *TaBx6* genes, namely, TaBx6-1–TaBx6-4, which consist of similar residue numbers, 373 residues, although the N-terminal residue of the naturally occurring TaBx6 is not confirmed. Tanwir et al. [14] reported a *TaBx6-like* gene with 86% identity with *ScBx6* at the amino acid level by searching for *Bx6-like* homologs in a wheat sequence database. However, the *TaBx6*s isolated in the present study share higher identity with *ScBx6* at both nucleotide and amino acid levels. Considering the high identity (>90%) between the known wheat and rye Bx-related genes, *TaBx6* genes reported here have to be the orthologs of rye and maize *Bx6*s.

All the four recombinant TaBx6s exhibited a 2-oxoglutarate-dependent dioxygenase activity that converted DIBOA-Glc into TRIBOA-Glc (Table 1). While their kinetic parameters did not vary considerably among the isozymes, TaBx6-4 exhibited the highest efficiency (k_{cat}/K_m), which was more than three times higher than in the others. Since their primary structures share high sequence identities, a detailed activity evaluation based on the primary-structure differences would offer some insights on the structural factors involved in their substrate recognition and activity.

3.4. Quantification of Bx-genes in JA-treated and etiolated wheat shoots

The transcript levels of the Bx-related genes (*TaBx1–TaBx6* and *TaBx8*) in individual plants (n = 4) were quantified using real-time PCR (Fig. 5). Notably, *TaBx8* was the only gene that was not influenced by any treatments. Although *TaBx6* expression in coleoptile was affected by JA and etiolation, its transcript levels in the 1st and 2nd leaves were

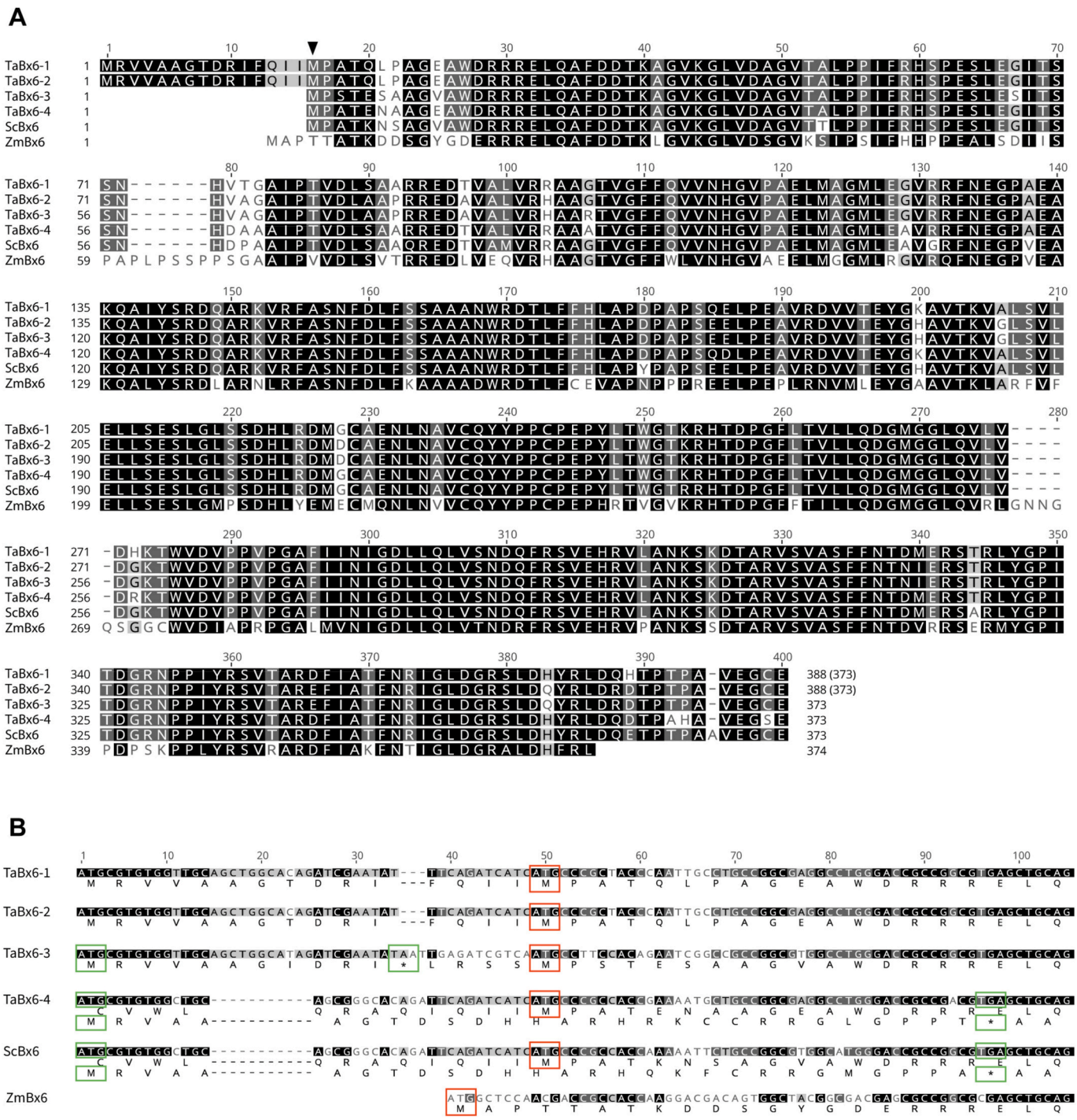

Fig. 4. Amino-acid sequence alignment of Bx6. The primary structures of Bx6 in wheat (TaBx6-1–TaBx6-4), rye (ScBx6; MG516219), and maize (ZmBx6; AF540907) were aligned using ClustalOmega. A, Alignment of the amino acid sequences that were deduced from the longest reading frames. The sequences of TaBx6-1 and TaBx6-2 used for heterologous expression in *Escherichia coli* start from the methionine indicated with closed triangle. B, Alignment of the nucleotide sequences corresponding to the N-terminal regions of Bx6. The sequences from the most upstream ATG in cDNA are shown. ATG sequence regarded as the initiation codon of each gene in this study is indicated in a red box. Note that translation from the most upstream ATG in *TaBx6-3*, *TaBx6-4*, and *ScBx6* (green box) results in production of short peptides. Their corresponding termination codons are indicated in green boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

remarkably lower than the coleoptiles throughout the experimental period regardless of the treatments. These expression profiles did not obviously reflect the Bx concentrations in wheat, suggesting that *TaBx6* and *TaBx8* were not the factors influencing Bx concentration.

In the coleoptiles, the expression levels of all the genes decreased significantly in a time-dependent manner, although the accumulation of DIMBOA-Glc did not change so largely (Fig. 3). The discrepancy between Bx concentration and gene expression levels was also notable in the effects of JA on the 72-h-old coleoptiles; *TaBx1–TaBx6* were upregulated by JA, although the effect on Bx concentration was not considerable. The effects of etiolation on 72-h-old coleoptiles varied depending on the genes; *TaBx2*, *TaBx5*, and *TaBx6* were upregulated

Table 1
Kinetic parameters of TaBx6s.

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}/mM)
TaBx6-1	217	0.433	2.00
TaBx6-2	318	0.508	1.60
TaBx6-3	282	0.524	1.86
TaBx6-4	104	0.715	6.86

more than 2-fold, which did not correspond with the rates of accumulation of the compound, while the others were not affected. In the 1st leaves, etiolation had greater effects on gene expression than JA at all growth stages, except for *TaBx6* and *TaBx8*. Such trends in *TaBx1–TaBx5* were consistent with the Bx accumulation levels. In the 2nd leaves, however, none of the genes exhibited correlative expression profiles with the Bx accumulation levels.

To examine whether the transcript levels corresponded with the amounts of enzymes, one of the Bx-associated enzymes, TaBx5, was detected by Western blot analysis. TaBx5 is the enzyme responsible for the last step of the four sequential oxygenation by cytochrome P450 enzymes (CYPs) required for the formation of the 2,4-dihydroxy-1,4-benzoxazin-3-one skeleton (Fig. 1) and whose activity was the lowest among the four CYPs [17]. Treatment with JA increased TaBx5 in both coleoptiles and leaves at 72 h (Fig. 6). In the 120-h old plants, the amounts in both tissues were lower and treatment with JA did not influence them. The data are consistent with the results of quantitative-PCR.

In the present study, the Bx concentration in wheat leaves were

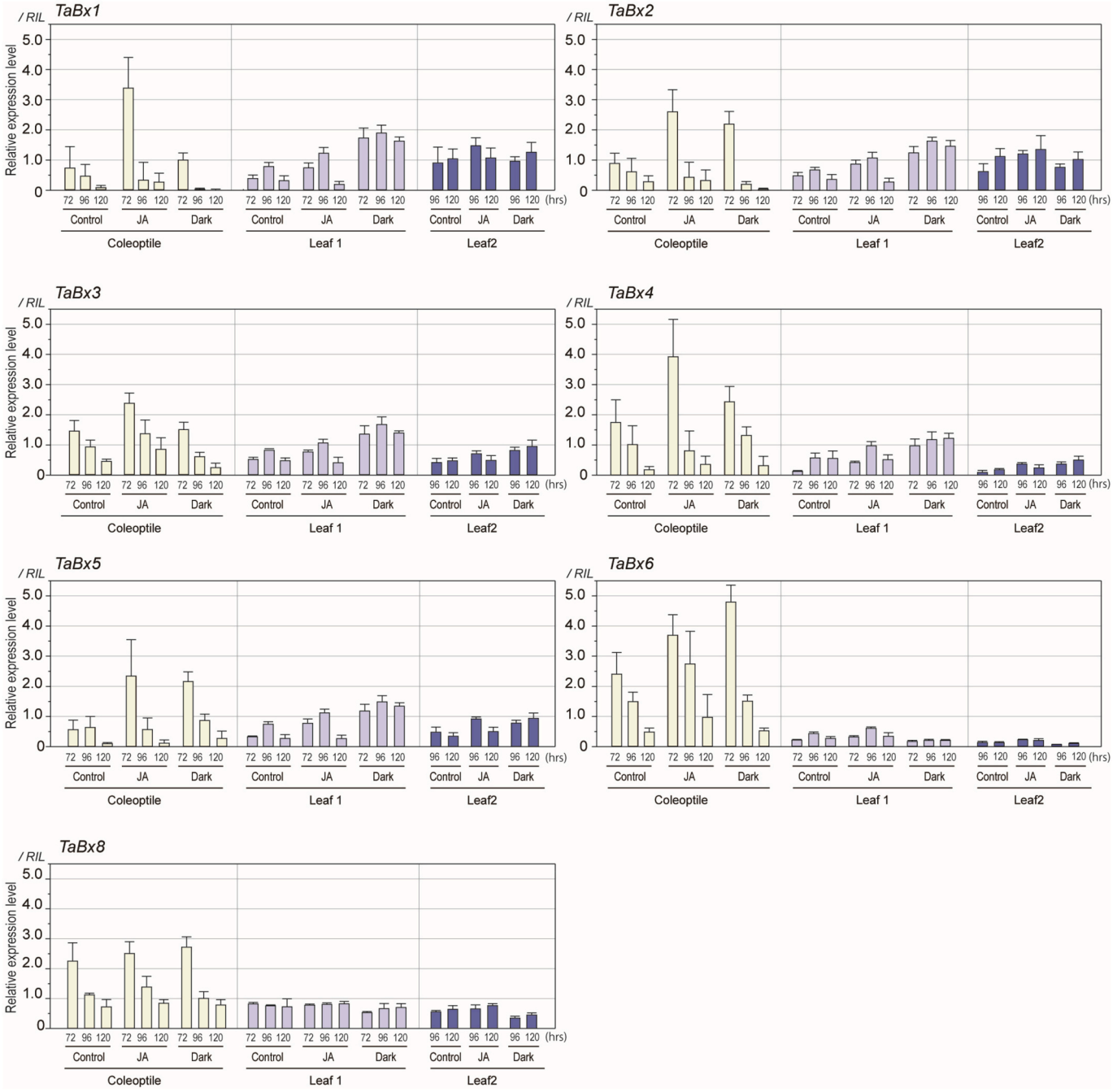

Fig. 5. Time course (72–120 h) of the transcript levels in jasmonate (JA)-treated and etiolated wheat shoot. RNase inhibitor-like protein (RLI) was used as a housekeeping gene. JA was administered to agarose at a concentration of 5 μM.

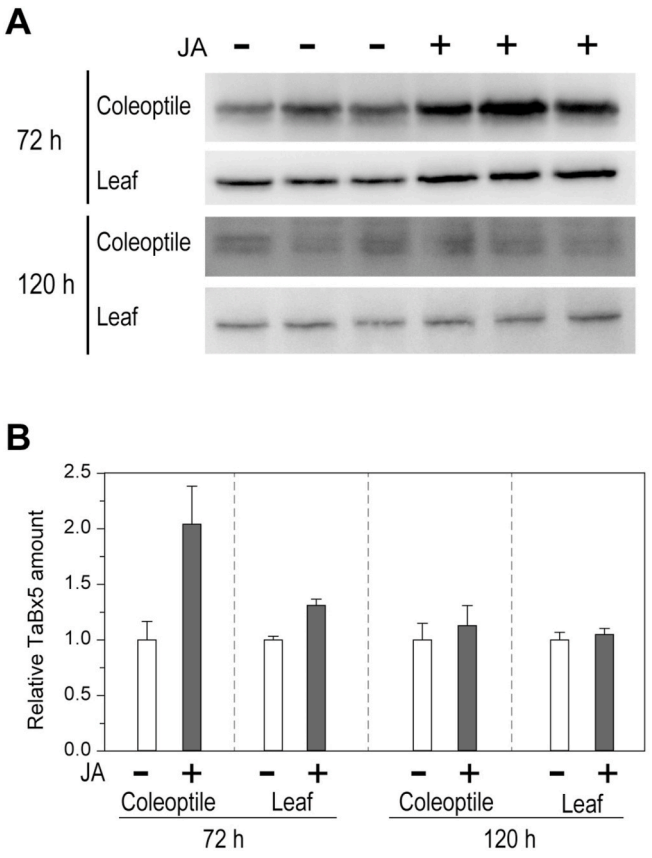

Fig. 6. Effect of JA on TaBx5 amounts in wheat shoots. TaBx5 in 72- and 120-h-old plants (coleoptiles and leaves) were analyzed using Western blot analysis. Microsomal fraction (30 μg/lane) was separated on a 1.2% polyacrylamide gel and blotted onto PVDF membranes. A, Western blot analysis using anti-TaBx5 antibody. B, Densitometric data from the Western blot analysis. The data were normalized using total protein at each lane and presented as values relative to the values of the control sample.

elevated by JA treatment and etiolation (Fig. 3), although the relationship between both the treatments has not been elucidated. In the leaf of rice seedling, it was shown that the expression of *GY1* which is involved in JA biosynthesis was upregulated by etiolation [24], indicating etiolation may also increase the JA level in wheat leaves. In the wheat seedlings, however, JA treatment and etiolation had different effects on Bx accumulation and the gene expression depending on the plant parts (Figs. 3 and 5), which suggests the Bx level in etiolated wheat is not controlled by JA dependent pathway alone.

The expression patterns of Bx-related genes were not synchronized entirely in wheat seedlings, which may be derived from their dispersed chromosomal locations. While it is still unclear which step in the DIMBOA-Glc biosynthesis pathway is rate-limiting, none of the biosynthetic enzymes examined in the present study seemed to influence Bx accumulation exclusively in the plants. Based on the primary structures, DIMBOA-Glc is synthesized by 8 enzymes localized in three subcellular compartments (chloroplast, endoplasmic reticulum, and cytoplasm) and is stored in the vacuole (Fig. 1). Therefore, efficient translocation among compartments and the appropriate enzyme organizations are required for efficient Bx biosynthesis. The Bx aglycones are toxic and unstable and require glucosylation before translocation to vacuoles. *Arabidopsis* over-expressing *ZmBx8* was reported to be able to grow on a medium containing a Bx aglycone [9], which demonstrated the significance of glucosylation. Co-localization and the organization of biosynthetic pathways into complexes of multiple enzymes, which are referred to as metabolons, are proposed as pathways for the synthesis of plant secondary metabolites such as cyanogenic glucosides [25]. This could also apply in the case of Bx biosynthesis. Further analyses, including investigation of enzyme organization in cells and the transport of the metabolites through membrane systems, are required for the comprehensive understanding of Bx accumulation mechanisms.

Credit author statement

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Declaration of competing interest

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Appendix A. Supplementary data

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