

Table S1 Primers used in gene cloning and vector construction

Gene	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
<i>ZmBX1</i>	CTAG <u>CTCGAG</u> CATGGCTTTCGCGCC CAAAAC (XhoI)	GACTGGATCCCATATGCGTACTGTA GTTTTTATTCAATC (BamHI)
<i>ZmMYB61</i>	<u>TCTAGAG</u> TATGGGGAGGCCACCGTG (XbaI)	<u>TCTAGAG</u> ACGATACAGCAACATGA AC (XbaI)
<i>ZmbHLH20</i>	<u>GAATTC</u> GCACCGTAACCTGATGGAG TTC (EcoRI)	<u>GGATCC</u> TCGTGTTTCGTCACAGCAT GTCG (BamHI)
<i>ZmbHLH20- eGFP</i>	<u>GAATTC</u> GCACCGTAACCTGATGGAG TTC (EcoRI)	<u>TCTAGAT</u> CGTGTTTCGCAGCATGTC GG (XbaI)
<i>ZmbHLH76</i>	<u>GAATTC</u> GTCGATCGAGATGAAGAGC CG (EcoRI)	<u>GGATCC</u> CTCAGCTCAGACGAGGAT CG (BamHI)
<i>ZmbHLH76- eGFP</i>	<u>GAATTC</u> GTCGATCGAGATGAAGAGC CG (EcoRI)	<u>TCTAGAC</u> TCAGCGACGAGGATCGT C (XbaI)

Note: The underlined sequences are the restriction enzyme digestion sites, and the corresponding enzymes are indicated in the brackets.

Table S2 Primers used for amiRNA plasmid construction

Primer name	Prime sequence (5' - 3')
amiRNA-ZmBX1-I	gaTATCGAATGAACGGCCCGCTAtctctcttttgattcc
amiRNA-ZmBX1-II	gaTAGCGGGCCGTTTCATTGATAtcaaagagaatcaatga
amiRNA-ZmBX1-III	gaTAACGGGGCCGTTCTTTTCGATTtcacaggtcgtgatatg
amiRNA-ZmBX1-IV	gaAATCGAAAGAACGGCCCGTTAtctacatatattcct
amiRNA-ZmbHLH20-I	gaTCTATGGTAACGATCGGTCTAtctctcttttgattcc
amiRNA-ZmbHLH20-II	gaTAGACCGATCGTTACCATAGAtcaaagagaatcaatga
amiRNA-ZmbHLH20-III	gaTAAACCGATCGTTTCCATAGTtcacaggtcgtgatatg
amiRNA-ZmbHLH20-IV	gaACTATGGAAACGATCGGTTTAtctacatatattcct
amiRNA-ZmbHLH76-I	gaTATACTAGAGTACTACTCCGCtctctcttttgattcc
amiRNA-ZmbHLH76-II	gaGCGGAGTAGTACTCTAGTATAtcaaagagaatcaatga
amiRNA-ZmbHLH76-III	gaGCAGAGTAGTACTGTAGTATTtcacaggtcgtgatatg
amiRNA-ZmbHLH76-IV	gaAATACTACAGTACTACTCTGCtctacatatattcct
pRS300-A	CTGCAAGGCGATTAAAGTTGGGTAAC
pRS300-B	GCGGATAACAATTTACACAGGAAACAG

Note: pRS300-A and pRS300-B are primers used for amplifying the amiRNA precursors and thereafter cloning into the pM999 vector by digestion with EcoRI and BamHI. The lowercase sequences match with the plasmid pRS300 sequences and the uppercase sequences are used to replace the sequences in the pRS300 vector with the target amiRNA sequences by overlapping PCR.

Table S3 HPLC-MS running parameters

Benzoxazinoid	Retention time (min)	(M+H)⁺/Z	Mode
DHBOA-Glc	3.521	365.95	Q3 SIM(+)
DIBOA	3.563	182.05	Q3 SIM(+)
DHBOA	4.648	182.05	Q3 SIM(+)
DIBOA-Glc	4.672	344	Q3 SIM(+)
M ₂ BOA	4.868	196	Q3 SIM(+)
HMBOA-Glc	4.942	380.1	Q3 SIM(+)
DIMBOA-Glc	5.107	373.95	Q3 SIM(+)
DIMBOA	5.208	212	Q3 SIM(+)
DIM ₂ BOA	5.236	241.95	Q3 SIM(+)
MBOA	5.766	166	Q3 SIM(+)
HDM ₂ BOA-Glc	5.834	440	Q3 SIM(+)
HDMBOA-Glc	5.851	388	Q3 SIM(+)
DIM ₂ BOA-Glc	5.863	405	Q3 SIM(+)

Table S4 Primers used for RT-qPCR analysis of gene expression

Gene	Accession Number	Primer (5' - 3')
<i>ZmIGL</i>	GRMZM2G046191	Forward: GCCTCATAGTTCCCGACCTC Reverse: GAATCCTCGTGAAGCTCGTG
<i>ZmBX1</i>	GRMZM2G085381	Forward: CCTGCTCGGACCCCTACAT Reverse: GGACCCCCGCCTCTTTCAT
<i>ZmBX2</i>	GRMZM2G085661	Forward: GACGAGGACGACGATAAGGACTT Reverse: GGCCATACTCCTTCTGAAGAGACAG
<i>ZmBX3</i>	GRMZM2G167549	Forward: TCCACATGAAGGGCAAGGAC Reverse: ATCTCCATGGTCGCGAATCC
<i>ZmBX4</i>	GRMZM2G172491	Forward: TGCTCGCGAACCTCATCTAC Reverse: GAAGCGTCATCCCGAACTGA
<i>ZmBX5</i>	GRMZM2G063756	Forward: AGATCATGCTCGCCAACCTC Reverse: GAACGTCTCGTCCATGCTCA
<i>ZmBX6</i>	GRMZM6G617209	Forward: TTCTTCAACACGGACGTGAG Reverse: GCCATCGAGTCCTATGGTGT
<i>ZmBX7</i>	GRMZM2G441753	Forward: CCAGCCACGACCCCGCCAAGG Reverse: AGTAGTGTTCCTTCGAGGCGCCG
<i>ZmBX8</i>	GRMZM2G085054	Forward: CAGCTGGAGAGAGGGGAGAT Reverse: TCCTCTTCCTGATGCCCTCC
<i>ZmBX9</i>	GRMZM2G161335	Forward: GCCAGCTGCGACGCCCCCTTCA Reverse: CGGTACGCCATGTAGTCGC
<i>ZmBX10/11</i>	GRMZM2G311036 GRMZM2G336824	Forward: CTGCAACCGCTGTTTTCTC Reverse: CCGTGGAGATATGGCTTGCT
<i>ZmGAPDH</i>	GRMZM2G180625	Forward: AGCAGGTCGAGCATCTTCG Reverse: CTGTAGCCCCACTCGTTGTC
<i>ZmbHLH20</i>	GRMZM2G414252	Forward: CGATATCCCCGATGCAGACC Reverse: CTTCTTTGGGTACCCGGTGG
<i>ZmbHLH76</i>	GRMZM2G112629	Forward: TTCAAGCTGCACGAGGTCAT Reverse: GCGAGTGGACGGTGTAGAAT
<i>ZmMYB61</i>	GRMZM2G108959	Forward: GTGGACCAACTACCTGAGGC Reverse: GGAGGTAGGAGGCTATGGCT

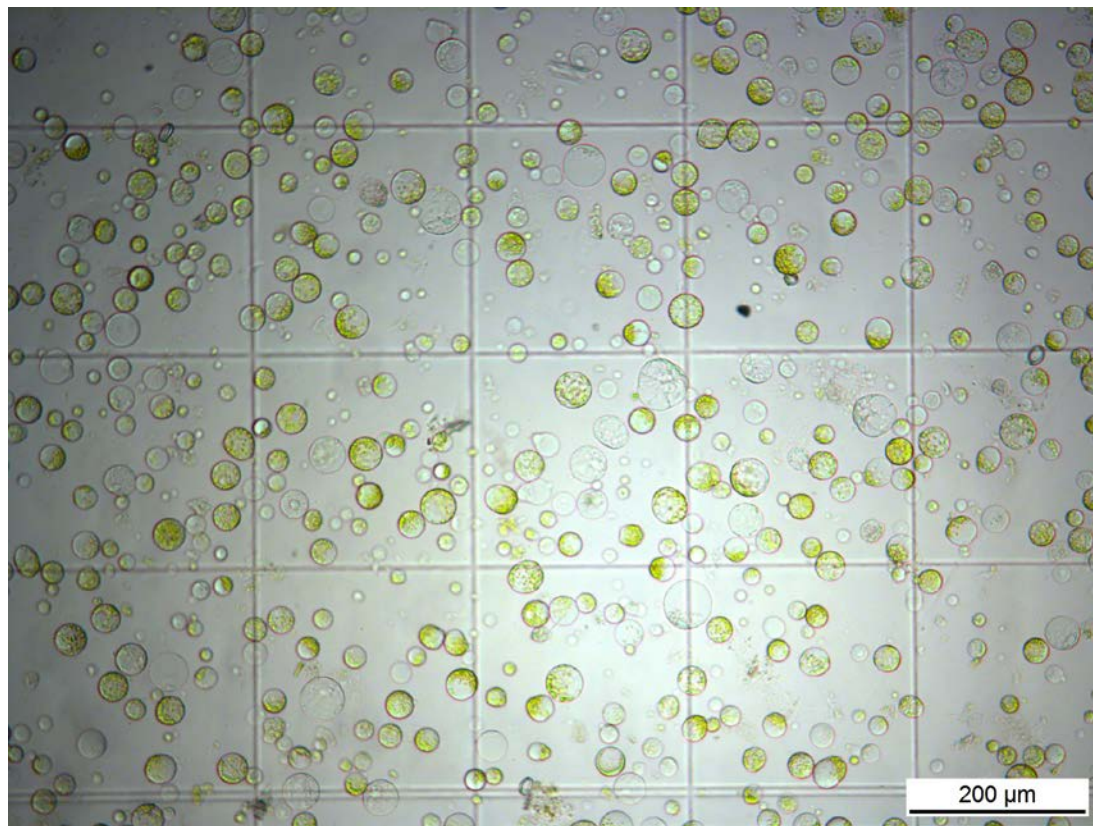
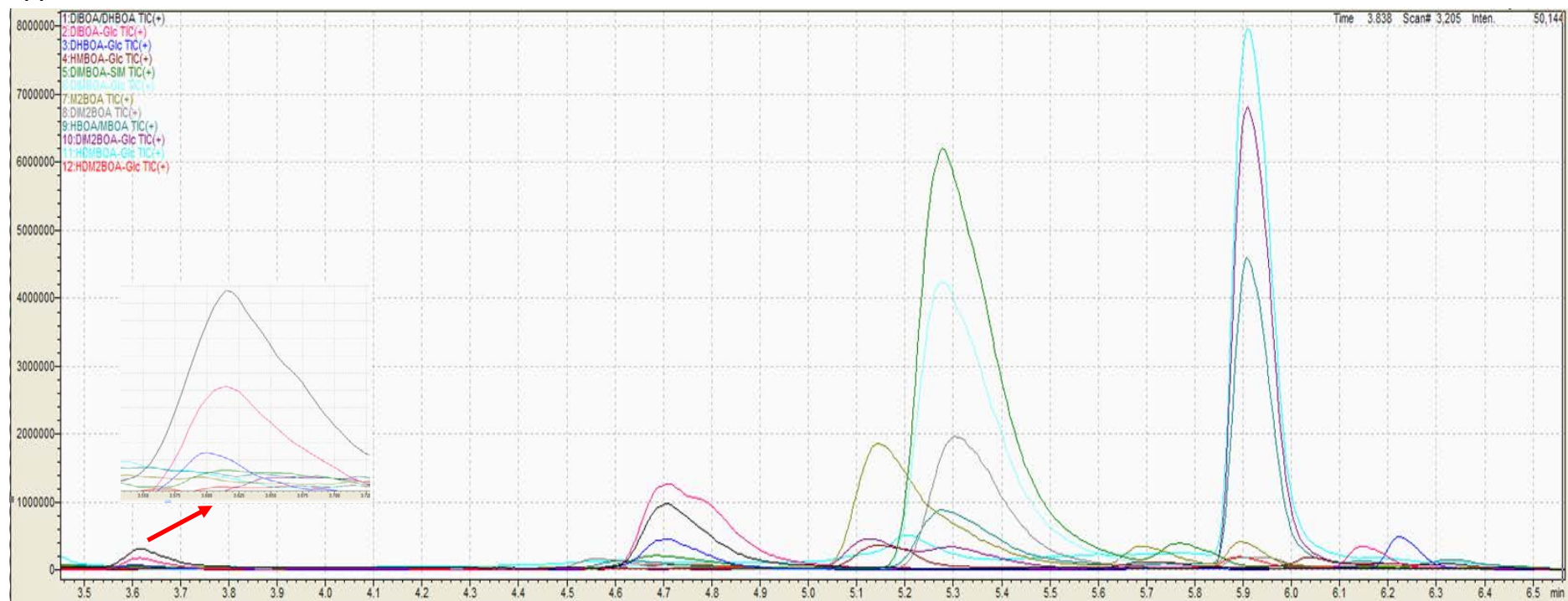


Fig. S1. Image of isolated maize protoplasts.

Isolated maize protoplasts were imaged with a microscope under a $100\times$ magnification.

Scale bar = 200 μm .

A

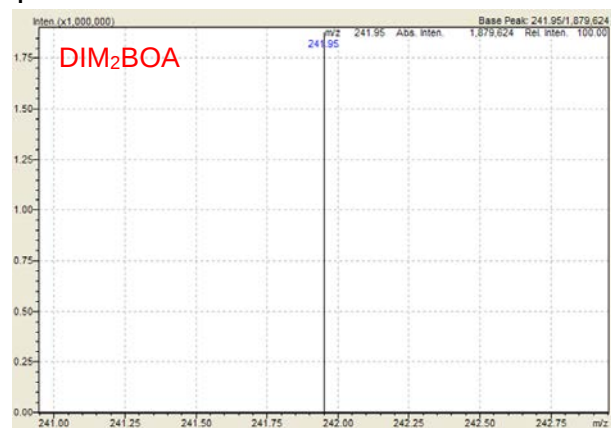




H



I



J



K



L



M

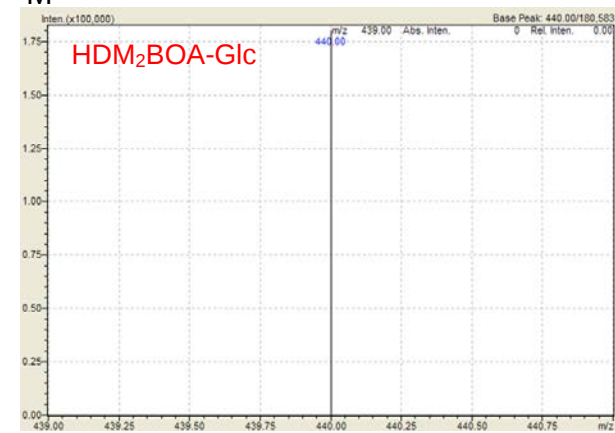




Fig. S2. The HPLC chromatogram and the MS profiles of all the benzoxazinoids.

(A) The HPLC chromatogram of the benzoxazinoids; (B-N) The MS profiles of the benzoxazinoids, (B) DHBOA, (C) DIBOA-Glc, (D) DHBOA-Glc, (E) HMBOA-Glc, (F) DIMBOA, (G) DIMBOA-Glc, (H) M₂BOA, (I) DIM₂BOA, (J) MBOA, (K) DIM₂BOA-Glc, (L) HDMBOA-Glc, (M) HDM₂BOA-Glc, (N) DIBOA. Y-axis indicates ionic intensity; X-axis indicates peak time.

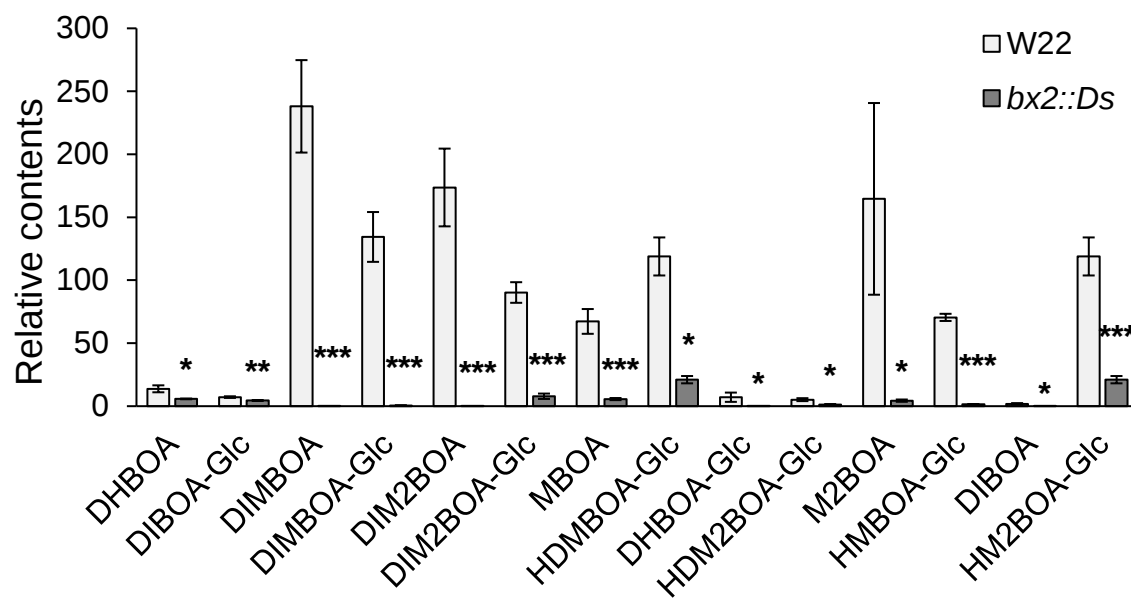


Fig. S3. Benzoxazinoid levels in W22 and *bx2::Ds* transposon knockout mutants.

Maize W22 and *bx2::Ds* mutants were grown until two-leaves stage. Leaves were harvested and benzoxazinoids were extracted for HPLC-MS analysis. Data are means $\pm$ SE. Asterisks indicate significant differences between WT and *bx2::Ds* (Student's t-test; $n = 4$; *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$).

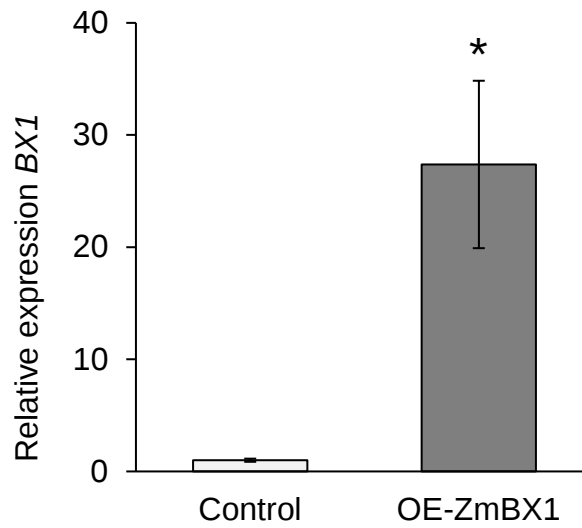


Fig. S4. *ZmBX1* transcript level in maize protoplasts after overexpressing target gene. Transcript values in controls are normalized to 1. Data = means $\pm$ SE. Asterisks indicate significant differences between control and overexpression or gene-silenced protoplasts (Student's t-test; $n = 4$; *, $P \leq 0.05$).

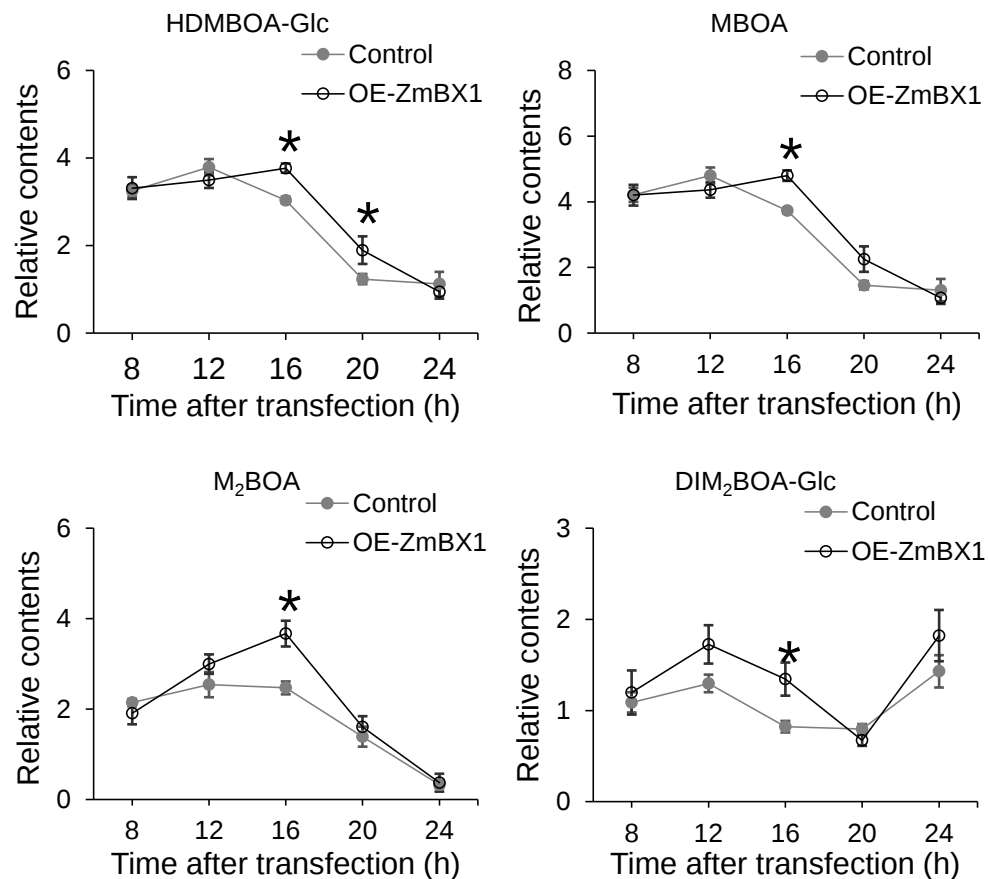


Fig. S5. The contents of HDMBOA-Glc, MBOA, M₂BOA, and DIM₂BOA-Glc in protoplasts transfected with *ZmBX1* over time.

Maize protoplasts were transfected with pM999-eGFP as the control or with pM999-*ZmBX1* to overexpress or silence *ZmBX1* (OE-*ZmBX1*). The relative contents of four main benzoxazinoids, HDMBOA-Glc, MBOA, M₂BOA, and DIM₂BOA-Glc in control and OE-*ZmBX1* maize protoplasts at different times were quantified. Asterisks indicate significant differences between control and OE-*ZmBX1* protoplasts (Student's t-test; n = 5; *, $P \leq 0.05$).

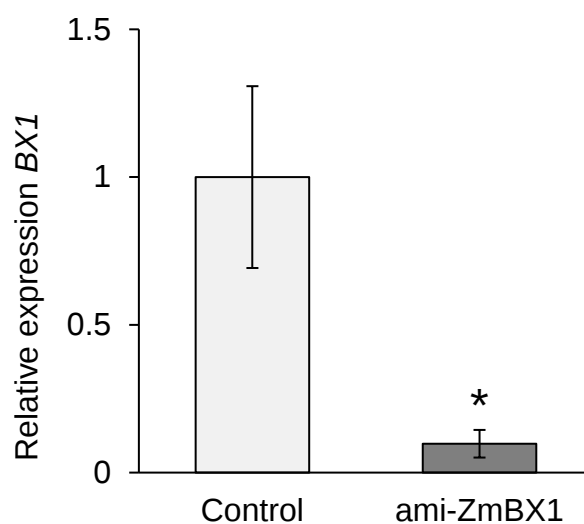


Fig. S6. *ZmBX1* transcript level in maize protoplasts after silencing with gene-specific amiRNA.

Transcript values in controls are normalized to 1. Data = means $\pm$ SE. Asterisks indicate significant differences between control and overexpression or gene-silenced protoplasts (Student's t-test; $n = 4$; *, $P \leq 0.05$).

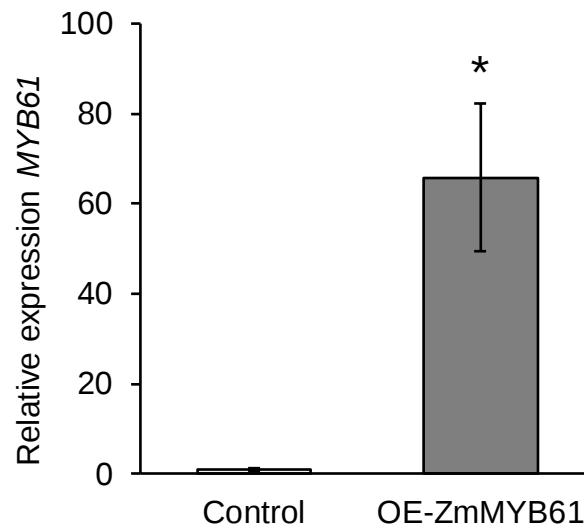


Fig. S7. *ZmMYB61* transcript level in maize protoplasts after overexpressing target gene.

Transcript values in controls are normalized to 1. Data = means $\pm$ SE. Asterisks indicate significant differences between control and overexpression or gene-silenced protoplasts (Student's t-test; $n = 4$; *, $P \leq 0.05$).

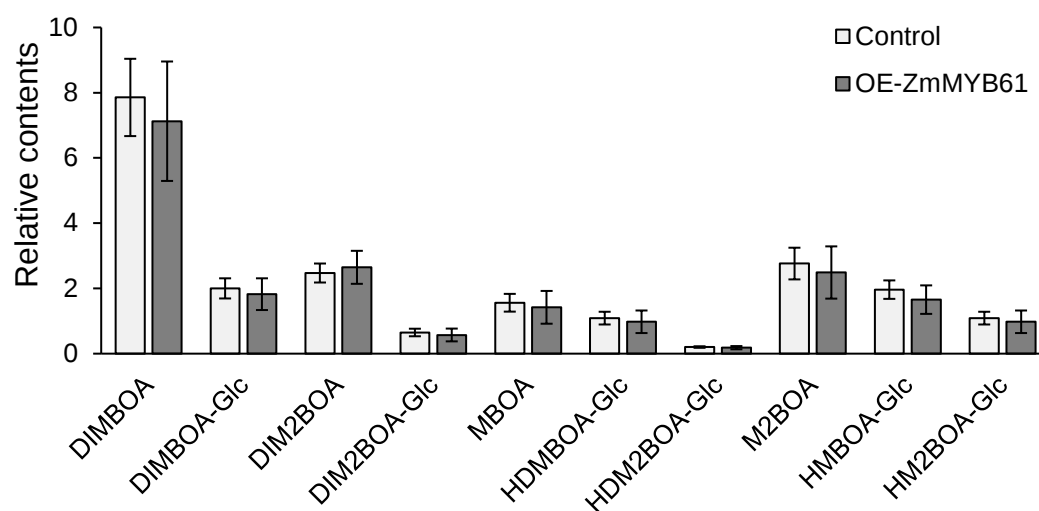


Fig. S8. The changes of benzoxazinoid contents induced by overexpressing *ZmMYB61*. Maize protoplasts were transfected with pM999-*ZmMYB61* (OE-*ZmMYB61*) or pM999-eGFP as the control. There are no significant differences between control and *ZmMYB61*-overexpression protoplasts (Student's t-test; $n = 5$).

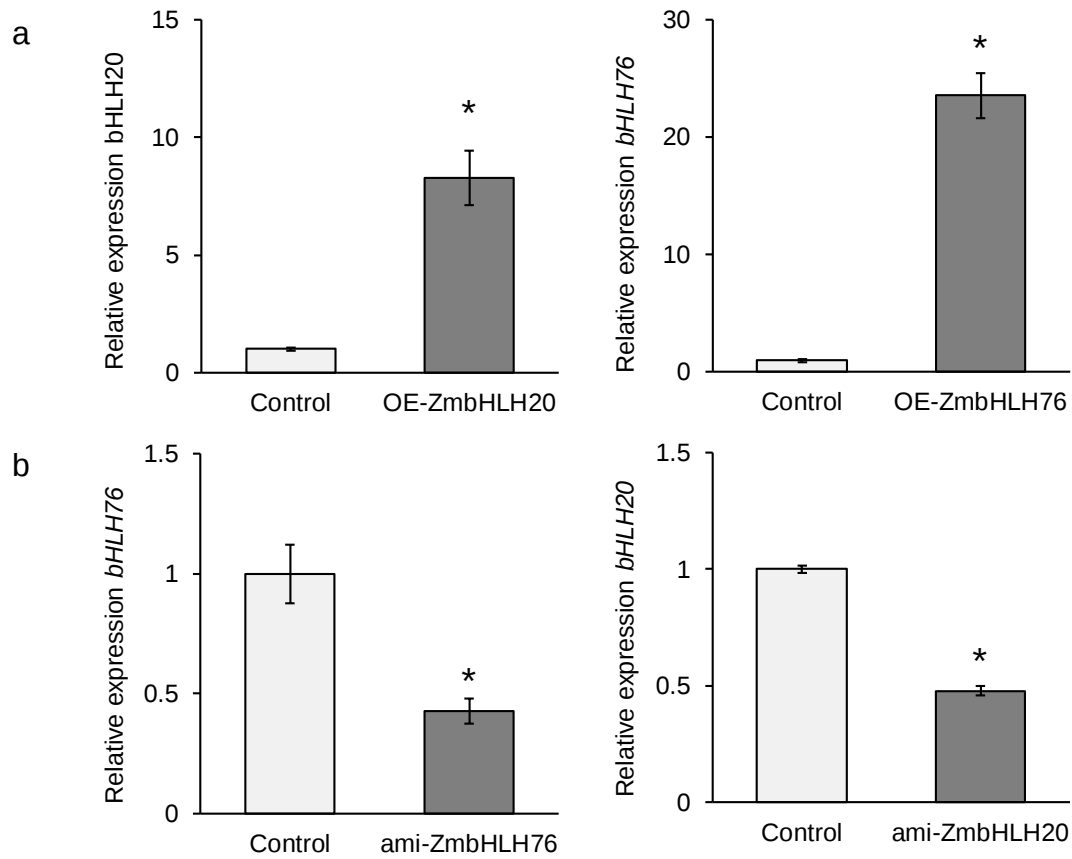


Fig. S9. Transcription levels of target genes after overexpression or silencing.

Maize protoplasts were transfected with empty vector (control) or vectors for overexpression of *ZmbHLH20*, and *ZmbHLH76* (OE-*ZmbHLH20*, and OE-*ZmbHLH76*, respectively) or silencing *ZmbHLH20*, and *ZmbHLH76* (ami-*ZmbHLH20* and ami-*ZmbHLH76*, respectively).

(a) *ZmbHLH20*, and *ZmbHLH76* transcript levels in maize protoplasts after overexpression.

(b) *ZmbHLH76* and *ZmbHLH20* transcript levels in maize protoplasts after silencing with gene-specific amiRNAs. Transcript values in controls are normalized to 1. Data = means $\pm$ SE. Asterisks indicate significant differences between control and overexpression or gene-silenced protoplasts (Student's t-test; n = 4; *, $P \leq 0.05$).

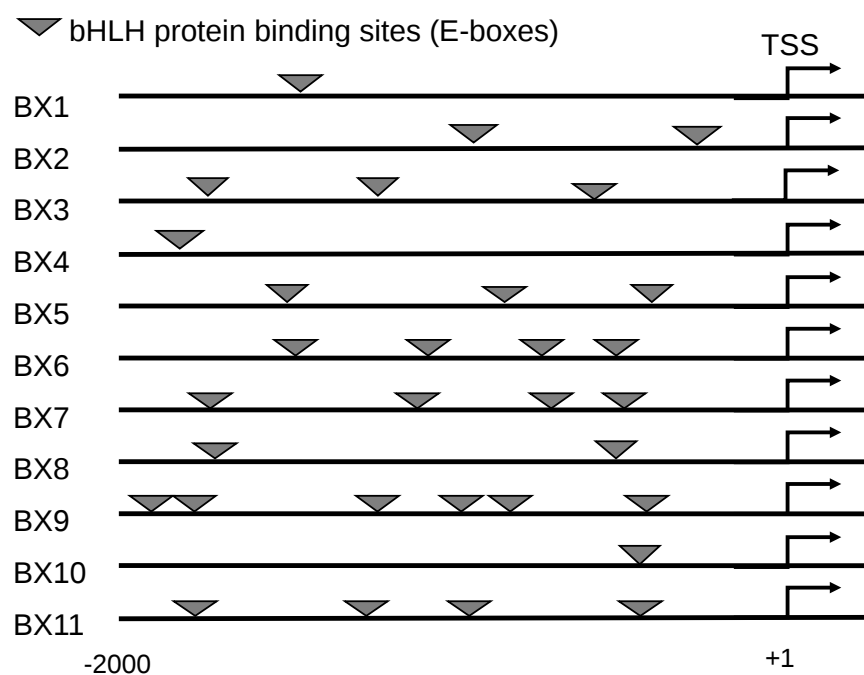


Fig. S10. Schematic representation of the predicted bHLH protein binding motifs (E-boxes) in the promoters of benzoxazinoid biosynthesis genes.

Two-kb regions upstream of the translation start sites (TSS, +1) was used for predicting the E-boxes, which are indicated by the arrow heads.