RESEARCH PAPER

Prolonged expression of the BX1 signature enzyme is associated with a recombination hotspot in the benzoxazinoid gene cluster in Zea mays

Linlin Zheng1, Michael D. McMullen2, Eva Bauer3, Chris-Carolin Schön3, Alfons Gierl1 and Monika Frey1,*

1 Lehrstuhl für Genetik, Wissenschaftszentrum Weihenstephan, Technische Universität München, 85354 Freising, Germany
2 USDA ARS, University of Missouri, Columbia, MO 65211, USA
3 Lehrstuhl für Pflanzenzüchtung, Wissenschaftszentrum Weihenstephan, Technische Universität München, 85354 Freising, Germany

* To whom correspondence should be addressed. E-mail: Monika.Frey@wzw.tum.de

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Abstract

Benzoxazinoids represent preformed protective and allelopathic compounds. The main benzoxazinoid in maize (Zea mays L.) is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). DIMBOA confers resistance to herbivores and microbes. Protective concentrations are found predominantly in young plantlets. We made use of the genetic diversity present in the maize nested association mapping (NAM) panel to identify lines with significant benzoxazinoid concentrations at later developmental stages. At 24 d after imbibition (dai), only three lines, including Mo17, showed effective DIMBOA concentrations of 1.5 mM or more; B73, by contrast, had low a DIMBOA content. Mapping studies based on Mo17 and B73 were performed to reveal mechanisms that influence the DIMBOA level in 24 dai plants. A major quantitative trait locus mapped to the Bx gene cluster located on the short arm of chromosome 4, which encodes the DIMBOA biosynthetic genes. Mo17 was distinguished from all other NAM lines by high transcriptional expression of the BX1 gene at later developmental stages. BX1 encodes the signature enzyme of the pathway. In Mo17×B73 hybrids at 24 dai, only the Mo17 BX1 allele transcript was detected. A 3.9 kb cis-element, termed DICE (distal cis-element), that is located in the Bx gene cluster approximately 140 kb upstream of BX1, was required for high BX1 transcript levels during later developmental stages in Mo17. The DICE region was a hotspot of meiotic recombination. Genetic analysis revealed that high 24 dai DIMBOA concentrations were not strictly dependent on high BX1 transcript levels. However, constitutive expression of BX1 in transgenics increased DIMBOA levels at 24 dai, corroborating a correlation between DIMBOA content and BX1 transcription.

Key words: Allele-specific expression, biosynthetic cluster, cis-element, DIMBOA, defence, QTL mapping, secondary metabolites.

Introduction

Benzoxazinoids, or cyclic hydroxamic acids, have a long record as defence chemicals in poaceous plants. In 1955, benzoxazinoids were described as antifungal compounds in rye seedlings (Virtanen and Hietala, 1955) and in 1959 benzoxazinoids were reported in maize and wheat (Wahlroos and Virtanen, 1959). In the following years, the impact of benzoxazinoids on tolerance against microbial pathogens, herbivores, and as an allelochemical has been documented (Niemeyer, 1988, 2009; Sicker et al., 2000). In maize, control of the European corn borer (Ostrinia nubilalis) is of great importance.
importance, and the content of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the main benzoxazinoid in maize, has been successfully increased by breeding programmes (Klun et al., 1970; Grombacher et al., 1989). DIMBOA represents a constitutive defence chemical that is produced without external challenge in the young plant, with concentrations of up to 30 mM in the seedling. In addition, benzoxazinoids are specifically induced by herbivory (Dafoe et al., 2013; Hufnaker et al., 2013). The constitutive concentration in the plant decreases with age, and high concentrations of DIMBOA (1.5 mM, equal to about 1 mg g⁻¹ of fresh weight; Long et al., 1975, 1977; Campos et al., 1989) are required for biological control. It would be beneficial for plant protection to extend significant DIMBOA levels to later developmental stages of the plant.

The biosynthesis of benzoxazinoids in maize has been elucidated (Frey et al., 1997; von Rad et al., 2001; Frey et al., 2003; Jonczyk et al., 2008; Meihls et al., 2013). The genes Bx1–Bx5 are sufficient to synthesize 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA). BX1 is the signature enzyme of the pathway and generates the branch off from the primary metabolism catalysing the formation of free indole that is consecutively hydroxylated by the four specific cytochrome P450 enzymes BX2–BX5. The unstable bioactive benzoxazinoid is stabilized by glucosylation catalysed by the UDP-glucosyl transferase BX8 or BX9. In some grasses, such as wild barley, DIBOA-glucoside is the major benzoxazinoid (von Rad et al., 2001) and can be considered the core genes of benzoxazinoid biosynthesis. Interestingly, these five genes constitute a biosynthetic cluster and are located within 264 kb (corresponding to 6 cM) on chromosome 4 in maize (Fig. 1). The local recombination rate in this region is 22.7 cm Mb⁻¹, which is more than 8-fold higher than the average genome-wide recombination rate of 2.7 cm Mb⁻¹, assuming a genome size of 2.3 Gb (Schnable et al. 2009) and a map length of 6243 cM (IBM302 map from MaizeGDB). In maize, DIBOA-glucoside is further modified by hydroxylation and methylation to yield DIBOA-glucoside. The respective genes, the 2-oxoglutarate-dependent dioxygenase Bx6 and the O-methyltransferase Bx7, locate 1.7 Mb upstream and 15 Mb downstream of the core cluster. The Bx8-homologous gene Bx9 resides on chromosome 1. All Bx genes are highly expressed in seedlings, which have, in parallel, the highest DIMBOA concentrations of any maize growth stage.

With the exception of the UDP-glucosyl transferases, the transcription level of all other Bx genes decreases within 3 weeks after imbibition (von Rad et al., 2001).

Gene clustering in plant secondary or specialized metabolism was first demonstrated for Bx genes (Frey et al., 1997) and since then has been discovered in several plant species; currently, at least 15 biosynthetic gene clusters have been reported (reviewed by King et al., 2014; Nützmann and Osbourn, 2014). It was speculated that gene clusters might have a selective advantage, since superior allelic combinations are inherited preferentially once established in the coupling phase (Field and Osbourn, 2012; Takos and Rook, 2012). Clustering might also facilitate co-ordinated regulation by domains for chromatin modification. Indeed, for theavenacin biosynthetic cluster, co-ordinated localized chromatin modification was revealed by DNA fluorescence in situ hybridization and correlated with expression of clustered genes (Wegel et al., 2009). Data about transcription factors and cis-elements that are employed in biosynthetic cluster regulation are scarce. A unique example is given for the momilactone and phytocassene gene cluster in rice (Okada et al., 2009) where transcription is regulated by a chitin oligosaccharide elicitor-inducible basic leucine zipper transcription factor. Nomura et al. (2008) identified by biomathematical comparison putative cis-elements for Bx gene regulation in wheat. However, experimental proof is limited. Regulatory cis-elements can be quite distal to the coding region of genes and therefore difficult to determine. Such an arrangement has been detected for the maize Vgt1, tgl1, and b1 genes, which have essential regulatory sequence elements at a distance of several 10 kb. These sequences have been detected by genetic analysis (Stam et al., 2002; Clark et al., 2006; Salvi et al., 2007).

Quantitative trait locus (QTL) analysis has been used to study maize resistance to European corn borer and aphids (Jampatong et al., 2002; Cardinal et al., 2006; Betsiaishvili et al., 2015). These studies indirectly addressed the variation in DIMBOA content at later developmental stages, and all detected a major QTL in the region of the Bx gene cluster on chromosome 4. In a recent QTL analysis, the DIMBOA content at 32 d after imbibition (dai) in maize plants was assayed directly (Butrón et al. 2010) across genetically divergent maize inbred lines. A major QTL was detected in the region of the Bx gene cluster with eight recombinant inbred line (RIL) populations of the nested association mapping (NAM) population array (Yu et al., 2008). The parallel association mapping with the Bx candidate genes placed a major polymorphism in proximity to the Bx1 gene.
In this study, we screened all 26 NAM parental lines to evaluate potential differences in DIMBOA content in 24 dai maize plants. QTL mapping was performed using lines with high (Mo17) or low (B73) 24 dai DIMBOA content as parental lines. We specifically characterized the Bxl-specific transcription pattern and detected a 3.9 kb cis-element approximately 140 kb upstream of Bxl that is required for high Bxl transcript levels during the later stages of plant development. A high recombination frequency was associated with the Bx gene cluster, suggesting a genetic mechanism that provides diversity in defence gene expression.

**Materials and methods**

**Standards, reference chemicals, oligonucleotides**
The benzoxazinoids were a gift from Professor D. Sicker, University of Leipzig, Germany, or were prepared as described by von Rad et al. (2001). Oligonucleotides were synthesized by Eurofins MWG Operon (Ebersberg, Germany), Microsynth AG (Balgach, Switzerland) and Biomers (Ulm, Germany).

**Bacterial artificial chromosome (BAC) resources**
BAC AC213878 (B73) was provided by the Children’s Hospital Oakland Research Institute, CA, USA. BAC b60506A16 (Mo17) was provided by Dr Bailin Li, DuPont Pioneer, USA.

**Plant material**
The maize inbred lines B73 and Mo17, the NAM founder lines, the IBM302 population, RILs, and near-isogenic lines (NILs) were provided by the Maize Genetic Stock Center, and Dr Nathan Springer (University of Minnesota). HiII parental lines were provided by Dr Patrick S. Schnable, Iowa State University, IA, USA.

**Molecular biology methods**
DNA and RNA isolation, cDNA synthesis, cloning, and PCR amplification was as described by Schullehner et al. (2008). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out with a Roche LightCycler 480 instrument using a Bioline SensiFAST™ SYBR No-ROX kit. Primer and PCR conditions are given in Supplementary Table S1, available at JXB online. DNA sequencing was by Source Bioscience, Berlin), GATC-Biotech AG (Konstanz, Germany), and Eurofins Genomics (Ebersberg, Germany). All primer pairs were checked for specificity against the maize B73 reference genome, version 2, and PCR products were sequenced to verify amplification of the respective Bx gene.

**Genotyping**
Genotypes were determined by the size difference of the PCR products, by using allele-specific primer pairs or by sequencing. Genotyping of the distal cis-element (DICE) was by a combination of allele-specific primers and sequencing. The PCR primer pair DICEFW and DICE2REV (Supplementary Table S1) were used for the Mo17 and B73 alleles of DICE and a fragment of about 527 bp was amplified independent of the genotype. The fragment included the border of DICE-A and DICE-B and sequence alterations that distinguished the B73 and Mo17 DICE sequences. If duplication was present, the PCR amplicon included two fragments. In this case, the sequencing led to double labelling due to the sequence difference in the two DICE-A parts in Mo17. If a single DICE was present, sequence analysis allowed determination of the DICE-B genotype and discrimination between B73 DICE-A and the second Mo17 DICE-A on the other hand. To discriminate between B73 DICE-A and the first Mo17 DICE-A, the marker M143 was used, which revealed the size differences existing between the two sequences.

**Determination of expression levels by sequencing of PCR-amplified cDNA**
Primer pairs Bx1QF2/Bx1QREV2 and Bx2QF/Bx2QR (Supplementary Table S1) were used for the amplification of cDNA and genomic DNA templates. Contamination of cDNA by genomic DNA was recognized by the presence of introns. As controls, Bxl was analysed in parallel, and genomic DNA of the B73×Mo17 hybrid was used as a control for both Bxl and Bx2 analyses.

**Maize transformation**
Transgenic maize was generated as described by Frame et al. (2002). The Bxl coding sequence under the control of a maize ubiquitin promoter (Christensen and Quail, 1996) was inserted into Ti-plasmid pTF101.1. pTF101.1 was kindly provided by the Plant Transformation Facility (Iowa State University, Ames, IA, USA). Selfed T1 progeny segregating for wild-type and transgenic plants was analysed. The genotype was confirmed by analysis of Bxl transcription (Supplementary Table S1).

**Plant growth and harvesting of plant material**
Maize seed was sterilized with 1.3% sodium hypochlorite, washed with tap water and germinated at 28 °C on germination paper for 2–5 d in the dark before analysis or planting (standard soil ED73 with 10%, w/w, sand, 8 × 8 cm pots). Twenty plants were grown in a tray in the plant growth chamber (Climate Chamber HPS 2000; Heraeus Vötsch Industrietechnik GmbH, Germany) for 16 h at 22 °C in the light and 8 h at 18 °C in the dark at 80% moisture. For each experiment, B73 and Mo17 plants were grown in parallel to RILs, NILs, recombinants, and hybrids, and the plants were placed randomly in the trays. The trays were circulated every 24 h.

Shoots of etiolated seedlings were harvested at 4 dai. For the 24 dai material, the blade of the third leaf was cut at the border to the leaf sheath with the scissors. Three to four harvested leaves of one line were pooled, the weight was determined, and the material was frozen immediately in liquid nitrogen and stored at −70 °C.

**Biochemical analysis of benzoxazinoids**
Benzoxazinoid extraction was carried out as described by Grün et al. (2005). Briefly, the fresh weight of the plant material used was determined (about 400 mg of ground powder per assay). The DIMBOA amount was determined by high-performance liquid chromatography (HPLC) using a standard curve. DIMBOA (1 mmol per 1 kg of fresh weight) was considered to be 1 mM DIMBOA. The ground material was suspended in 3.3 vols of H2O (w/v) and incubated for 1 h at room temperature to allow the maize β-glucosidase to generate the benzoxazinoid aglucone. No attempt was made to analyse aglucone and glucoside separately. The probes were dissolved in methanol.

HPLC analysis of DIMBOA was carried out on a LiChroCART® 250–4 LiChrospher® 100 RP-18e (5 µm) column (Merck, Darmstadt, Germany) using Dionex 2284 SoftIon SP2 (Thermo Scientific Dionex). As mobile phases, 0.3% formic acid and methanol were used at a flow rate of 1.0 ml min−1. The HPLC was run for 60 s in 80% 0.3% formic acid, 20% methanol, and the methanol concentration was then increased to 40% within 300 s and to 42.5% in the following 360 s. Washing of the column was for 60 s with 100% methanol and equilibration was for 180 s with 80% 0.3% formic acid and 20% methanol. Analysis was done with the chromatography data system Chromeleon version 6.80 (Thermo Scientific Dionex).
Benzoxazinoid concentration was calculated using a calibration curve. The values were normalized to material fresh weight.

**Statistical analysis tools**

Microsoft Excel was used to calculate the average values, standard deviations of the DIMBOA contents, and late *Bx1* expression level. Composite interval QTL mapping (Jansen and Stam, 1994; Zeng, 1994) for the IBM population and selected IBM subpopulation was performed using Windows QTL Cartographer 2.5. Significance thresholds were determined by 1000 permutations (Basten et al., 1997). A high-density genetic map with 1435 markers combining the genotypes of the IBM302 population (Lee et al., 2002) provided by the maize mapping project (http://curation.maizegdb.org/ibm-302scores.html) with single-nucleotide polymorphism (SNP) scores from University of Missouri was used (Supplementary material, available at *JXB* online). The total length of this genetic map was 6242.7 cM.

Analysis of variance was performed, and differences between genotypic means were tested with a Kruskal–Wallis test accounting for multiple comparisons at the 0.05 significance level. Allele-specific effects of hybrids were tested using Student’s *t*-test with *P*<0.05 considered significant. Spearman’s correlation coefficient between late DIMBOA content and *Bx1* expression in selected RILs and the two NILs was calculated in R (http://www.r-project.org).

BLAST analysis and genome browsing were performed with the tools of MaizeGDB (http://www.maizegdb.org/ Monaco et al., 2013). All sequence positions are given according to the Arabidopsis Genome Initiative (AGI) B73 RefGen_v2.

**Results**

**Effective benzoxazinoid levels beyond the seedling stage are rare in maize**

The first analyses of benzoxazinoid levels in maize (e.g. Long et al., 1975) revealed that, at the seedling stage, concentrations are generally high and significant line differences do not become obvious until the plant reaches heights of about 25 cm. We used the NAM panel of maize lines (Yu et al., 2008) to define the range of benzoxazinoid content in older plants that exists due to genetic diversity in maize. To minimize environmental differences, the plants were placed in growth chambers and analysed at 24 dai. At this time point, all lines had a similar size and for all genotypes the third leaf was fully expanded; the blade of this leaf was harvested for benzoxazinoid analysis and RNA extraction. In parallel, 4 dai seedlings of the NAM inbred lines were analysed. At 4 dai, all lines had high benzoxazinoid content in seedling shoots and roots (16.8±9.4 and 7.6±5.7 mM, respectively), and the variation within the lines was high (Supplementary Fig. S1, available at *JXB* online). Differences between the lines become obvious for the 24 dai leaf material. For most lines, the concentration was below 1 mM (Fig. 2); only lines B97, M37W, and Mo17 reached levels of 2 mM or higher. Mo17 had a consistently high benzoxazinoid concentration at 24 dai (3.8±2.0 mM; Fig. 2A).

![Fig. 2.](attachment:image.png) Analysis of 24 dai plants of the NAM founder line panel. (A) DIMBOA content. (B) *Bx1* transcript level. (C) *Bx4* transcript level. (D) *Bx8* transcript level. Transcript levels were normalized to the cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) mRNA level. Two sets of material were grown and at least one sample of pooled leaf material (three to four plants; results show means±standard deviation) was analysed.

High late transcript level of the signature gene *Bx1* is a unique feature of Mo17

To obtain a first insight into the transcriptional expression of the biosynthetic genes (*Bx* genes) within the NAM panel, the 24 dai transcript levels of *Bx1* (Fig. 2B), *Bx4* (Fig. 2C), and *Bx8* (Fig. 2D) were determined by qRT-PCR. The three genes represent the signature enzyme (branch-point reaction), a pathway-specific P450 mono-oxygenase, and the UDP-glucosyltransferase required for stabilization of the benzoxazinoid aglucone. Sequence information was available for these genes from all 26 lines of the NAM panel, and primer pairs for all the different alleles were designed. *Bx4* and *Bx8* transcript levels varied between the lines but...
displayed no correlation with benzoxazinoid levels (Fig. 2C, D; \(P > 0.05\)). \(Bx1\) mRNA was present at low concentrations in most lines [<0.1 pg pg\(^{-1}\) of cytosolic glyceraldehyde 3-phosphate dehydrogenase (GAPC) mRNA] and the level did not correlate significantly with DIMBOA content (\(P > 0.5\)). Mo17 was exceptional in having about a 10-fold higher \(Bx1\) transcript level compared with all other lines (Fig. 2B, and see below). The data indicated that the mRNA level of the pathway genes is not necessarily correlated with elevated benzoxazinoid concentrations at 24 dai. However, the relatively high \(Bx1\) transcript level in 24 dai Mo17 could indicate an influence on DIMBOA content in this line.

**\(Bx1\) transcription is Mo17 allele specific at later developmental stages**

Benzoxazinoid content and transcript levels of the \(Bx\) genes in Mo17 and B73 as representatives of high and low 24 dai benzoxazinoid-containing lines were analysed in more detail (Fig. 3 and Supplementary Fig. S2, available at JXB online). Primer pairs for all alleles of all \(Bx\) genes in the two lines were designed (Supplementary Table S1). In seedlings (4 dai) of both lines, the pattern for all \(Bx\) genes was almost identical (Supplementary Fig. S2). In B73, the \(Bx1\) level decreased to the detection limit by 14 dai. By contrast, \(Bx1\) levels were high in Mo17 at 24 dai. The high \(Bx1\) transcription level at this ‘late’ stage was the major difference between the lines in transcript pattern and correlated with the difference in DIMBOA content determined for the two lines at 24 dai (Fig. 3B). SNPs in the coding region of the Mo17 and B73 \(Bx1\) alleles do not change the amino acid sequence and catalytic properties should be equivalent.

This difference might be caused by the presence of linespecific transcription factors and variation in cis-elements. To distinguish between these possibilities, allele-specific primers were designed (Supplementary Table S1), and hybrid plants were analysed (Fig. 3E, F) to determine the contribution of the parental genomes to \(Bx1\) expression. In 4 dai seedlings, the \(Bx1\) transcript level was similar in the parental lines and the reciprocal hybrids (Fig. 3C). Allele-specific analysis shows that both alleles were expressed at the same level (Fig. 3E). The picture changed for 24 dai material. In both reciprocal
hybrids, the Mo17 allele was expressed almost exclusively (Fig. 3F). Hence, cis-element(s) contribute to the 24 dai expression of the Mo17 Bx1 allele. Since the mid-parent Bx1 transcript level was not fully reached in the hybrids (Fig. 3D), a negative effect by trans-acting factors cannot be excluded.

In summary, 24 dai Mo17 and B73 plants differed in DIMEOA concentration and Bx1 mRNA levels. The 24 dai expression of the Mo17 allele is probably the result of the interaction of trans-factors with Mo17-specific cis-element(s). To analyse the impact of transcriptional regulation on benzoxazinoid concentration and to reveal additional mechanisms, a QTL analysis based on the B73×Mo17 intermated RIL population IBM302 (Lee et al., 2002) was performed.

Mapping reveals a major QTL for prolonged DIMBOA content close to the Bx gene cluster

Late DIMEOA concentrations were measured for the IBM302 RILs. As expected for quantitative traits, continuous values of benzoxazinoid content were displayed. The lowest line (MO382) had a benzoxazinoid level close to the detection limit. The value of the highest line (MO147) exceeded the level of the high parent Mo17 by a factor of 4 (Supplementary Fig. S3, available at JXB online).

The genotype scores of the IBM302 population provided by the maize mapping project (http://curation.maizegdb.org/ibm302scores.html, Schaeffer et al., 2011) were used to construct a high-density genetic map with 1435 markers (Supplementary material) for QTL mapping. Three QTLs, QTL1 (chromosome 1), QTL4-1 (chromosome 4), and QTL5 (chromosome 5), were detected and were consistent in 1000 permutations (Supplementary Fig. S4, available at JXB online). A further QTL on chromosome 4 (QTL4-2) had a LOD of 2.7. QTL4-1 mapped to the short arm of chromosome 4 and overlapped with the Bx gene cluster; the major peak was around 200 kb upstream of Bx1 (position 3 109 838). QTL4-1 (LOD value 15.2) explained 21.5% of the phenotypic variation (Supplementary Table S2, available at JXB online). The other three QTLs accounted for 3.3–6.1% of the phenotypic variation (Supplementary Table S2). For QTL4-1, QTL4-2, and QTL5, Mo17 alleles increased the trait value, while at QTL1 the positive contribution was from B73 (Supplementary Fig. S4 and Table S2, available at JXB online). In the following, the analysis concentrated on the major QTL4-1.

A hotspot of recombination is detected in the intergenic interval between Bx8 and Bx5

In order to determine whether QTL4-1 comprised the cis-element(s) required for high 24 dai Bx1 expression, RIL MO038 (Supplementary Table S3, available at JXB online) was crossed to B73 for fine mapping. MO038 had a late DIMEOA content that was more than twice the Mo17 level. With respect to Bx1 expression, MO038 had the same properties as Mo17: the transcript level in 24 dai plants was as high as that in Mo17, allele-specific expression was displayed in the hybrid, and the transcript level in the hybrid was about 30% lower than the mid-parent value (Supplementary Fig. S5, available at JXB online). In contrast to Mo17, the RIL MO038 had homozygous B73 conformation for QTL-1 and the largest part of QTL4-2. These two QTL regions will be homozygous in the F1 and all selfed progeny.

Two B73×MO038 hybrid plants were selfed and the progeny used for fine mapping in the interval between marker M210 (chromosome 4: 3 045 994–3 046 270) and M5 (chromosome 4: 3 250 998–3 251 489), which is the central region of QTL4-1. For the analysis, 11 co-dominant polymorphic markers were generated (Supplementary Table S4, available at JXB online). Twenty recombination events (Supplementary Fig. S6) were detected by screening of 750 plants representing 1500 gametes. Two hotspots of recombination were revealed. The borders are given by the markers M210 and M148 (seven recombination events within 62 kb), and M148 and M137 (13 recombination events within 11 kb) (Supplementary Fig. S6), respectively. No recombination was detected in the 132 kb DNA stretch between markers M137 and M5. The majority of recombination was located close to (eight recombinations) or within (five recombinations) the region 3 112 492–3 116 365. This 3.9 kb region was termed the ‘distal cis-element’ (DICE). It was present as a duplication in Mo17 (Supplementary Fig. S7A, available at JXB online). While one DICE copy in Mo17 was almost identical to that of B73, the other copy had several sequence alterations (Supplementary Fig. S7A, B). The structure of the DICE sequence in Mo17 was verified by Southern blot analysis (Supplementary Fig. S7C) and sequencing of the genomic and BAC DNA. The recombination rate in the DICE element was 74 cm Mb−1, which is almost 30 times higher than the genome’s average and even above the values generally found for genomic regions (Dooner et al., 1985). At present, it is unclear whether a specific sequence segment of DICE or its duplication in Mo17 triggers recombination in this region.

Mo17-specific cis-elements required for prolonged and allele-specific Bx1 transcription are located within 141 kb upstream of the gene

The recombinants made it possible to estimate the contribution of the sequences upstream and downstream of DICE, and the effect of the DICE duplication on late DIMEOA content, Bx1 gene expression, and allele-specific expression. To generate homozygosity with respect to the QTL4-1 region, the recombinants were selfed and the F3 genotyped (Fig. 4A). Either the homozygous F3 plants or F4 progeny thereof were analysed for DIMEOA content, Bx1 transcript level. For allele-specific Bx1 expression analysis, these lines were crossed with either Mo17 or B73 to generate heterozygosity with respect to Bx1. It was expected for the F3 and F4 material analysed that loci unlinked to QTL4-1 would segregate independently. Recombinants with exchanges in the same region were grouped together in the following analyses (Fig. 4, groups A–G).

The DIMEOA content differed by a factor of 4.5 (Fig. 4B) in the homozygous recombinants. The high value of the parental MO038 line was not reached, but all lines had values higher than B73. The DIMEOA concentration tended
to increase with the presence of the Mo17 genotype in and around the DICE region. The highest late DIMBOA content was detected in group F. The reciprocal exchange (group A) had reduced DIMBOA levels and a significant decrease was found for group G, which was distinguished from group F by loss of the Mo17 conformation of the DICE sequence (Fig. 4B).

The variation in the late Bx1 expression level in the recombinants was more than 60-fold (Fig. 4C) and was not absolutely correlated with the DIMBOA content. The Bx1 transcript level of Mo17 was reached by the recombinants that had both the Mo17 constitution for DICE and Mo17 sequences downstream thereof (group F). If the DICE duplication was missing (Fig. 4C, group G), the transcript level was significantly reduced. This result delimited upstream sequences influencing Bx1 transcript level to the DICE sequence. However, presence of the duplication was not sufficient to confer high late Bx1 mRNA levels if the downstream

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**Fig. 4. Analysis of recombinants.** (A) Schematic presentation of recombination breakpoints. Marker positions are indicated in the header line and the distance of the marker to Bx1 is indicated by the number, e.g. M137 is located 137 kb upstream of Bx1. The size of gaps between the markers demonstrating recombination in the neighbourhood of DICE is given in the light grey fields. The DICE region is delimited by grey lines. Grey, Mo17 genotype, white, B73 genotype, light grey: unknown genotype. (B, C) DIMBOA content at 24 dai (B) and Bx1 transcript level at 24 dai (C) in the respective groups of recombinants. All homozygous recombinants of group A–D were crossed with Mo17 and all recombinants of group E–G with B73, to yield heterozygous conformation for Bx1. B73 or Mo17 in (C) indicates whether the transcript level of the recombinant chromosome’s Bx1 gene is the lower (B73-type) or the higher (Mo17-type) one. Statistical analysis was by Kruskal–Wallis test and multiple comparison of treatments. Mean values and standard deviation are indicated. Identical letters above columns indicate no statistical difference (P>0.5).
sequences came from the B73 genome (group D). In hybrids, the $Bx1$ transcript level of the allele encoded by the recombinant chromosome was according to its genotype (groups A–D, B73-like, groups E–G, Mo17-like; Fig. 4). Hence, the recombination breakpoint closest to $Bx1$ (DICE, 141 kb upstream of $Bx1$) gave the upstream limit for localization of the cis-element(s) conferring allele-specific transcription. However, although in the hybrid B73 recombinant 222 (group G) the Mo17 allele of $Bx1$ contributed by the recombinant had a higher transcript level than the B73 counterpart, the absolute $Bx1$ transcript level was low compared with B73×Mo17 hybrids (Supplementary Fig. S8, available at JXB online). This implied that the Mo17 DICE sequence that was missing in recombinant 222, in conjunction with a further cis-element, was required for high 24 dai $Bx1$ expression.

To refine the mapping of the cis-elements, RILs were analysed (Fig. 5), which represent sets with opposing genotypes upstream of $Bx1$ (set 1, Mo17 genotype: MO038, MO141,

**Fig. 5.** Analysis and genotypes of selected lines. (A, B) DIMBOA content (A) and $Bx1$ mRNA level (B) in 24 dai plants. Statistical analysis was by Kruskal–Wallis test and multiple comparison of treatments. Mean values and standard deviation are indicated. Identical letters above columns indicate no statistical differences ($P>0.5$). (C) Schematic representation of the genotypes of these lines for chromosome 4 and the QTL regions on chromosomes 1 and 5. White, B73; black, Mo17; hatched, hybrid genotype. The positions of $Bx1$ and of QTLs are indicated. For genotyping, the markers listed in Supplementary material were used. The position of the QTL is given in Supplementary Table S2.
MO024, MO035, MO033, MO067, and MO276; set 2, B73 genotype: MO161, MO0331, MO061, MO076, MO039, and MO382) and harbour different proportions of the B73 genome downstream thereof (Fig. 5C). Set 1 RILs indicated that chromosome 4 sequences downstream of Bx1 might also modulate Bx1 transcript levels. With the exception of MO276, all of these lines had DIMBOA and Bx1 levels that were significantly higher than B73 and DIMBOA levels and Bx1 expression was correlated (Spearman correlation coefficient=0.75, P<0.001; Fig. 5A, B). By contrast, all lines of set 2 had low DIMBOA and low Bx1 transcript levels. The data corroborated the impact of DICE and downstream sequences on Bx1 expression detected in the recombinant lines.

Representative RILs and two B73×Mo17 NILs (Eichten et al., 2011; see below) were crossed with Mo17 or B73 to generate progeny with heterozygous conformation for the Bx1 gene. These hybrids were analysed for allele-specific expression (Supplementary Fig. S8). All sequences downstream of Bx1 were present in the B73 conformation in NIL B184 and RIL MO276 (Fig. 5C). For both lines, allele specificity was detected. Therefore, the responsible cis-elements were located between DICE and the Bx1 coding sequence. Allele-specific expression of all other lines was in accordance (Supplementary Fig. S8). In MO067 and MO276, Bx1 had a B73 genotype and the upstream sequences were Mo17. In the hybrids generated by crossing with Mo17, both alleles were expressed at the same level. Hence, the cis-elements relevant for allele specificity were present in these RILs and no sequences within the gene itself were required. MO057 and the recombinant 222 (see above), which both had the recombination breakpoints within the DICE sequence leading to a monomeric hybrid DICE sequence, displayed allele-specific expression. This result revealed that it was the downstream sequences and not the duplication of the DICE sequence conferring allele specificity. The DICE sequence therefore mainly influences the Bx1 transcript level, while allele specificity is mediated by additional cis-element(s) located in the DNA stretch of 141 kb between DICE and Bx1 in Mo17.

**High late DIMBOA levels can be established independent of the transcription of the biosynthetic genes**

B73×Mo17 NILs were included in the study (Fig. 6). From the NIL collection, the lines B184 (B73 background) and M31 (Mo17 background) were chosen, which had Mo17 and B73 introgressions in the QTL4-1 region (Fig. 6A). Surprisingly, M31, which had the B73 conformation for QTL4-1 including the whole Bx gene cluster, had a high 24 dai DIMBOA content. The benzoxazinoid level in the reciprocal NIL B184 was increased relative to B73 but was significantly lower than in Mo17 and M31 (Fig. 6B). With respect to Bx1 transcript levels, the NILs behaved as expected: M31 had levels as low as B73, and B184 had an increased Bx1 transcript level compared with B73 (Fig. 6C). This showed that in Mo17 a mechanism exists that confers the prolonged presence of elevated DIMBOA concentrations independent of transcriptional activity of the Bx1 gene. The finding was in line with the data for RILs and recombinants that showed no absolute correlation between Bx1 mRNA levels and benzoxazinoid content.

**Influence of trans-acting factors on Bx1 transcript levels**

The previous analysis located cis-elements of Bx1 gene transcription to the QTL4-1 region. Transcriptional regulation is the result of the interaction between cis-elements and trans-acting factors. The finding that in B73×Mo17 hybrids the mid-parent value of the Bx1 transcript level was not reached (e.g. Fig. 3D) could be explained by a dose-dependent activator(s) present in Mo17 and by a negative factor(s) in the B73 genome. Investigation of the NIL hybrids was carried out to reveal whether, in addition to the cis-elements, trans-factors were present in the QTL 4-1 region (Fig. 7). B184×Mo17 hybrids had a homozygous Mo17 constitution at QTL4-1 and were heterozygous for the rest of the genome. The Bx1 transcript level was twice as high as in the B73×Mo17 hybrids but significantly lower than in Mo17 (Fig. 7A). The difference in transcript level compared with Mo17 had to be ascribed
A QTL for variation of DIMBOA content on chromosome 1 revealed by Meihls et al. (2013) using B73×CML322 RILs (bin 1.04) did not coincide with the QTL found here (bin 1.06).

**Bx2 transcript level is not influenced by cis-elements**

The Bx genes form a biosynthetic cluster. It has been speculated that clustering of the Bx genes favours co-ordinated regulation (Gierl and Frey, 2001). Co-ordinate regulation by shared elements was expected especially for Bxl and Bx2 since both genes are separated by only 2.5 kb. Late Bx2 levels for B73 and Mo17 were not significantly different, but this did not exclude the possibility that allele specificity exists. There was no sequence difference for the two Bx2 alleles that would allow differentiation of expression by RT-qPCR. Instead, an analysis by cDNA sequencing was performed. Three SNPs were analysed for Bxl and four for Bx2. The predominant expression of the Mo17 allele of Bxl was obvious (Supplementary Fig. S9, available at JXB online). In contrast, both Bx2 alleles were expressed at the same level. The cis-elements defined in the Bxl upstream region hence were not effective for Bx2 expression.

**Increased Bx1 expression is sufficient to increase DIMBOA content in older plants**

The previous analyses revealed a major QTL for high late benzoxazinoid concentration in the region of the Bx cluster and further analysis showed that the main feature correlated with QTL4-1 was elevated late Bxl expression mediated by cis-elements. However, the investigations demonstrated that further mechanisms exist that result in high late DIMBOA concentrations and are independent of Bxl transcription. To assay whether prolonged Bxl gene expression was sufficient to elevate the benzoxazinoid content in older plants, we attempted to increase Bxl expression by transgenic expression of the gene driven by the ubiquitin promoter. The hybrid maize line HiII (Zhao et al., 1998) was used for transformation with Agrobacterium tumefaciens (Frame et al., 2002). There was no obvious phenotypic difference between transgenic plants and the non-transformed siblings at the seedling and adult stage. In seedlings, the amount of Bxl mRNA detected by qRT-PCR in HiII was similar to B73 (data not shown). In transgenic progeny, the Bxl transcript levels consistently exceeded glyceraldehyde 3-phosphate dehydrogenase values at 24 dai and were more than 20-fold increased compared with the non-transgenic sibling plants (Supplementary Table S5, available at JXB online). No correlation of transgenic Bxl expression and transcript levels of the other Bx genes was detected (Supplementary Table S5). Segregating T1 progeny were analysed for benzoxazinoids at 24 dai. Individuals with the transgenic Bxl allele had DIMBOA concentrations of up to 3.9 mM and could be considered high lines. The average value reached in the transgenics was 1.8 mM, which was significantly higher than the level of 0.7 mM displayed in average by the non-transgenic sibling plants (Fig. 8).
Prolonged Bx1 expression, as detected in Mo17, therefore seems to be an effective mechanism to sustain effective benzoxazinoid concentrations during the later stages of plant development.

Discussion

Transcriptional regulation of Bx1 affects DIMBOA concentration

The starting point of the study was the survey of genetic variability in DIMBOA content in maize beyond the seedling stage (24 dai). In the NAM panel, which represents 80% of the variability in maize, prolonged presence of the benzoxazinoid was displayed by three out of 26 lines; among these, Mo17 was prominent (Fig. 1). B73 belongs to the majority of lines with low DIMBOA level at 24 dai and therefore the IBM302 mapping population offers a solid basis for QTL mapping. The major QTL for DIMBOA content in 24 dai plants mapped to the cluster of Bx genes (QTL4-1; Supplementary Fig. S4). A QTL in this position has been determined in different studies aiming directly (Butrón et al., 2010) or indirectly via the impact of benzoxazinoids on insect resistance (Jampatong et al., 2002; Cardinal et al., 2006; Betsiashvili et al., 2015) to detect loci influencing DIMBOA concentration. Mo17 was also exceptional within the NAM diversity panel with respect to high Bx1 transcript levels in 24 dai plants. The genetic analysis demonstrated that high Bx1 transcript levels were correlated with relatively high DIMBOA concentrations at later developmental stages (24 dai) of the maize plant. The alteration of the Bx gene transcription pattern was limited to Bx1; the pattern for all other Bx genes was similar for Mo17 and B73 (Supplementary Fig. S2). The increase in DIMBOA content in 24 dai plants by transgenic overexpression of Bx1 also indicated that prolonged Bx1 transcription was sufficient to generate elevated DIMBOA levels beyond the seedling stage. Furthermore, the overexpressing plants demonstrated that the correlation between Bx1 mRNA level and DIMBOA concentration in the older plant was not restricted to the Mo17 genetic background. Hence, it can be speculated that BX1, the branch-point enzyme of the biosynthetic pathway, has a bottleneck function in DIMBOA biosynthesis of maturing plants.

This finding is in line with the result of a recent candidate association analysis investigating the correlation of Bx gene sequence polymorphisms on DIMBOA content in older (32 dai) plants (Butrón et al., 2010). A significant association was determined for Bx1 sequence polymorphisms but for none of the other Bx genes.

Late Bx1 transcription depends on DICE

In the candidate gene association study by Butrón et al. (2010), the causal polymorphism connected to Bx1 in the QTL for DIMBOA content could not be detected. The analysis was restricted to the gene sequence and proximal regions. Fine mapping of the IBM302 QTL4-1 in our study included 250 kb of the Bx1 upstream region and revealed the sequence DICE as required for increased Bx1 transcript levels at 24 dai. The DICE element is located 141 kb upstream of Bx1 and the conformation that promotes Bx1 transcription in Mo17 is a tandem duplicate. This is reminiscent of the long-range effect of the upstream approximately 107 kb regulatory elements of the bl1 gene of maize, which is dependent on multimerization of the sequence element. Here, an impact of the multimers on binding of regulatory proteins (Brzeska et al., 2010) and formation of regulatory small RNA (Belele et al., 2013) was hypothesized. At present, it is unknown how the increase in Bx1 transcript level is mediated by the Mo17 haplotype. In addition to DICE, further cis-element(s) that influence Bx1 transcript levels have been delimited to the region between the Bx1 gene and DICE. These sequences are responsible for the allele-specific expression of Bx1. No recombinants that would allow more precise positioning of the additional cis-elements were available from the fine mapping and IBM302 RILs.

Strikingly, allele-specific and increased transcription was restricted to Bx1 and included neither Bx2, which is located only 2.5 kb downstream, nor any other Bx gene in the cluster (Supplementary Fig. S9). In hybrids, both Bx2 alleles were expressed at the same level in seedlings and late plants. Allele specificity could not be analysed for Bx5 and Bx8 since polymorphisms are lacking, but both genes, which flank DICE (Fig. 1), were expressed at comparable low rates in Mo17 and B73 24 dai plants (Supplementary Fig. S2). This was unexpected since Bx5 is in close proximity to DICE (0.5 kb), and Bx8 is located between DICE and Bx1. These results demonstrated that clustered genes are not necessarily co-regulated and rather indicates an individual regulation, in this case of the gene encoding the signature enzyme of the pathway.

A possible contribution of trans-factors located within the QTL4-1 region was investigated by analysis of hybrid NIL lines. The results excluded major trans-factors in this genomic region (Fig. 7). The minor QTLs QTL3, QTL4-2, and QTL5 might coincide with trans-factors. The genotypes of the IBM302 RILs analysed in detail (Fig. 5), however, showed no correlation of the genotype in the minor QTL and 24 dai Bx1 transcript levels.

Fig. 8. DIMBOA content in 24 dai transgenic plants overexpressing Bx1. Statistical analysis was by Kruskal–Wallis test and multiple comparison of treatments. Mean values and standard deviation are indicated. Identical letters above columns indicate no statistical differences (P>0.5).
High DIMBOA content at 24 dai is not strictly dependent on high Bx1 transcript levels

DIMBOA analysis of the NAM panel lines demonstrated that high 24 dai DIMBOA levels could also occur when Bx1 transcription was low. An analogous result was displayed for the NIL M31. M31 is of Mo17 genotype with the exception of the region around QTLa-1, which confers a low Bx1 mRNA level at 24 dai due to the B73 genotype of DICE and downstream sequences; nevertheless, M31 has an extremely high DIMBOA content (Fig. 6). Similar findings of unexpectedly high DIMBOA levels in the presence of the B73 genotype in the Bx gene region were reported in a recent study (Betsiashvili et al., 2015) on correlation between aphid control and DIMBOA content using analogous NILs from the same collection of lines (Eichten et al., 2011). Since benzoxazinoid steady-state levels are the product of formation, storage, and decay, other processes in addition to Bx1 transcription, can cause high DIMBOA levels in 24 dai maize plants.

The Bx gene cluster contains hotspots of recombination

The DIMBOA biosynthetic genes in maize are organized as a cluster. The core harbours six Bx genes and spans 264 kb of genomic DNA (Fig. 1). In maize, this is considered a gene-rich region in relation to the 2.3 Gb genome (Schnable et al., 2009). Recombination frequencies are generally higher in unique, mostly genic regions compared with regions with repetitive sequences (Yao et al., 2002; Dooner and He, 2008). Several hotspots of recombination have been detected by the fine mapping within the 3.9 kb DICE element and in the adjacent genomic regions (Fig. 4 and Supplementary Fig. S6). The recombination rate in the DICE element is significantly higher than expected for genic regions. DICE is a unique sequence that is flanked by the Bx5 gene and a long stretch of low repetitive sequence (MIPS annotation of repetitive sequence motifs, Kurtz et al., 2008; MaizeGDB browser: http://www.maizegdb.org/gbrowse.php). DICE and the adjacent downstream genomic regions are hypomethylated in the third leaf of 18-d-old plants (Eichten et al., 2011; MaizeGDB browser: http://www.maizegdb.org/gbrowse.php), indicating open chromatin conformation, as expected for recombinationally hyperactive chromosomal regions (Goodstadt and Ponting, 2011).

Clustering of genes for secondary or specialized metabolic pathways is common in plants (reviewed by Osbourn, 2010). It was speculated that one of the evolutionary forces for the formation and maintenance of gene clusters is the suppression of recombination by close physical linkage of the genes. Thereby, gene clusters might have a selective advantage since superior allelic combinations are inherited preferentially once established in the coupling phase (Frey et al., 2009; Field and Osbourn, 2012; Takos and Rook, 2012). The finding of recombination hotspots in the Bx gene cluster revealed this hypothesis in a different light. Also, transcriptional co-regulation of the clustered genes, a further hypothesis, does not apply to our findings.

We demonstrated allelic variation of Bxl gene expression and DIMBOA content at the 24 dai stage of leaf development for B73 and Mo17 and detected a recombination hotspot connected with a cis-element involved in allele-specific transcription. A direct correlation between a recombination hotspot and variation of allelic expression has been demonstrated for eight genes in the Sh1–Bz1 chromosomal region of maize (Hawkins et al., 2014) and seems to be a more common feature. The high recombination frequency in the Bx gene cluster is consistent with the hypothesis that diversity in defence gene expression should be beneficial at the population level. It would be surprising if there were only a small number of optimal haplotypes, given the wide variety of environments in which maize (or teosinte) grows. This does not seem to be inconsistent with the hypothesis that it is beneficial to have a genetically linked gene cluster that can be inherited as a biosynthetic unit.

Potential to increase maize protection by elevated DIMBOA concentration at later growth stages

The analyses revealed that high Bxl transcript levels in older plants prolonged the presence of effective DIMBOA concentrations. Several IBM302 RIL lines, e.g. MO038, had significantly higher 24 dai DIMBOA content than Mo17. All of these lines had the Mo17 haplotype for the DICE and the downstream cis-element region. The values for MO038 (Supplementary Fig. S5) demonstrated that the higher DIMBOA content compared with Mo17 was not caused by a further increase in Bx1 transcript level but rather by independent mechanisms. This offers the possibility of using high late Bx1-expressing lines and breeding on this background to further increase the long-lasting protection by DIMBOA.

The feature of high late DIMBOA content was relatively rare among the 26 NAM panel lines. Only three had a significant DIMBOA concentration at 24 dai. The NAM lines reflect a certain amount of manmade selection. Besides representation of large variation, the NAM lines were chosen to include public lines of importance to temperate breeding and important tropical and subtropical lines (Liu et al., 2003), and inbreds were selected that produce seed in the summer in the USA (Yu et al., 2008). This could have reduced the time interval for DIMBOA biosynthesis for the benefit of increased yield or other agronomic traits. It is assumed that plant defence, especially constitutive mechanisms like the preformed toxic benzoxazinoids of the grasses and glucosinolates of the Brassicaceae, are costly by detracting resources from growth and reproduction (Herms and Mattson, 1992). Analysis in Arabidopsis thaliana using mutants revealed that glucosinolate biosynthesis indeed evokes growth costs (Züst et al., 2011). The cost in biosynthesis might render high late DIMBOA levels beneficial only in certain environments.

Supplementary data

Supplementary data are available at JXB online.
Supplementary Fig. S1. DIMBOA concentration of seedlings of the NAM panel lines.
Supplementary Fig. S2. Bx gene transcript levels of B73 and Mo17.
Supplementary Fig. S3. DIMBOA concentration in the IBM302 RILs.
Supplementary Fig. S4. QTL analysis for DIMBOA.
Supplementary Fig. S5. Characterization of the RIL MOO38.
Supplementary Fig. S6. Genotype of recombinants.
SupplementaryFig. S7. Characterization of the distal cis-element (DICE).
Supplementary Fig. S8. Analysis of Bxl transcript levels in 24 dai heterozygous plants.
Supplementary Fig. S9. Analysis of Bxl and Bx2 allele-specific expression.
Supplementary Fig. S10. QTL analysis in IBM302 subset.
Supplementary Fig. S11. Bxl expression analysis and genotype of selected IBM lines.
Supplementary Table S1. Primer and PCR conditions.
Supplementary Table S2. Composite interval mapping.
Supplementary Table S3. MOO38 genotype.
Supplementary Table S4. Marker for fine mapping.
Supplementary Table S5. Bx gene transcript levels in transgensics at 24 dai.
Supplementary material. List of markers.

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