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A 2-oxoglutarate-dependent dioxygenase is integrated in DIMBOA-biosynthesis☆

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Benzoxazinoids are secondary metabolites of grasses that function as natural pesticides. While many steps of DIMBOA biosynthesis have been elucidated, the mechanism of the introduction of OCH_3-group at the C-7 position was unknown. Inhibitor experiments in *Triticum aestivum* and *Zea mays* suggest that a 2-oxoglutarate-dependent dioxygenase catalyses the hydroxylation reaction at C-7. Cloning and reverse genetics analysis have identified the *Bx*6 gene that encodes this enzyme. *Bx*6 is located in the *Bx*-gene cluster of maize.

Keywords: *Zea mays*; *Triticum aestivum*; DIMBOA biosynthesis; 2-Oxoglutarate dependent oxygenase; Gene cluster

1. Introduction

The benzoxazinoids DIBOA [2,4-di-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and its methoxy derivative DIMBOA are found predominantly in Gramineae. Benzoxazinoids are natural pesticides and serve as important factors of host plant resistance against microbial diseases and insects and as allelochemicals (reviewed in Niemeyer, 1988; Sicker et al., 2000). In maize, a series of five genes is sufficient to encode the enzymes to synthesize DIBOA (Frey et al., 1997). The first gene in this pathway, *Bx1*, encodes an enzyme function that catalyses the formation of indole (Frey et al., 1997). All five oxygen atoms of DIMBOA are incorporated from molecular oxygen (Glawischnig et al., 1997). In plants, mainly two classes of enzymes, cytochrome P450-dependent monooxygenases (P450 enzymes) (for review Chappel, 1998) and 2-oxoglutarate-dependent dioxygenases (De Carolis and De Luca, 1994), are involved in oxidation reactions using molecular oxygen. Both types of enzymes are found in the biosynthetic pathways of a large variety of primary and secondary metabolites. The introduction of four oxygen atoms into the indole moiety that yields DIBOA is catalyzed by four P450 enzymes termed BX2–BX5. In certain grasses like rye, DIBOA is glucosylated and stored in the vacuole. In other species like maize and wheat, conversion of DIBOA to its 7-methoxy derivative DIMBOA via hydroxylation and consecutive methylation, precedes vacuolar storage of the glucoside (Fig. 1, Gierl and Frey, 2001). A comprehensive survey of maize P450 enzymes excluded the possibility that a further P450 enzyme is responsible for the hydroxylation at position C-7 of DIBOA. Here we present evidence that this reaction is catalyzed by the 2-oxoglutarate-dependent dioxygenase *Bx6*. In maize *Bx6* is located in the cluster of *Bx*-genes on the short arm of chromosome 4.

2. Results

2.1. A 2-oxoglutarate-dependent dioxygenase is involved in conversion of DIBOA to TRIBOA in maize

Prohexadion-Ca (calcium 3-oxido-4-propionyl-5-oxo-3-cyclohexen-carbonic acid) is a specific inhibitor of the

Abbreviations: DIBOA, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DIMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; EST, Expressed sequence tag; TRIBOA, 2,4,7-trihydroxy -2*H*-1,4-benzoxazin-3(4*H*)-one.

☆ Accession number: AF540907.

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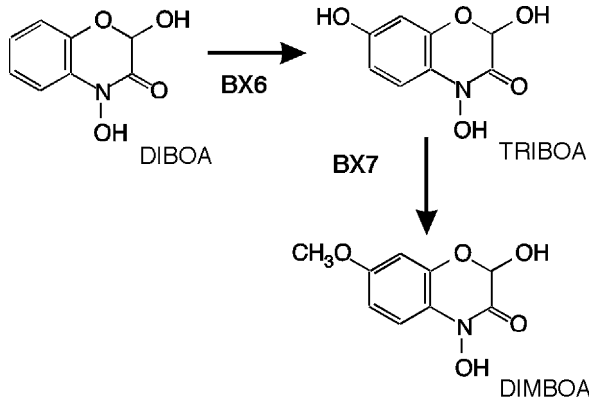

Fig. 1. Conversion of DIBOA to DIMBOA. The 2-oxoglutarate-dependant dioxygenase BX6 confers hydroxylation of DIBOA at position 7. The resultant TRIBOA is finally methylated to generate DIMBOA by the *O*-methyltransferase BX7. This enzyme has not yet been identified.

catalysis of 2-oxoglutarate-dependent dioxygenases. The inhibition is due to the structural similarity of prohexadion-Ca to the co-substrate 2-oxoglutarate and has been first recognized by its effect on 2-oxoglutarate-dependent dioxygenases involved in the biosynthesis of gibberellins. (Rademacher et al., 1992). To investigate the participation of this type of oxygenase in the biosynthesis of DIMBOA in maize and wheat, seedlings were grown in the presence of prohexadion-Ca. Benzoxazinoids were isolated from control plants and plants grown in a solution of the inhibitor and analyzed by chromatography (Fig. 2). In control plants DIMBOA is the major benzoxazinoid. Only traces of DIBOA can be recognized. The inhibition of 2-oxoglutarate-dependent dioxygenases by prohexadion-Ca leads to an almost complete loss of DIMBOA in the seedlings and the main benzoxazinoid is the precursor in the biosynthetic pathway, DIBOA. Hence, in both grasses, the hydroxylation of DIBOA at position 7 is most probably catalyzed by an 2-oxoglutarate-dependent dioxygenase.

2.2. A candidate gene for Bx6 is identified by its expression pattern and its identity is verified by a reverse genetics approach

Plant 2-oxoglutarate-dependent dioxygenases involved in biosynthesis of ethylene, gibberellin, and secondary metabolites have been cloned recently (e.g. Sato and Theologis, 1989; Lange et al., 1994; De Carolis and De Luca, 1994; VasquesFlota et al., 1997; Saito et al., 1999). BLAST analysis of these sequences revealed diagnostic amino acid sequence motifs (see Experimental section) that can be easily detected in EST libraries. These motifs were used to screen the maize EST-data collection of Pioneer Hi-Bred. Since all cloned genes of the DIMBOA biosynthetic pathway are predominantly expressed in the seedling stage, ESTs with

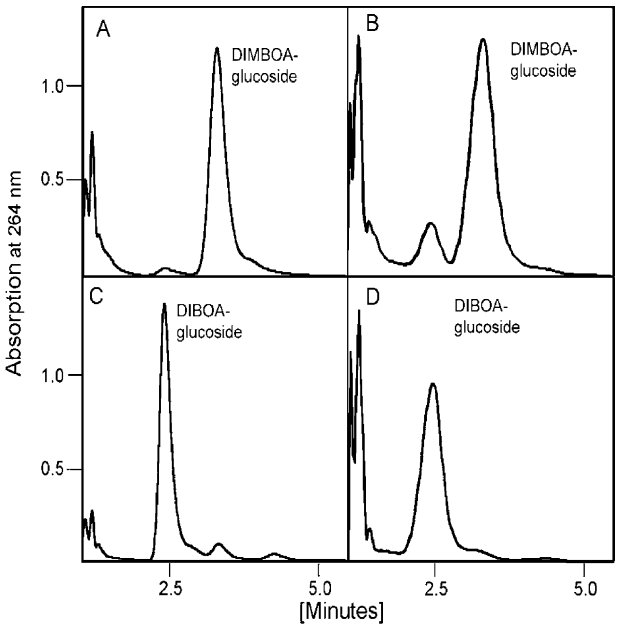

Fig. 2. A 2-oxoglutarate-dependant dioxygenase is necessary for DIMBOA biosynthesis in maize and wheat. **A, B** Analysis of benzoxazinones isolated from 4 days old etiolated maize seedlings (A) or wheat seedlings (B) grown in water. The main substance is DIMBOA. **C, D** Maize seedlings (C) or wheat seedlings (D) were grown 4 days etiolated in the presence of the inhibitor prohexadion-Ca. The main benzoxazinone is DIBOA.

the specific signature that were derived from seedling tissue libraries were taken as candidate sequences (10 sequences). Genes that obviously represent homologs of 2-oxoglutarate-dependent dioxygenases of known function or that were induced after application of biotic or abiotic stress to the seedlings, were not analyzed further (6 sequences). For the other genes full-size cDNA clones were retrieved and assayed by northern analysis. The P450-gene *Bx2* was employed as a representative gene of the DIMBOA biosynthetic pathway (Fig. 3). One candidate gene displayed an almost identical expression pattern as *Bx2*. The candidate cDNA encoded an enzyme of 375 amino acids, no intron was present in the genomic sequence. The sequence has been deposited at GenBank (Accession number AF540907).

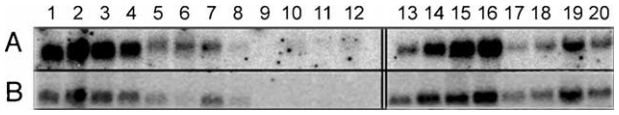

Fig. 3. Northern analysis. **A** Transcript levels displayed by hybridization with the radiolabeled dioxygenase *Bx6* cDNA. **B** the same northern blot was re-probed with the *Bx2* cDNA. 1, 2: 2 days old etiolated seedlings; 3, 4: 3 days old etiolated seedlings; 5, 6 roots of 5 days old etiolated seedling; 7, 8: roots of five days old light-grown seedlings; 9, 10: roots of 10 days old etiolated seedlings; 11, 12: roots of 10 days old light-grown seedlings; 13, 14: shoots of 5 days old etiolated seedlings; 15, 16: shoots of 5 days old light-grown shoots; 17, 18: shoots of 10 days old etiolated seedlings; 19, 20: shoots of 10 days old light-grown seedlings. Odd numbers: maize line A 619, even numbers: *bx1, bx1* mutant line.

Fig. 4. Distribution of the integration sites of the transposable element *Mutator* in the dioxygenase gene *Bx6*. The ATG start-codon and the TAG stop-codons are given.

A reverse genetic approach was taken in order to verify the function of this 2-oxoglutarate-dependent dioxygenase named *Bx6*. Four independent integrations of the transposable element Mutator (Mu) that are scattered across the cDNA sequence (Fig. 4) were uncovered in the TUSC population (Benson et al., 1995). Since the dioxygenase gene has no introns all Mu integrations locate in the coding region. Hence the transposon integrations were expected to have a major influence on the enzymatic function of the encoded enzyme. Four maize lines representing the different Mu-integration events were analyzed for the presence of benzoxazinoids. Mutant individuals that did no longer synthesize DIMBOA but include DIBOA as the main benzoxazinoid were identified in the F2 progeny of three lines (Table 1, integration sites 1,2,4). The analysis of the genetic constitution of the individual plants was made by genomic Southern analysis (Mu insertion 3,4) and by quantitative PCR (Mu-insertion 1,2). A total of 42 plants was analyzed and in all cases wildtype phenotype (DIMBOA production) was connected with the presence of at least one wildtype allele of the dioxygenase gene and the mutant phenotype was displayed only by plants that were homozygous for the integration of a Mu-element into *Bx6*. This perfect co-segregation provides compelling evidence that the gene responsible for the hydroxylation of DIBOA, *Bx6*, in maize has been identified.

Bx6 displayed no strong sequence similarity to other 2-oxoglutarate-dependent dioxygenases. An *Oryza sativa* gene of unknown function (Accession GI:19927942, Yu et al., 2002) exhibiting 52% identity and 63% similarity on amino acid level, was the closest relative in BLAST analysis (Altschul et al., 1997). Desacetoxyvindoline-4-hydroxylase (Vasques Flota et al., 1997) shared 40% identity and 58% similarity with BX6 on amino acid level and is the closest related 2-oxoglutarate-dependent dioxygenase with defined enzyme function.

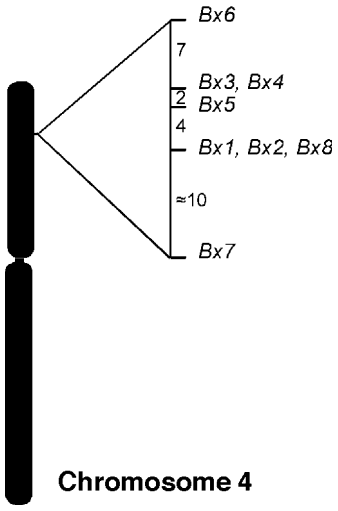

Fig. 5. Genomic location of *Bx6*. Seven genes of the DIMBOA biosynthetic pathway map within 13 centiMorgan on the short arm of chromosome 4. *Bx6* is a gene without intron.

2.3. *Bx6* maps to the short arm of chromosome 4 and is closely linked to the cluster of *Bx* genes

A distinct feature of the *Bx*-genes of maize is the clustering on the short arm of chromosome 4 (Gierl and Frey, 2001). Therefore, the location of the *Bx6* gene in the maize genome is an interesting feature. Mapping was done by Southern analysis using the *Bx6*-cDNA as a probe on the recombinant inbred population CM37xT232 (Burr and Burr, 1991). The data position *Bx6* in close proximity of the cluster of *Bx* genes on chromosome 4 (Fig. 5). *Bx6* and *Bx3* and *Bx4* are separated by 7 centiMorgan. These results are in good accordance with previous mapping data for the hydroxylation of the C-7 position in DIMBOA-biosynthesis (K. Lobos and P. Sisco, personal communication).

3. Discussion

3.1. Two functionally different classes of oxygenases are involved in the biosynthesis of DIMBOA

P450 enzymes and 2-oxoglutarate-dependent dioxygenases catalyze among other reactions oxidation

Table 1
Cosegregation of mutant phenotype with homozygous Mu-insertion in *Bx6*

	Mu-Integration			
	Position 1	Position 2	Position 3	Position 4
Number of F2 plants investigated	9	10	12	10
Number of phenotypic mutants	1	5	0	2
Number of Homozygous *Bx6*::*Mu* plants	1	5	0	2

reactions that lead to the incorporation of oxygen atoms from molecular oxygen (Halkier, 1996; Que and Ho, 1996). These enzymes represent fairly large gene families. In *Arabidopsis thaliana,* 286 P450 genes (The Arabidopsis Genome Initiative, 2001) and 54 2-oxoglutarate-dependent dioxygenases (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html) have been annotated. It has been demonstrated recently that apparent gene duplication and diversification of 2-oxoglutarate-dependent dioxygenases genes have a significant impact on diversity of the secondary metabolism in plants (Kliebenstein et al., 2001). Both classes of enzymes are involved in the biosynthesis of plant hormones and various secondary metabolites (Sato and Theologis, 1989; Lange et al., 1994; Heller and Forkmann, 1993; De Carolis and De Luca, 1994). Enzymes of one or the other class can virtually catalyze the same reaction. It has been demonstrated in pumpkin seed that gibberellin 7-oxidase activity is executed by a P450 enzyme and by a 2-oxoglutarate-dependent dioxygenase (Lange et al., 1994). The branch point reaction in the flavonoid pathway towards flavones or flavonols, 2,3-desaturation of flavanones, is catalyzed alternatively by P450 enzymes or 2-oxoglutarate-dependent dioxygenases in different plant species (Lukacin et al., 2001). Although plants and the fungus *Gibberella fujikuroi* produce structurally identical gibberellins, it appears that P450 enzymes and 2-oxoglutarate dependent dioxygenases have replaced each other with respect to the oxidation at identical C-positions (Hedden et al., 2001).

The biosynthesis of DIMBOA in maize and wheat represents another example of the integration of these two classes of enzymes in one biosynthetic pathway. We have previously shown that the P450 enzymes BX2-5 catalyze the conversion of indole to DIBOA by four consecutive hydroxylation reactions (Frey et al., 1997; Spiteller et al., 2001). A set of P450 genes has been isolated from wheat and shown to encode the orthologous functions (Nomura et al., 2002). The hydroxylation of C-7 of the benzoxazinone to yield TRIBOA is catalyzed in both cereals by a 2-oxoglutarate dependent dioxygenase that we have termed *Bx6*. The substrate of this enzyme is not yet known and might be DIBOA or any of the precursors in the pathway starting with indole. The repeated incorporation of oxygen atoms into the indole backbone enhances the hydrophilicity of the resulting intermediates. A possible scenario would be therefore that indole is converted to DIBOA by the P450 enzymes BX2-BX5 located in the endoplasmatic reticulum (ER). DIBOA is released to the cytosol from the relatively hydrophobic ER environment and then converted to TRIBOA by hydroxylation of C-7 by BX6. However, a flavonol-6-hydroxylase, recently characterized as a novel flavonol 2-oxoglutarate dependent dioxygenase from *Chrysosplenium americanum* (Anzellotti and Ibrahim, 2000), exhibits a preference for hydrophobic substrates. Enzymatic assays with BX6 will be required to reveal the sequence of hydroxylation reaction in DIMBOA biosynthesis.

3.2. Evolution of benzoxazinoid biosynthesis

Benzoxazinoids are widely distributed in grasses and are also found in several dicotyledoneous species like Acanthaceae, Ranunculaceae and Scrophulariaceae (Sicker et al., 2000), suggesting that the acquisition of this pathway occurred relatively early in the evolution of Gramineae and probably even before monocots and dicots diverged (Gierl and Frey, 2001). The P450 enzymes that convert indole to DIMBOA are functionally conserved within cereal species. Maize and rye microsomes catalyze identical reactions (Glawischnig et al., 1999). Orthologous genes for *Bx2–Bx5* have been described in wheat (Nomura et al., 2002). The C-7 position is hydroxylated by a 2-oxoglutarate-dependent dioxygenase in maize and wheat, each representing two relatively unrelated phylogenetic lineages within the cereals (Devos and Gale, 1997).

A striking finding was that the DIMBOA biosynthetic genes are clustered on the short arm of chromosome 4 in maize (Fig. 5). *Bx1*, an indole-glycerolphosphate lyase, and the P450 genes *Bx2–Bx5* are separated by 6 centiMorgan (Frey et al., 1997). *Bx8*, the DIMBOA-specific glucosyltransferase maps at the *Bx1* position (von Rad et al., 2001). Here we show that *Bx6*, encoding the C-7 specific oxygenase is also part of the gene cluster. The gene encoding the methyltransferase converting TRIBOA to DIMBOA (tentatively designated as *Bx7*) was genetically mapped to the same cluster (K. Lobos and P. Sisco, personal communication). It is unclear how gene clustering is related to the evolution of benzoxazinoid biosynthesis and whether for example, gene clustering has any influence on the expression of the *Bx* genes. However, the fact that three *Bx* genes from wheat are clustered in a syntenic position (Devos and Gale, 1997) on chromosome 5 A (Nomura et al., 2002) in each of the three wheat genomes would suggest that the *Bx*-gene cluster represents the ancestral chromosomal organization of the DIMBOA-biosynthetic pathway. DIMBOA is the predominant benzoxazinoid in wheat and maize, while its precursor DIBOA is dominating in rye and some wild barley species (Sicker et al., 2000). If the maize gene cluster is considered to be an original feature it can be argued that DIMBOA is the original secondary metabolite. In rye and barley benzoxazinoid biosynthesis was possibly reduced to DIBOA as a final metabolite by gene loss (*Bx6* and *Bx7*). Further characterization of the benzoxazinoid biosynthesis genes in other monocot and dicot species is needed for a better understanding of the evolution of this secondary metabolic pathway.

4. Experimental

4.1. Plant materials and application of prohexadion-Ca

The maize line LG22 and the wheat line Trakos were used for the analysis of benzoxazinoid patterns. The seed were incubated for about 4 h in water and then grown in wet germination paper in the dark. Prohexadion-Ca (BASF, Germany) at a concentration of 0.1% (weight/volume) in water was used instead of water in the inhibition experiment. Incubation was for 4 days at 28 °C (maize) and 20 °C (wheat).

The maize plants used in the reverse genetics approach (Trait Utility System for Corn, TUSC) were as described by Benson et al. (1995).

4.2. Isolation and analysis of benzoxazionoids

The standard material for the isolation of benzoxazinoids is etiolated seedling tissue that contains low levels of phenolic compounds but high concentration of benzoxazinoids (DIMBOA concentration is up to 0.1% of the fresh weight). The extraction of benzoxazinoids from etiolated seedlings was essentially as described by Hartenstein et al. (1992). Maize and wheat seedlings were frozen in liquid nitrogen, immediately ground to a fine powder and extracted with methanol, filtered using filter paper and vacuum dried. The dry pellet was extracted four times with acetone/methanol (1:2). The acetone/methanol extracts were pooled and vacuum-dried. The dry pellet was dissolved in methanol and applied to HPLC using a LiChroCART RP 18 column (125×4, Merck, Darmstadt). An isocratic gradient of 85% acetic acid (10%)/15% methanol was applied for 7 min at a flow rate of 1 ml per min. Analytes were detected at 264 nm and characterized by the retention time and the absorption spectrum in comparison to the respective benzoxazinoids.

4.3. Materials and molecular biology methods

All chemicals used were of pro analysis or HPLC grade. All molecular biology methods were as described by von Rad et al. (2001).

BLAST sequence analysis was performed as described by Altschul et al. (1997).

The amino acid sequence motifs used for the identification of putative 2-oxoglutarate-dependent dioxygenases in EST-data collections were:
LQVMT NGRFKSVKHRVLTNS
LQLITNDKFKSVEHRVLANK.

For the analysis of the Mu-tagged plants PCR was performed using the following primers:

Mu2: 5′AGAGAAGCCAACGCCAWGGCCTCYA TTTCG3′

F1: 5′CCTGAGCGACATAATTTCTCC3′
F2: 5′GAGGCGAAGCAGGCGCTCTACTCCA3′
F3: 5′TCTTCACCATCCTCCTGCAGGA3′
F4: 5′CAACAAGAGCAGCGACACGGCGA3′
R2: 5′TCCTGCAGGAGGATGGTGAAGA3′
R3: 5′AAACGTAGCCAGGTAGCTAGAGCCT3′

The presence of a wildtype allele of *Bx 6* was assayed by PCR with the primer pairs F1/R2 (position 1), F2/R2 (position 2), F3/R3 (position 3), F4/R3 (position 4). The integration of Mu into the gene was revealed by PCR with the primer pairs Mu2/F1 (position 1), Mu2/F2 (position 2), Mu2/F3 (position 3), Mu2/F4 (position 4). For single F2 plants with Mu-insertions the generated PCR fragments including the Mu-termini were sequenced. For these plants a further PCR was performed employing the primer pairs Mu2/R2 (position 1), Mu2/R2 (position 2), Mu2/R3 (position3) and Mu2/R3 (position 4). The resulting fragments that included the Mu-termini were sequenced. For every integration site a 9 base pair target site duplication that is characteristic for the integration of Mu was revealed by comparison of these two Mu-flanking sequences with the sequence of the wildtype *Bx6* sequence.

4.4. Gene mapping

Data were generated by Southern analysis using the *Bx6* cDNA as a probe. A unique restriction fragment length polymorphism (RFLP) was revealed for the CM37xT232 recombinant inbred tagging population (Burr and Burr, 1991).

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