

the biological effects of the JNK signaling pathway. We investigated the effect of JIP-1 on the promotion of apoptosis by JNK after the withdrawal of nerve growth factor (NGF) from PC12 cells (6). Expression of the JBD of JIP-1 (residues 127 to 281) reduced the NGF withdrawal-induced apoptosis by more than 90% (13). These data demonstrate that JIP-1 suppresses the biological actions of the JNK signal transduction pathway.

We also examined the effects of JIP-1 on oncogenic transformation. The *Bcr-Abl* leukemia oncogene causes activation of JNK, but not ERK (5). Thus, transformation by *Bcr-Abl* may be mediated, in part, by the JNK signaling pathway. Expression of *Bcr-Abl* or the viral oncogene *v-Abl* caused constitutive activation of JNK (approximately fivefold), which was blocked by co-expression of JBD (Fig. 4A). We prepared bi-cistronic retroviruses expressing *Bcr-Abl* alone or together with JBD in the sense and antisense orientations (Fig. 4B). Immunoblot analysis of *Bcr-Abl* and JBD confirmed that the retroviral constructs expressed the appropriate proteins. The recombinant retroviruses were used to infect primary marrow cells, and the transformation of precursor B cells (pre-B cells) was monitored in culture. *Bcr-Abl* caused transformation of pre-B cells (Fig. 4C). JBD inhibited transformation when it was expressed in the sense but not in the anti-sense orientation. These data implicate the JNK pathway in pre-B cell transformation by *Bcr-Abl*. Because *Bcr-Abl* has a role in human disease, both JNK and JIP-1 are candidate targets for the design of therapeutic strategies for the treatment of chronic myeloid leukemia.

The results of this study demonstrate that one role of JIP-1 may be to suppress signal transduction by the JNK pathway. For example, JIP-1 may compete with substrates that bind JNK. Alternatively, JIP-1 may have a more direct role in targeting JNK to specific regions of the cell or to specific substrates. Indeed, overexpression of JIP-1 caused retention of JNK in the cytoplasm. Therefore, in addition to other possible physiological functions, JIP-1 may act as a cytoplasmic anchor for JNK.

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19. Luciferase reporter assays in CHO cells were performed 48 hours after transfection with the use of β-galactosidase (β-Gal) to measure transfection efficiency (14). The cells were activated by treatment with fetal calf serum (10%). The data are presented as the relative luciferase activity [mean ± SEM of the activity ratio of luciferase/β-Gal (*n* = 3)].
20. We performed bone marrow transformation assays using recombinant retroviruses [C. L. Sawyers, J. McLaughlin, O. N. Witte, *J. Exp. Med.* **181**, 307 (1995)] that were packaged with 293T cells. We constructed the bi-cistronic retroviruses expressing the JBD and *Bcr-Abl* by subcloning p185*Bcr-Abl* in the Cla I site and the JBD in the Eco RI site of pSRαTK.
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Analysis of a Chemical Plant Defense Mechanism in Grasses

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In the Gramineae, the cyclic hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) form part of the defense against insects and microbial pathogens. Five genes, *Bx1* through *Bx5*, are required for DIBOA biosynthesis in maize. The functions of these five genes, clustered on chromosome 4, were demonstrated in vitro. *Bx1* encodes a tryptophan synthase α homolog that catalyzes the formation of indole for the production of secondary metabolites rather than tryptophan, thereby defining the branch point from primary to secondary metabolism. *Bx2* through *Bx5* encode cytochrome P450-dependent monooxygenases that catalyze four consecutive hydroxylations and one ring expansion to form the highly oxidized DIBOA.

A substantial number of secondary metabolites in plants are dedicated to pathogen defense. These include the cyclic hydroxamic acids, which are found almost exclusively in Gramineae. For example, DIMBOA and its precursor DIBOA are present in maize. DIMBOA confers resistance to first-brood European corn borer (*Ostrinia*

nubilalis), northern corn leaf blight (*Helminthosporium turcicum*), maize plant louse (*Rhopalosiphum maydis*), and stalk rot (*Diplodia maydis*), as well as to the herbicide atrazine (1). DIBOA is the main hydroxamic acid in rye, whereas DIMBOA is the predominant form in wheat and maize (1). The DIMBOA and tryptophan biosynthetic

pathways share certain intermediates. Labeled tryptophan precursors such as anthranilic acid and indole are incorporated into DIMBOA, although labeled tryptophan is not incorporated (2). The maize mutation *bx1* (*benzoxazineless*) abolishes DIMBOA synthesis (3). Plants homozygous for *bx1* grow normally but are extremely susceptible to the pathogens mentioned above.

To clone the *Bx1* gene, we used the *Mutator* (*Mu*) transposon tagging system (4). Approximately 150,000 seeds were produced from a cross of a *Mu* female line with the pollen from plants homozygous for the recessive *bx1* mutant allele (3). Seventeen putative mutants were identified and outcrossed to an inbred *Bx1/Bx1* line. The segregation of the *bx1* alleles in the progeny of the crosses was followed by a cleaved amplified polymorphic sequence (CAPS) marker (5) derived from the linked *Bx4* gene (6). One of the putative mutants showed the expected 1:1 segregation for the *bx1* allele and the newly *Mu*-induced recessive *bx1* allele with respect to the linked marker. From this material, the *Mu* element cosegregating with the new *bx1::Mu* allele was identified and a flanking genomic DNA fragment was isolated by a polymerase chain reaction (PCR)-based method (7). This fragment was used to isolate the wild-type *Bx1* and recessive *bx1* (3) alleles from genomic λ libraries (8) as well as a full-length cDNA clone (Fig. 1). DNA sequence analysis revealed that the *bx1* allele harbors a deletion of 924 base pairs (bp) comprising 355 bp of the 5' nontranscribed region, the first exon, the following intron, and 53 bp of the second exon. The position of the *Mu* element in the *bx1::Mu* allele isolated in the transposon-tagging experiment was determined by PCR amplification of the flanking genomic sequences.

DNA sequence analysis revealed the exact *Mu* insertion site and the characteristic 9-bp host sequence duplication associated with integration of the transposon (Fig. 1). The exon sequences of *Bx1* were found to be identical to a gene previously described (9) that is homologous to tryptophan synthase α (TSA) and, when expressed in *Escherichia coli*, complemented a tryptophan synthase α mutation. TSA catalyzes the

conversion of indole-3-glycerol phosphate to indole, the penultimate reaction in tryptophan biosynthesis. However, indole was also implicated as an intermediate in DIMBOA biosynthesis (2). Consequently, the *bx1* mutant should also be defective in the production of free indole.

The immersion of shoots of *bx1* seedlings (4 days after imbibition) into a 1 mM solu-

tion of indole in the dark for 1 day restored the formation of DIMBOA (Fig. 2A). Hence, the biosynthetic block in the *bx1* mutant cannot be downstream of indole formation. Addition of tryptophan did not result in DIMBOA accumulation (Fig. 2B). When [^{13}C]indole was administered to maize shoots, [^{13}C]DIMBOA (75% incorporation) was recovered (Table 1). This

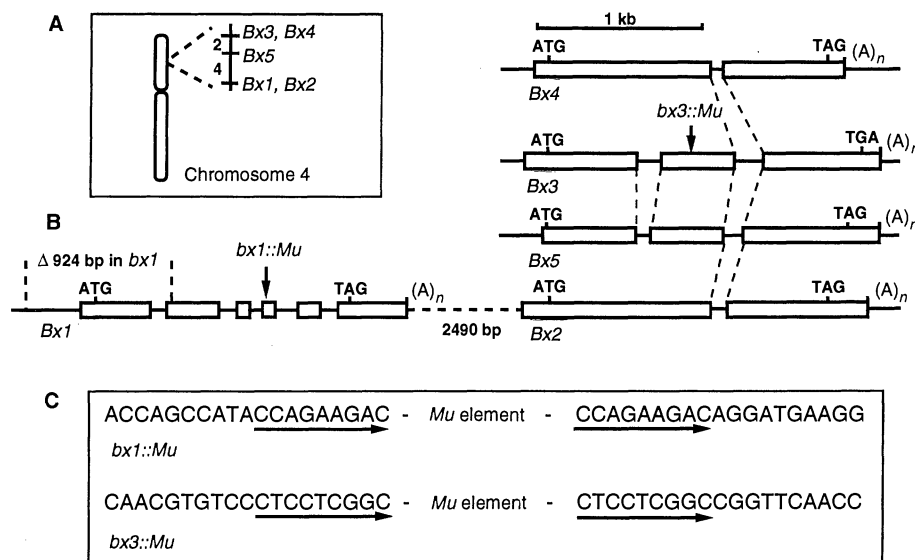


Fig. 1. Structure and chromosomal location of the *Bx* genes. (A) Schematic representation of the *Bx* gene cluster on chromosome 4. Genetic distances are indicated (in centimorgans). (B) Exon-intron structure of *Bx1* through *Bx5*. Exons are represented by boxes. Translation start and stop codons and polyadenylate addition sites are shown. Arrows represent insertion of a *Mu* element in the *bx1::Mu* and *bx3::Mu* alleles. The deletion in the *bx1* standard allele is indicated; it comprises nucleotides 1366 to 2289 of the published sequence (9). The distance from *Bx1* to *Bx2* (2490 bp) is not drawn to scale. The complete sequences of the genes have been deposited in the European Molecular Biology Laboratory data bank [accession numbers X76713 (*Bx1*), Y11368 (*Bx2*), Y11404 (*Bx3*), X81828 (*Bx4*), and Y11403 (*Bx5*)]. (C) Insertion sites of *Mu* in *bx1::Mu* and *bx3::Mu*. The characteristic 9-bp host sequence duplication associated with *Mu* insertion is underlined with an arrow. The insertion occurred at position 2826 of the genomic DNA sequences in *bx1::Mu* and at position 1260 in *bx3::Mu*.

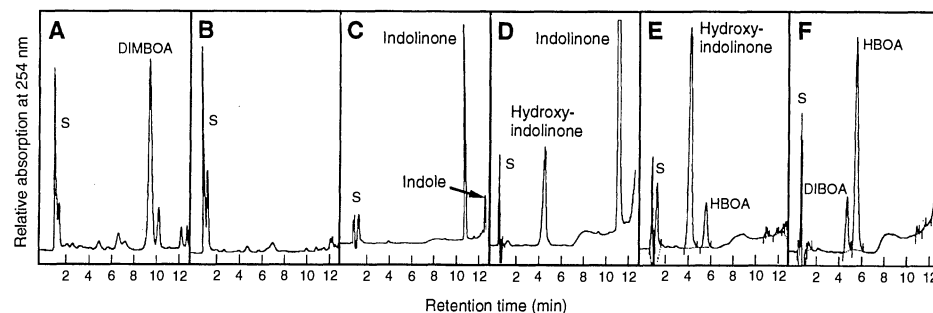


Fig. 2. Detection of metabolites of the DIMBOA pathway by HPLC. Metabolites are indicated at the position of chromatographic peaks. S represents the solvent peak. (A and B) Feeding of *bx1* standard mutant seedling shoots with 1 mM indole (A) or 1 mM tryptophan (B). Seedling material (1 g) was extracted (27) and analyzed on a Merck LiChroCART RP-18 HPLC column (4 $\times$ 125 mm). Elution was for 5 min under isocratic conditions with solvent A (H_2O /acetic acid, 9:1) followed by a linear gradient from 100% solvent A to 100% solvent B (methanol/ H_2O /acetic acid, 70:27:3) over 7 min. (C to F) Analysis of maize P-450 enzymes expressed in yeast microsomes. Reaction mixtures of 0.2 ml contained 50 mM potassium phosphate buffer (pH 7.5), 0.8 mM NADPH, 0.1 to 0.5 mM of the respective substrates, and 1 mg of microsomal protein. Incubation was for 30 min at 25°C. HPLC analysis was as described above. Shown are results for (C) BX2 microsomes incubated with indole, (D) BX3 microsomes incubated with indolin-2-one, (E) BX4 microsomes incubated with 3-hydroxyindolin-2-one, and (F) BX5 microsomes incubated with HBOA.

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confirms that indole is an intermediate in DIMBOA biosynthesis (2).

In bacteria, TSA activity is almost completely dependent on formation of an active $\alpha_2\beta_2$ complex (10) with tryptophan synthase β (TSB), and indole is usually not released during tryptophan synthesis. An analogous heterosubunit complex exists in *Arabidopsis* (11). If the TSA homolog BX1 catalyzes the formation of free indole from indole-3-glycerol phosphate, BX1 should function independently of TSB. To test this assumption, we expressed BX1 in *E. coli*, then purified and assayed it for steady-state kinetic constants (12). We determined a Michaelis constant K_m indole-3-glycerol phosphate of 0.013 mM and a catalytic rate constant k_{cat} of 2.8 s^{-1} . Comparison of these values with the constants for conversion of indole-3-glycerol phosphate to indole by *E. coli* $\alpha_2\beta_2$ complex (K_m indole-3-glycerol phosphate = 0.027 mM, $k_{cat} = 0.2 \text{ s}^{-1}$) (13) demonstrates that BX1, independent of TSB, is ~ 30 times as efficient as the bacterial complex in catalyzing the production of indole. Tryptophan is essential for the maize plant (14). Because the *bx1* mutants are viable, *Bx1* cannot be the only maize gene encoding TSA activity. We suggest that there are at least two TSA genes in maize: one that is involved in tryptophan biosynthesis, forming the $\alpha_2\beta_2$ complex, and a second gene, *Bx1*, that is required for the production of free indole and secondary DIMBOA synthesis.

Four maize cytochrome P-450-dependent monooxygenase genes, one of which was isolated by subtractive cDNA cloning from high versus low DIMBOA-accumulating lines (6), are in the CYP71C subfamily of plant cytochrome P-450 genes. These genes are strongly expressed in young maize seedlings, share an overall amino acid identity of 45 to 60%, and are clustered on the short arm of chromosome 4 (Fig. 1). The finding that all oxygen atoms of DIMBOA

are incorporated from molecular oxygen (15) led to the speculation that these cytochrome P-450 enzymes might be involved in this pathway. The genes encoding these enzymes are here designated *Bx2*, *Bx3*, *Bx4*, and *Bx5* (6).

Direct evidence for the involvement of *Bx3* in DIMBOA biosynthesis is provided by a mutant allele (*Bx3::Mu*) isolated by a reverse genetic approach to screen for *Mu* insertions in the P-450 genes (16). Sequencing of the PCR-amplified *Mu*-flanking genomic DNA fragments showed that *Bx3::Mu* has a *Mu* transposon inserted in the second exon of the gene (Fig. 1). In maize seedlings homozygous for the recessive mutant allele, no DIMBOA could be detected by high-performance liquid chromatography (HPLC) analysis. In contrast, DIMBOA was detected in seedlings that were either heterozygous or homozygous wild-type. Cosegregation of the recessive mutant phenotype was established by genomic blotting analysis of 27 F_2 individuals (four homozygous recessives). These results demonstrate that an intact *Bx3* gene is required for DIMBOA biosynthesis.

Genomic clones of *Bx2* through *Bx5* were isolated from a λ library and sequenced (8), and the exon-intron structure of the genes was determined (Fig. 1). The position of one intron is identical for *Bx2* through *Bx5*, indicating a common evolutionary origin. The position of a second intron is conserved in *Bx3* and *Bx5*. DNA sequence comparison showed that *Bx1* and *Bx2* are separated by only 2490 bp.

To investigate the function of the four P-450 enzymes in DIMBOA biosynthesis, we used a yeast expression system (17). The cDNAs of *Bx2* through *Bx5* were inserted into the pYeDP60 expression vector (18). These constructs were used to transform the WAT11 yeast strain. In WAT11, a galactose-inducible *Arabidopsis thaliana* microsomal NADPH (reduced form of nicotin-

amide adenine dinucleotide phosphate)-P-450 reductase (ATR1) replaces the yeast reductase (19).

Microsomes were isolated from the transgenic yeast strains and tested for enzymatic activity (17, 19). Indole was converted to DIBOA by the stepwise action of the four cytochrome P450 enzymes (Fig. 2, C to F). When [$3\text{-}^{13}\text{C}$]indole was incubated with yeast microsomes containing BX2 protein, [$3\text{-}^{13}\text{C}$]indolin-2-one was produced in the reaction assay. A sufficient amount of [$3\text{-}^{13}\text{C}$]indolin-2-one was produced by this enzyme-catalyzed reaction to test for subsequent enzymatic conversions. Incubation of [$3\text{-}^{13}\text{C}$]indolin-2-one with microsomes containing BX3 resulted in the production of [$3\text{-}^{13}\text{C}$]hydroxyindolin-2-one. For further analysis, unlabeled 3-hydroxyindolin-2-one was obtained by reduction of isatin (20). The conversion of 3-hydroxyindolin-2-one to 2-hydroxy-1,4-benzoxazin-3-one (HBOA) was catalyzed by microsomes containing BX4. The reac-

Table 1. ^1H -NMR analysis of indole-derived enzymatic products in maize (δ , chemical shift; J_{HH} , coupling constant). The signal assignments are based on two-dimensional NMR analysis (28). We used ^1H -NMR spectroscopy to monitor ^{13}C enrichment from [^{13}C]indole and subsequent metabolites. The ^{13}C label from [$3\text{-}^{13}\text{C}$]indole was incorporated into position 3 of indolin-2-one (100% incorporation) and position 2 of DIMBOA (75% incorporation). The label from [$3\text{-}^{13}\text{C}$]indolin-2-one was incorporated into position 3 of 3-hydroxyindolin-2-one (100% incorporation). Some values of J_{HH} are dual because coupling acts on both protons over scalar bonds; others are missing because the signals were characterized by singlet multiplicity and J_{HH} could not be extracted.

Indolin-2-one			3-Hydroxyindolin-2-one			DIMBOA		
Position	δ (ppm)	J_{HH} (Hz)	Position	δ (ppm)	J_{HH} (Hz)	Position	δ (ppm)	J_{HH} (Hz)
4	7.22	7.5	4	7.42	7.5	5	7.26	8.8
6	7.18	7.8, 0.9	6	7.26	7.7	6	6.67	8.8, 2.6
5	6.97	7.5, 0.8	5	7.07	7.5	8	6.63	2.6
7	6.86	7.8	7	6.84	7.7	2	5.67	
3	3.49		3	5.05		O-CH ₃	3.75	

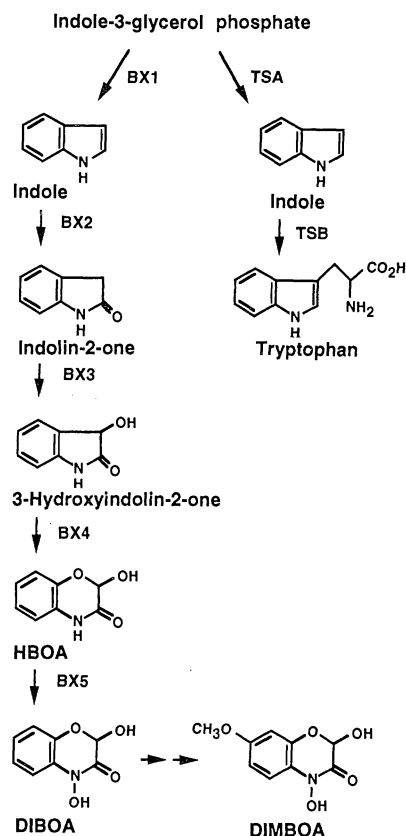


Fig. 3. Biosynthetic pathways to DIMBOA and tryptophan. Names of gene products are indicated for each of the reactions. BX1 represents a tryptophan synthase α activity. BX2 through BX5 represent cytochrome P-450-dependent monooxygenases of the CYP71C subfamily. The sequence of N-hydroxylation and introduction of the methoxy group at C-7 of DIMBOA has not yet been elucidated.

tion mechanism for this unusual ring expansion is as yet unknown. Finally, HBOA was converted to DIBOA by microsomes containing BX5. This reaction was previously described for maize microsomes (21). The identity of the reaction products was confirmed by cochromatography with the authentic substances and by their ultraviolet spectra. The reaction products indole-2-one and 3-hydroxyindolin-2-one were further identified by their ^1H nuclear magnetic resonance (NMR) spectra (Table 1). The identity of HBOA and DIBOA was corroborated by gas chromatography-mass spectrometry (GC-MS) analysis (22).

Although the four cytochrome P-450 enzymes are homologous proteins, they are substrate-specific. Only one substrate was converted by each respective P-450 enzyme to a specific product. No detectable conversions occurred in other enzyme-substrate combinations. Enzymatic reactions identical to the reactions with the different yeast microsomal preparations could be performed with maize microsomes, which indicates that these reactions occur natively in maize. These findings suggest an *in vivo* reaction sequence in maize from indole to HBOA (Fig. 3). According to this scheme, benzoxazinone would not be a natural intermediate for DIMBOA synthesis, as proposed earlier on the basis of feeding experiments (23). Whether alternative routes for DIMBOA synthesis exist remains to be determined.

Bx1 and the four cytochrome P-450 genes represent a sufficient set of genes for the conversion of indole-3-glycerol phosphate to DIBOA (Fig. 3). DIMBOA is the 7-methoxy derivative of DIBOA. Because the oxygen atom at C-7 is incorporated from molecular oxygen (15), hydroxylation by another cytochrome P-450 enzyme followed by a methyltransferase reaction would be expected for the conversion of DIBOA to DIMBOA.

We estimate that the DIMBOA concentration in maize seedlings is $\sim 0.1\%$ of the fresh weight. This value exceeds the total tryptophan content of the seedling by a factor of about 10 to 20 (24). Hence, most of the metabolites from the early steps of the tryptophan pathway would end up in

the DIMBOA pathway. Indole-3-glycerol phosphate represents the branch point from tryptophan biosynthesis, and BX1 enzyme would catalyze the committing step.

The synthesis of several other secondary metabolites in plants, such as the indole glucosinates, anthranilate-derived alkaloids, and tryptamine derivatives (24, 25), depends on the tryptophan pathway. Indole-3-glycerol phosphate was also proposed as a branch point from the tryptophan pathway for the synthesis of the indolic phytoalexin camalexin (3-thiazol-2'-yl-indole) in *Arabidopsis thaliana* (26, 27). It will be interesting to see whether a BX1 homologous enzyme also catalyzes the first specific step in camalexin synthesis. Such an observation would shed light on the role of indole-3-glycerol phosphate as an intermediate in a wide range of secondary metabolites in plants.

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