The maize *viviparous15* locus encodes the molybdopterin synthase small subunit

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**Summary**

A new *Zea mays* viviparous seed mutant, *viviparous15* (*vp15*), was isolated from the UniformMu transposon-tagging population. In addition to precocious germination, *vp15* has an early seedling lethal phenotype. Biochemical analysis showed reduced activities of several enzymes that require molybdenum cofactor (MoCo) in *vp15* mutant seedlings. Because MoCo is required for abscisic acid (ABA) biosynthesis, the viviparous phenotype is probably caused by ABA deficiency. We cloned the *vp15* mutant using a novel high-throughput strategy for analysis of high-copy Mu lines: We used MuTAIL PCR to extract genomic sequences flanking the Mu transposons in the *vp15* line. The Mu insertions specific to the *vp15* line were identified by *in silico* subtraction using a database of MuTAIL sequences from 90 UniformMu lines. Annotation of the *vp15*-specific sequences revealed a Mu insertion in a gene homologous to human MOCS2A, the small subunit of molybdopterin (MPT) synthase. Molecular analysis of two allelic mutations confirmed that *Vp15* encodes a plant MPT synthase small subunit (ZmCNX7). Our results, and a related paper reporting the cloning of maize *viviparous10*, demonstrate robust cloning strategies based on MuTAIL-PCR. The *Vp15/CNX7*, together with other CNX genes, is expressed in both embryo and endosperm during seed maturation. Expression of *Vp15* appears to be regulated independently of MoCo biosynthesis. Comparisons of *Vp15* loci in genomes of three cereals and *Arabidopsis thaliana* identified a conserved sequence element in the 5′ untranslated region as well as a micro-synteny among the cereals.

**Keywords:** MoCo, MuTAIL, seed development, transposon, viviparous.

**Introduction**

Abscisic acid (ABA) is a key regulator of seed maturation and plant-stress responses (Giraudat, 1995; Zeevaart and Creelman, 1988). The *viviparous* (*vp*) mutants of *Zea mays* cause precocious germination through blocks in ABA synthesis or perception (McCarty, 1995). Most of the ABA-deficient mutants (*vp2*, *vp5*, *vp7*, *vp9*, *w3*, *y3* and *y9*) block synthesis of the carotenoid precursors of ABA. These mutants produce albino or pale green, non-viable seedlings. The *Vp5* and *Vp7* genes encode enzymes in the carotenoid biosynthetic pathway (Hable et al., 1998; Singh et al., 2003). The *vp14* mutant (Schwartz et al., 1997a; Tan et al., 1997), on the other hand, is blocked in the first committed step in ABA biosynthesis, cleavage of epoxy-carotenoids to xanthoxin. Xanthoxin is subsequently converted to ABA by two steps (Schwartz et al., 1997b). Reduction of xanthoxin to ABA-aldehyde is catalyzed by a short-chain alcohol dehydrogenase defined by the *aba2/gin1* mutant of *Arabidopsis thaliana* (Cheng et al., 2002; Gonzalez-Guzman et al., 2002). The final step, oxidation of ABA-aldehyde to ABA by an aldehyde oxidase, is controlled by *Arabidopsis AAO3* (Seo et al., 2000). The aldehyde oxidase requires molybdenum cofactor (MoCo). Hence the *aba3* and *flacca* mutants of *Arabidopsis* and *Lycopersicon esculentum*, respectively, are deficient in ABA synthesis as well as another MoCo-dependent enzyme,
Molybdobium cofactor is present in both prokaryotes and eukaryotes, and is a cofactor for several classes of enzyme (Mendel and Hansch, 2002). In plants, at least four enzymes (aldehyde oxidase, xanthine dehydrogenase, nitrate reductase and sulfite oxidase) require MoCo for enzymatic activity (Mendel and Hansch, 2002). The MoCo-biosynthesis pathway has been well studied in *Escherichia coli*. Six genes have been proposed in the plant pathway by analogy to *E. coli* MoCo genes (Mendel and Hansch, 2002). The primary precursor, GTP or a derivative, is converted into a pterin compound called precursorZ in a reaction catalyzed by the plant CNX2 and CNX3 proteins, which are functional homologs of *E. coli* molybdobium cofactor-biosynthesis proteins (Figure 1). Second, two sulfur molecules are incorporated into the pterin compound, generating molybdopterin (MPT). Further sulfuration of the MoCo molecule is required for aldehyde oxidase and xanthine dehydrogenase, whereas nitrate reductase and sulfite oxidase do not require any additional modifications of the cofactor.

Mutants deficient in MoCo biosynthesis have been isolated in various plant species (Gabard et al., 1988; LaBrie et al., 1992; Sato et al., 1996; Walker-Simmons et al., 1989). In *Nicotiana plumbaginifolia*, six mutant loci for MoCo production have been isolated, suggesting that these mutants correspond to the CNX genes (Gabard et al., 1988). Physiological studies and feeding experiments have mapped the probably defective steps of biosynthesis in these mutants. In addition to the extensive genetic and physiological characterization of the MoCo-biosynthesis mutants performed in tobacco, progress has been made in identifying the CNX genes of Arabidopsis, including the *chl-6/cnx1* mutant (Schwarz et al., 2000). Interestingly, at least four of six *sir* mutants identified in a chemical genetics screen for novel auxin-signaling mutants in Arabidopsis have mutations in CNX genes (Dai et al., 2005; Zhao et al., 2003). However, the loss-of-function phenotypes and regulation of MoCo-pathway genes are not well understood in plants.

In maize we have recently developed a systematic functional genomics approach to molecular analysis of seed development mutants based on the high-copy *Robertson’s Mutator* (*Mu*) transposon (http://www.uniformmu.org). Here we report isolation and characterization of a novel seedling lethal viviparous mutant, *viviparous15* (*vp15*), from the UniformMu population. We show that the mutant is deficient in multiple MoCo-dependent enzymes including aldehyde oxidase, xanthine dehydrogenase and sulfite oxidase. We cloned the *Vp15* gene using a novel strategy based on MuTAIL-PCR coupled with *in silico* subtraction and annotation of *Mu* flanking sequences to show that the gene encodes the MPT synthase small subunit (ZmCNX7).

**Results**

**Isolation of a *vp15* mutant from the UniformMu population**

The *vp15* mutant was isolated in a large-scale screen of the UniformMu transposon population (http://www.uniformmu.org) for seed mutations. The *vp15* mutant kernels had strongly viviparous, green embryos with apparently normal endosperm (Figure 2a). Mutant seedlings rescued in soil or agar media were typically smaller than the wild type, with spindly leaves (Figure 2b). When grown in soil, mutant seedlings ceased growth and became necrotic following the emergence of two true leaves. Mutant seedlings grown on agar medium developed slightly further than soil-grown seedlings, producing one or additional leaves before necrosis. These phenotypes suggested that the function of *Vp15* gene product is essential to early post-germinative growth. Among maize viviparous mutants, the *vp15* phenotype was indistinguishable from the *vp10* mutants (Porch et al., 2006). Crosses to *vp10* alleles resulted in complementation, indicating two separate loci. We mapped the *vp15* to chromosome 5L by uncovering the mutant with the T-B5Lb

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*xanthine dehydrogenase* (Sagi et al., 1999; Schwartz et al., 1997b). The *aba3* and *flacca* mutants are blocked in the molybdopterin sulfuration step of MoCo biosynthesis (Bittner et al., 2001; Sagi et al., 2002; Xiong et al., 2001).

**Figure 1.** Molybdobium cofactor biosynthesis pathway (adapted from Mendel and Hansch, 2002). Arabidopsis CNX1 and recently cloned Maize VP10 catalyze the last step of the synthesis (Porch et al., 2006; Stallmeyer et al., 1995). Whereas sulfite oxidase (SO) and nitrate reductase (NR) utilize di-oxo MoCo, xanthine dehydrogenase (XDH) and aldehyde oxidase (AO) require sulfurated mono-oxo Moco.
translocation stock, whereas the vp10 is located on chromosome 10 (McCarty, 1995). We designated this novel viviparous mutant as vp15-umu1.

**MoCo-dependent enzyme activities are reduced in the vp15 mutants**

The Vp10 gene was recently cloned and shown to encode ZmCNX1, the maize ortholog of CNX1, which catalyzes the last step of the MoCo biosynthesis pathway (Porch et al., 2006). As expected, MoCo-dependent enzymes showed reduced activity in the vp10 mutants. Because of the similarity of the vp15 phenotype, we assayed MoCo-dependent enzyme activities in extracts of seedlings of vp15-umu1 and two allelic mutants, vp15-DR1126 and vp15-MJ7546 (Figure 3). Activities of aldehyde oxidase and xanthine dehydrogenase were undetected in the mutants by gel-activity assays. Sulfite oxidase activity was also reduced sharply in vp15 mutants. These results indicate that the Vp15 gene probably also encodes a factor essential for MoCo biosynthesis.

**Cloning of Vp15 gene by in silico filtering of MuTAIL-PCR sequences**

Settles et al. (2004) adapted TAIL-PCR to amplify flanking DNA sequences of high-copy Mu transposons (MuTAIL) efficiently in the maize genome. To ensure that Mu insertions represented in the MuTAIL products were derived exclusively from germinal insertions in the mutant line, Mu-inactive vp15 heterozygous plants were selected using the bz1-mum9 marker. In Mu-active individuals, the bz1-mum9 seeds are densely spotted, indicating a high frequency of somatic transposition. We back-crossed the vp15-umu1 isolate twice to the W22 inbred selecting ears that segregated both a stable bronze aleurone phenotype (non-spotted seed) and vp15-umu1. Genomic DNA was prepared from a pool of nine seedlings grown from the non-viviparous seeds obtained from self-pollinated heterozygotes, and used to construct a library of cloned MuTAIL products.

We obtained single-pass sequence reads from 280 randomly selected MuTAIL clones in the vp15 library (Table 1). To identify candidate clones for the vp15-umu1 locus, we performed in silico subtraction against a large collection of MuTAIL sequences derived from 90 different UniformMu lines, by assembling all sequences in the data set into contigs using the PHRAP program (Ewing and Green, 1998; Ewing et al., 1998). The assembly identified three classes of sequence: shared contigs that contained MuTAIL sequences from multiple libraries; unique contigs comprised solely of sequences from the vp15 library; and singleton clones that were unique to vp15. Of the 280 vp15 MuTAIL clones, 251 were assembled to a total of 63 contigs. Of the 63, only five
contigs were unique to the vp15 library. Including 29 singletons, we identified 34 Mu flanking sequences as being unique to the vp15 library, and therefore potential candidates for causative insertion in the Vp15 gene.

In a second stage of analysis, the 34 unique MuTAIL clones were analyzed by BLASTN search (Altschul et al., 1990) to extract all available maize genome sequence and cereal expressed sequence tag (EST) sequences that matched the MuTAIL sequences. These sequences were analyzed by BLASTX of the nr protein database to identify potential genes (Figure 4). In this analysis, one contig showed a significant homology to Homo sapiens MOC2A, the small subunit of MPT synthase in the MoCo biosynthetic pathway (Stallmeyer et al., 1999) corresponding to plant CNX7. This alignment, together with biochemical evidence that the vp15 mutant is MoCo-deficient, identified the presumptive insertion linked to Vp15.

To confirm the presence of the Mu insertion in the vp15-umu1 MOC2A homolog, we performed PCR analysis of a segregating family using vp15 gene-specific primers and a partially degenerate Mu terminal inverted repeat (TIR)-specific primer. We detected expected PCR products from pooled DNA of 10 vp15-umu1 heterozygotes as well as 20 homozygous mutant individuals, but not from the W22 inbred. Both 5' and 3' gene-specific primers produced the expected size of PCR products with the TIR primer (data not shown). Products were sequenced to confirm that they contained bona fide Mu flanking sequences matching the MuTAIL products with a 9-bp host duplication (5'-CCGCC-CATC-3') typical of a Mu insertion.

Analysis of independent vp15 alleles

To confirm the identity of the Vp15 gene, we determined the lesions in two allelic mutants, vp15-DR1126 and vp15-MJ7546. We detected PCR products from vp15-DR1126 with the same primer pairs used for analysis of vp15-umu1. Analysis of the PCR products confirmed that vp15-DR1126 has a Mu insertion in the 5' portion of the coding region of the gene (Figure 5a). We failed to detect Mu flanking PCR products from vp15-MJ7546 using either combination of TIR and gene-specific primers. We therefore sequenced the MPT synthase small subunit alleles from homozygous vp15-MJ7546 mutant plants and homozygous wild-type siblings. The analysis revealed a 15-bp in-frame deletion in the coding region in the homozygous mutant plants. The molecular defects in these three alleles confirmed that the Vp15 gene encodes a CNX7 homolog (ZmCNX7).

Gene and protein structure of Vp15

To determine the structure of the Vp15 gene, we designed primers based on overlapping maize GSS sequences. A 1296-bp genomic region containing the complete protein-coding region was determined by sequencing overlapping PCR products (Figure 5a). Interestingly, a serine-tRNA gene was found immediately upstream of the Vp15-coding region in the opposite orientation.

We identified several maize EST clones that are nearly identical to the Vp15 gene. Two ESTs (Genbank accessions CKJ347894 and CK347582) from a maize root cDNA library (H. J. Bohnert and co-workers, University of Illinois, IL, USA, unpublished data) were from near full-length clones. The 5' end of the longest Vp15 cDNA is only 108 bp distant from the 5' end of the tRNA gene. The transcript includes the first potential ATG codon located distal to the tRNA gene, suggesting that it contains the full-length coding sequence comprised of two exons. Based on the Vp15 genomic sequence, as well as the EST sequences, we conducted RT-PCR with the primers spanning the entire coding sequence. We obtained the expected size product, and confirmed that the sequences of the RT-PCR products are identical to the predicted CDS by the W22 Vp15 genomic sequence (Figure 5a). The cDNA sequences predict a protein of 106 amino acids that align with MPT synthase small-subunit orthologs from other organisms (Figure 5b).

Amino acid alignment of the MPT synthase small subunits from various organisms, including E. coli, highlighted the C-terminal conserved domain (Figure 5b). This conservation is consistent with the essential function of the C-terminal glycine as a covalent acceptor of a sulfur molecule in the catalytic reaction (Rudolph et al., 2001). The N-terminal
segments show a limited degree of conservation. The phenylalanine residue at position seven of *E. coli* MoaD has been implicated in interactions with MoaE (Rudolph et al., 2003) and appears to be highly conserved in both prokaryotes and eukaryotes. The predicted plant MPT synthase small-subunit proteins, especially rice and maize VP15, possess N-terminal extensions that may affect subcellular localization of the protein; however, software predictions of localization indicated a cytosolic location as most probable (data not shown).

We performed Northern analysis of VP15 in developing maize seedlings (Figure 5c). We detected transcripts for the VP15 gene from the W22 inbred, but not *vp15-umu1* and *vp15-DR1126* mutants, suggesting that the transposon insertions disrupted transcription or mRNA processing/stability. In contrast, a nearly normally sized mRNA was detected from the *vp15-MS7546* in-frame deletion allele, suggesting possible translation of an internally deleted protein. These results were consistent with the structures of the three *vp15* alleles.

**Developmental regulation of maize Cnx genes**

To address the potential for developmental regulation of the MoCo biosynthesis pathway, we analyzed expression of *Vp15*, *Vp10* and maize CNX2, CNX3, CNX5 and CNX6 orthologs in developing embryo and endosperm tissues. Partial or entire sequences of maize *ZmCnx2*, *ZmCnx3*, *ZmCnx5* and *ZmCnx6* genes were identified by BLAST searches of the MAGI genome assembly sequences (Fu et al., 2005) and public EST databases. RT–PCR primers based on these sequences were used to analyze mRNA levels in total RNA sampled from dissected embryo and endosperm tissues at 18, 26 and 34 days post-pollination. As shown in Figure 6(a), transcripts of all six MoCo-pathway genes are detected in developing embryo and endosperm tissues. ZmCnx2 expression showed marked upregulation in both embryo and endosperm late in development, whereas the other MoCo-pathway genes were expressed at relatively constant levels throughout development. Expression levels of individual genes in
embryo and endosperm were strongly correlated at all three stages, indicating an absence of differential regulation between tissues in the seed. Quantitative comparisons between genes were not considered definitive, due to possible differences in primer efficiencies. Although MoCo-pathway genes were expressed at comparable levels in embryo and endosperm, \textit{vp15} mutants lacked a discernible endosperm phenotype. Interestingly, genetically non-concordant seed with mutant embryo and wild-type endosperm, created by crossing a \textit{vp15} heterozygote to the chromosome 5L T-B translocation stock, produced viviparous embryos (data not shown), indicating that endosperm expression of the pathway was incapable of rescuing the mutant embryo.

To address possible feedback regulation of MoCo-pathway genes, we examined their expression in \textit{vp15} mutant seedlings. As shown in Figure 6(b), expression of \textit{Vp10}, \textit{ZmCnx2}, \textit{ZmCnx3}, \textit{ZmCnx5} and \textit{ZmCnx6} mRNAs was comparable in wild-type and \textit{vp15} mutant seedlings. This result suggested that MoCo biosynthesis is probably regulated independently of the substrate–product level, at least at the level of transcription. Consistent with the Northern analysis, no \textit{Vp15} transcript was detected by RT–PCR in the \textit{vp15-umu1} and \textit{vp15-DR1126} homozygous mutants, whereas a PCR product slightly smaller than normal was observed in the \textit{vp15-MJ7546}, consistent with the 15-bp deletion.

**Figure 6.** Expression of \textit{ZmCnx} genes.
(a) RT–PCR analysis for \textit{ZmCnx} gene expression during seed maturation. The W22 inbred was self-pollinated and the developing ears were harvested at 18, 26 and 34 days after pollination. Seeds were quickly dissected to embryo and endosperm for total RNA isolation.
(b) RT–PCR analysis of \textit{ZmCnx} genes in seedling leaves of W22 control and \textit{vp15} mutants.

**Hormone synthesis in the \textit{vp15} mutant seedlings**

MoCo is required by aldehyde oxidases that function in biosynthesis of ABA and possibly auxin (Mendel and Hansch, 2002). While the ABA deficiency of MoCo-pathway mutants is well documented in barley, tomato and Arabidopsis (Sagi \textit{et al}., 1999; Schwartz \textit{et al}., 1997b; Walker-Simmons \textit{et al}., 1989), much less is known about possible affects on auxin biosynthesis. To address the role of hormone alterations in conditioning the \textit{vp15} seedling phenotype, we analyzed levels of multiple signaling molecules in W22 wild-type and \textit{vp15} mutant seedlings that were sampled prior to visible signs of stress or necrosis. As expected for well watered seedlings, low basal amounts of ABA detected in wild type were only slightly higher than levels in mutant seedlings. The amounts of indole acetic acid (IAA) also did not differ significantly in wild-type and \textit{vp15} mutant seedlings (Figure 7). In contrast, levels of jasmonic acid (JA) precursor, 12-oxo-phytodienoic acid (OPDA), were markedly elevated in mutant seedlings compared with wild-type seedlings. Moreover, a pronounced increase in JA occurred within 20 min following detachment of mutant leaves, consistent with rapid conversion of OPDA to JA. The presence of high levels of OPDA in leaves prior to excision was a strong indication that \textit{vp15} mutant seedlings were responding to an internal stress or injury condition before the onset of visible stress.

**Figure 7.** Synthesis of key signaling molecules in \textit{vp15} mutant seedlings.
Concentrations of indole acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and 12-oxo-phytodienoic acid (OPDA) were determined in wild type and two \textit{vp15} mutants using the method of Schmelz \textit{et al}., 2003. Detached seedling leaves were extracted immediately (\(t = 0\), open and hatched bars) or after 20 min incubation in ambient air (\(t = 20\), solid grey and black bars). Upper panel: IAA open and gray filled bars; ABA, hatched and black bars. Bottom panel: JA, open and grey filled bars; OPDA, hatched and black bars. Error bars indicate mean deviation of replicate experiments.
distress. However, the stress condition was not correlated with substantial differences in ABA or auxin levels.

**Structure of Vp15 orthologs in cereal and Arabidopsis genomes**

Southern hybridization analysis suggested that the maize genome possesses a second locus closely related to Vp15 (Vp15b, data not shown). Consistent with this analysis, we searched maize GSS sequences (Palmer et al., 2003; White-law et al., 2003) and identified a second locus with high similarity to the Vp15 gene (Figure 8a), with nearly identical sequence in the coding region. The second gene was also adjacent to a serine-tRNA gene in the opposite orientation, suggesting that Vp15 and Vp15b are in duplicated segments of the maize genomes. However, the nucleotide sequence of the 5′ portion of the duplicate gene does not include an in-frame ATG, indicating that Vp15b is probably a non-functional pseudogene consistent with the single-factor inheritance of the vp15 mutant. We confirmed this frame shift by sequencing 268 bp of the 5′ portion of the vp15b locus that spanned the expected translation start site (data not shown).

In addition, Northern blot analysis of vp15-umu1 and vp15-DR1126 mutants using the Vp15-coding region as a probe confirmed that the duplicate gene is not expressed in seedling leaves (Figure 5c).

Using database searches, we extracted and compared genomic sequences of the probable Vp15 orthologs of Oryza sativa, Sorghum bicolor and Arabidopsis (Arabidopsis Genome Initiative, 2000; Bedell et al., 2005; International Rice Genome Sequencing Project http://rgp.dna.affrc.go.jp/IRGSP). The structural comparison of these loci revealed that all three cereal orthologs have a conserved gene structure including the adjacent serine-tRNA genes in the 5′ proximal region (Figure 8a). Like maize, the rice genome contains at least one duplicate region that is structurally similar to the Vp15 locus. The duplicate rice gene is unlikely to encode a functional CNX7 protein, due to the rearrangements including a deletion and an insertion. It also lacks the adjacent tRNA gene. In contrast to cereal orthologs, the Arabidopsis CNX7 locus (At4g10100) is a single gene and unlinked to a tRNA gene.

Closer inspection of the Vp15 orthologs identified at least one non-coding sequence element that is evidently conserved among the four plant species (Figure 8b). The CTCGGCGA sequence is present in the 5′ untranslated region close to the translation initiation site, suggesting that this may be a cis element involved transcriptionally or translationally in the regulation of Vp15 expression.

**Discussion**

Our results demonstrate a novel and efficient transposon-tagging strategy based on bioinformatic analysis of Mu transposon insertion sites identified by high-throughput MuTAIL sequencing. Two bioinformatics criteria were sufficient to identify the causative insertion in the vp15-umu1 allele isolated from the UniformMu population: (i) *in silico* subtraction of parental insertions shared by multiple UniformMu lines identified the subset of Mu insertions in the mutant genome that were unique to the mutant; and (ii) automated annotation of the unique insertion sites identified a strong candidate gene that was consistent with the biochemical phenotype of the mutant and known characteristics of the ABA-biosynthetic pathway.

In implementing selection based on gene annotation, we encountered some limitations due to incomplete annotation of plant genomes and the current highly fragmented state of maize genome sequence resources. A primary BLASTX annotation of the MuTAIL sequences failed to detect the MPT synthase small-subunit homology due to insufficient coverage of the Mu flanking sequence. A secondary annotation of the fragmentary maize genomic sequences detected homologs from human as well as Arabidopsis; however, at that time only the human accession was annotated as a MoCo-related protein. Hence for a less conserved gene, the informative match might have escaped immediate detection. Fortunately, we can expect such limitations to diminish sharply in near future as more plant genome sequences are completed and fully annotated.

In the case of vp15, inferences based on prior biochemical knowledge of the ABA pathway played a key role in the bioinformatic analysis. In applications to a broader range of tagged mutants, a variety of additional bioinformatics filters...
may be leveraged, including genetic map information based on in silico mapping of maize genomic and cDNA sequences; detection of allelic insertions; similarity to potentially orthologous mutants defined in other species; and correlation with expression profiles of candidate genes based on EST or microarray databases. The overall efficiency of this approach for forward genetics applications depends on the number of tagged mutations available for high-throughput analysis (e.g. http://www.uniformmu.org). While, in principle, tagged alleles derived from any Mu transposon population may be analyzed by this strategy, backgrounds that provide genetic control of transposon activity are strongly recommended in order to suppress somatic insertions. In UniformMu lines, selection of Mu-inactive derivatives is facilitated by incorporation of the bz1-mum9 marker.

Our genetic analysis indicates that the maize genome contains a single functional gene for the MPT synthase small subunit. The absence of detectable aldehyde oxidase and near-background levels of sulfite oxidase activity indicate that all three vp15 alleles are nearly or completely null for small subunit activity. Northern analysis confirms that vp15-umu1 and vp15-DR1126 lack detectable stable transcripts. In contrast, the vp15-MJ7546 allele produces transcripts at levels comparable with the wild type. This in-frame deletion allele is indistinguishable from the other two alleles in the phenotype, as well as the MoCo enzyme activities. Based on the structure of MoaD, the catalytically important C-terminal tail is exposed on the surface of the subunit and projected into a fold of the MoeE subunit to form the active complex (Rudolph et al., 2001). The alignments suggest that the 5-aa deletion in the predicted vp15-MJ7546 protein is located immediately upstream of an alpha–beta fold that may have a role in positioning the C-terminal peptide in the MoaD–MoeE complex (Rudolph et al., 2003). Moreover, the amino acid alignments indicate that plant CNX7 protein has the N-terminal extension. Hoff et al. (1995) reported that Arabidopsis also possess longer N-terminal regions of CNX2 and CNX3, compared with the E. coli proteins). The CNX6 protein may also possess C-terminal extension compared with homologs of other species (data not shown). These extended regions of the CNX proteins may have specialized functions in plant cells.

Our results indicate that genes in the MoCo biosynthetic pathway, including Vp15 (CNX7), are widely expressed in maize tissues, with relatively small changes in expression that are attributable to developmental regulation. The notable exception is ZmCnx2 which encodes the earliest defined step in the pathway. The RT–PCR products for ZmCnx2 are more abundant late in seed development, suggesting that regulation of CNX2 may be a key control point of the pathway in plants. Interestingly, sequence analysis of the RT–PCR products for the ZmCnx genes revealed that the PCR primers for ZmCnx2 gene amplified two distinct products, whereas the others detected only unique sequences. The two ZmCnx2 transcripts appear to be generated by alternative splicing, although we did not determine quantitative differences between the two variants in gene expression. Consistent with this observation, alternatively spliced forms of the CNX2 orthologs are identified in both rice and Arabidopsis (LOC_Os12g32230 and At2g31955, respectively; http://www.tigr.org; http://www.genoscope.cns.fr; M.S. and D.R.M., unpublished data). The altered splicing inserts GGTAA at the junction of the third exon and fourth intron in the rice and Arabidopsis transcripts, thereby generating a truncated form missing the C-terminal domain with iron–sulphur binding sites (Hänzelmann and Schindelin, 2004). This 5-nucleotide sequence is not present in one of the two types of the ZmCnx2 PCR products at the exon–intron junction. The function of the truncated CNX2 protein is unknown.

Although the MoCo pathway is expressed at comparable levels in embryo, endosperm and seedling tissues, the vp15 mutant indicates that MoCo is not essential for embryogenesis or endosperm formation. Because mutant seeds develop on a heterozygous maternal plant, we cannot rule out the possibility of maternal cross-feeding resulting in partial rescue of embryo and endosperm development. Interestingly, shoot aldehyde oxidase in tomato flacca mutant plants (Sagi et al., 2002) is not restored by root-specific expression of MoCo sulfurylase, suggesting that the sulfated mono-oxo MoCo, at least, is not transported long distances in plants. Moreover, genetically non-concordant seed created by crossing vp15 heterozygotes to the T-B5Lb translocation stock indicates that a wild-type (hyperploid) endosperm is insufficient to rescue a vp15 mutant embryo. Therefore cross-feeding of MoCo intermediates within the seed appears to be limited.

The viviparous phenotypes of vp10 (Porch et al., 2006) and vp15 are attributed to a block in the MoCo-dependent ABA-aldehyde oxidase step in ABA biosynthesis in the developing seed. The tomato flacca and Arabidopsis aba3 mutations in MoCo sulfurylase also lack aldehyde oxidase, but are viable. Thus the lethal phenotype of the maize mutants is probably due to one of several pathways that require the di-oxo form of MoCo. Consistent with that hypothesis, lethality of vp15 mutants in seedling development is not correlated with alterations in the ABA or IAA content of soil-grown seedlings. Instead, mutant seedlings exhibit a striking elevation in JA and OPDA synthesis, indicating that a severe stress or injury response precedes the onset of necrosis. On the other hand, our data do not fully rule out a complex interaction between multiple affected pathways that may include subtle hormone imbalances. Porch et al. (2006) found a modest reduction in auxin content of vp10 mutant seedlings grown in culture (30% less than wild type), suggesting that auxin synthesis may be partly MoCo-dependent in maize seedlings. The basis for the apparent difference in vp15 and vp10 effects on IAA levels is not known.

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Maize Vp15 is located on the long arm of chromosome 5. That arm contains at least two other linked loci (Vp2, Ps1/VP7; bin 5.04) that also affect ABA synthesis (McCarty, 1995; Neill et al., 1986; Singh et al., 2003). Several QTL studies have implicated chromosome 5 with ABA-related traits (Reymond et al., 2003; http://www.gramene.org). Interestingly, a syntenous region on chromosome 2 of rice includes the probable orthologs of CNX5 and CNX6 in addition to OsVP15/CNX7 and OsPS1/VP7. Hence multiple genes in this syntenic block may contribute to regulation of MoCo and ABA biosynthesis in cereals.

Comparative structures of functional Vp15 orthologs in other plant genomes reveal a conserved sequence in the 5’ UTR that may be a transcriptional or translational regulatory element. In all cases, the CTCCGGC motif is closely associated with the translation initiation site. Interestingly, this sequence resembles the A-box regulatory motif that is conserved in tRNA genes. The A box is located internally in tRNA genes and is required for assembly of the RNA polymerase III transcription initiation complex (Geiduschek and Kassavetis, 2001; Hofstetter et al., 1981; Marck et al., 1993; Shi and Tyler, 1991). The similarity of the motif to the A-box regulatory elements of tRNA genes is intriguing in the light of the close association of functional Vp15 orthologs of cereals with serine tRNA genes. While microsynteny is common among cereals, the separation of transcription units by just approximately 100 nucleotides is striking. Interaction or involvement of RNA polymerase III-associated factors in regulation of plant MPT synthase small subunit is an interesting possibility. Human retro-element L1, which encodes a long transcript with a polyA tail, is evidently transcribed by RNA polymerase III through the A box binding factor YY1 (Kurose et al., 1995). Likewise, the Vp15/CNX7 may be transcribed by RNA polymerase III, or by a complex involving subunits shared by TFIll and TFI complexes.

Experimental procedures

Plant material

The vp15-umu1 was isolated from the UniformMu transposon population constructed in the W22 inbred background. The mutant was back-crossed twice to the W22-inbred, self-pollinated, and screened for loss of Mu activity by selection of stable bronze kernels. The allele was isolated as a spontaneous mutant by Martha James. For RNA isolations and hormone quantification, the homozygous seeds were sown in pots with soil and grown for 10 days at 22°C before harvest.

Preparation of crude extracts for MoCo-enzyme assays

For the MoCo-dependent enzyme analysis, developing kernels (16 days after pollination) were removed from segregating ears and sterilized in 70% ethanol for 2 min, followed by treatment with 20% commercial bleach for 15 min. Seeds were rinsed with sterile water, and embryos were excised aseptically and cultured on MS medium (pH 5.7) with 0.4% Phytagel (Sigma, St Louis, MO, USA). The seedlings were incubated at 25°C in a 12-h light/dark cycle for 15 days before sampling leaf tissues. Crude protein extracts for aldehyde oxidase, xanthine dehydrogenase and sulfite oxidase were prepared as previously described (Eilers et al., 2001; Mendel and Müller, 1976; Walker-Simmons et al., 1989).

Assays of MoCo-dependent enzymes

For in-gel assays of aldehyde oxidase and xanthine dehydrogenase, native gel electrophoresis (7.5% polyacrylamide separation gel) was performed with in-running buffer (25 mM Tris, 250 mM glycine) at 8 mA. After electrophoresis the gels were equilibrated in 0.1 M sodium pyrophosphate buffer (pH 8.0) and transferred to staining buffers in the dark. The staining buffer used for aldehyde oxidase contained 0.1 mM Tris–HCl (pH 8.5), 1 mM 1-naphthaldehyde, 1 mM nitrotetrazolium blue and 0.1 mM phenazine methosulphate. The staining buffer for xanthine dehydrogenase contained 0.1 mM sodium pyrophosphate (pH 8.0), 2 mM hypoxanthine, 1 mM nitrotetrazolium blue and 0.1 mM phenazine methosulphate. For sulfite oxidase assays, 100 μl extract was added to 900 μl assay buffer (20 mM Tris–acetate (pH 8.0), 400 μM potassium ferriyanide and 0.1 mM EDTA). The catalytic reaction was monitored at room temperature by a Beckman D4-40 spectrophotometer (Fullerton, CA, USA). One unit of sulfite oxidase activity was measured as the conversion of 1 μmol sulfite min⁻¹.

MuTAIL library construction

MuTAIL-PCR and plasmid library construction was performed as described previously (Settles et al., 2004). Briefly, nine non-virulent seeds were chosen from a selfed heterozygous ear 02F-2009-8 that segregated vp15-umu1. Approximately equally sized leaf samples from each plant (approx. 1 g total) were mixed and used for the genomic DNA extraction. Leaf tissues were ground in liquid nitrogen and the DNA was extracted in urea buffer [3.67 M urea, 50 mM Tris–HCl (pH 8.0), 0.3125 mM NaCl, 20 mM EDTA (pH8.0), 1% (w/v) N-lauroyl sarcosine]. Four MuTAIL PCR reactions were performed using four different sets of arbitrary primers. The products were size-selected by passage through Sephacryl-400 spin columns and cloned into the pCR4-TOPO vector (Invitrogen, Fullerton, CA, USA). One unit of sulfite oxidase activity was measured as the conversion of 1 μmol sulfite min⁻¹.

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Northern blot analysis

The total RNA was prepared from maize seedlings with an RNAeasy kit (Qiagen, Valencia, CA, USA). Fifteen micrograms of the total RNA was resolved in a 1.2% agarose gel and transferred onto a nylon membrane. Hybridization was performed as described previously (Suzuki et al., 2001).

RT-PCR analysis

The total RNA from the seedling leaves was DNase-treated and purified with the RNAeasy kit. The total RNA from maize embryo and
endosperm was extracted as described previously (McCarty, 1986). The RNA was further purified and DNaseI-treated with the RNAse kit. Then 100 ng of the total RNA was used for RT–PCR reactions (30 cycles) in total volume of 15 μl with a One-Step RT–PCR kit (Qiagen). The primers used for RT–PCR were: 5′-ATCAATAGAGCTGT-GTAAAGCTGG-3′ and 5′-AATATGCTGCAACATGCTATC-TG-3′ for ZmCnx2; 5′-CTATAGCTGCTGAGGTTTTCTCAGG-3′ and 5′-TATATACAACCTGAGTGACAGCAGCTC-3′ for ZmCnx3; 5′- TTCAAAAGGAAGCATGTTGGCAATC-3′ and 5′-GACCCACCTG-TCTGAGCATCTCTGGCAG-3′ for ZmCnx5; 5′-CTACGTCTGAGCCAG-AGCTAAGGCGTCC-3′ and 5′-TCAAGCGCTCTGAGCACCCTGC- TTC-3′ for ZmCnx6; 5′-TTCCTCCGGCCGAGCGGAAAGATCC-3′ and 5′-TCAGCGCCACTGATGCGGCTGAGA-3′ for Vp15; 5′-TGC-GCAATGTCGTCGTCGCTGTCGTCG-3′ and 5′-CTGAAAGACAGACATAGTGAGACAGCAACAT-3′; 5′-TCAAGCTTGTTGATGATAAGTGTTG-3′ for Vp10.

Quantification of hormones

Leaves were harvested from seedlings and frozen with liquid nitrogen. Quantifications of IAA, ABA, JA and OPDA were performed as described by Schmelz et al. (2003).

Sequence assembly and analysis

Sequence processing and assembly were conducted as described previously (Settles et al., 2004). Briefly, sequences were vector-trimmed and repeat-masked to remove repetitive maize sequences and the residual Mu-TIR sequences using cross-match and assembly with MuTAIL sequences from 90 other seed mutant libraries using phrap (Ewing and Green, 1998; Ewing et al., 1998). Custom software written in Java was used to parse contigs and singletons that were unique to the Vp15 library. The unique sequences were analyzed with BLASTN (Altschul et al., 1990) searches of databases containing all available maize GSS sequences (excluding MuTAIL sequences) and cereal EST sequences. Database accesses that matched MuTAIL sequences were analyzed by BLASTX searches of the NCBI nr (non-redundant protein) database. The BLASTN and BLASTX annotations were parsed into a mysql database (www.mysql.com) using custom Java software. The genomic sequence of Vp15 has been deposited in the GenBank (accession number DQ273133).

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References


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