Running Head

Leucaena enzyme for mimosine degradation

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Research Area

Biochemistry and metabolism
A C-N lyase from *Leucaena leucocephala* catalyzes the first step of mimosine degradation

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Summary

Biochemical characterization of a C-N lyase from the tree-legume *Leucaena leucocephala* (leucaena) showed that it degrades the leucaena toxin mimosine into 3-hydroxy-4-pyridone, pyruvate and ammonia.
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Abstract

The tree-legume *Leucaena leucocephala* (leucaena) contains a large amount of a toxic non-protein aromatic amino acid, mimosine, and also an enzyme, mimosinase, for mimosine degradation. In this study, we isolated a 1520-bp cDNA for mimosinase from leucaena and characterized the encoded enzyme for mimosine-degrading activity. The deduced amino acid sequence of the coding region of the cDNA was predicted to have a chloroplast transit peptide. The nucleotide sequence, excluding the sequence for the chloroplast transit peptide, was codon-optimized and expressed in *E. coli*. The purified recombinant enzyme was used in mimosine degradation assays, and the chromatogram of the major product was found identical to that of 3-hydroxy-4-pyridone (3H4P), which was further verified by ESI-MS/MS. The enzyme activity requires pyridoxal 5’-phosphate (PLP) but not α-keto acid and therefore the enzyme is not an aminotransferase. In addition to 3H4P, we also identified pyruvate and ammonia as other degradation products. The dependence of the enzyme on PLP, and production of 3H4P with the release of ammonia indicate that it is a C-N lyase. It was found to be highly efficient and specific in catalyzing mimosine degradation with the apparent $K_m$ and $V_{max}$ values of $1.16 \times 10^{-4}$ M and $5.05 \times 10^{-5}$ mole$\cdot$s$^{-1}$$\cdot$mg$^{-1}$, respectively. The presence of other aromatic amino acids, including L-tyrosine, L-phenylalanine and L-tryptophan, in the reaction did not show any competitive inhibition. The isolation of the mimosinase cDNA and the biochemical characterization of the recombinant enzyme will be useful in developing transgenic leucaena with reduced mimosine content in the future.
Introduction

Leucaena leucocephala (leucaena) is an important agroforestry tree-legume of the tropics and its foliage can be used as a protein-rich fodder (Garcia et al., 1996; Soedarjo and Borthakur, 1998). Leucaena is highly tolerant to drought (Shelton and Brewbaker, 1994) and resistant to many pests and diseases. The protein-rich foliage and tolerance to various abiotic and biotic stresses make leucaena a promising legume for use as a fodder. In spite of these desirable attributes, the use of leucaena as a fodder is rather limited because its foliage also contains an N-heterocyclic non-protein amino acid, known as mimosine, which is toxic to both prokaryotic (Soedarjo et al., 1994) and eukaryotic cells (Lalande, 1990). Mimosine inactivates a variety of enzymes either by chelating bivalent metallic ions and thereby limiting their availability for use as cofactors by several metallic ion-dependent enzymes, such as ribonucleotide reductase, alkaline phosphatase, and dopamine β-hydroxylase (Chang, 1960; Hashiguchi and Takahashi, 1977; Dai et al., 1994) or by forming a stable complex with pyridoxal-5′-phosphate (PLP) leading to inactivation of PLP-dependent enzymes, such as cystathionine synthetase, cystathionase, aspartate-glutamate transaminase, tyrosine decarboxylase, tyrosinase, and L-dopa decarboxylase (Crounse et al., 1962; Lin et al., 1962; Lin et al., 1963; Hylin, 1969). The inactivation of important enzymes by mimosine causes various physiological abnormalities including enlarged thyroid glands, infertility, birth defects, and loss of hairs (Crounse et al., 1962; Hamilton et al., 1968; Joshi, 1968; Dewreede and Wayman, 1970; Reis et al., 1975; Jones et al., 1976).

Mimosine is abundant in all parts of leucaena and on a dry weight basis leucaena leaves contain ~5% of mimosine (Soedarjo and Borthakur, 1998). Such high mimosine content in the foliage indicates that mimosine may have some functional role in the plant. Previously, mimosine has been shown to inhibit DNA synthesis in many DNA viruses by chelating iron required by ribonucleotide reductase (Dai et al., 1994) suggesting its role in defense against virus attacks. Besides this, other possible roles of mimosine in leucaena are not well established. Considering its biochemical properties of inactivating various enzymes that require either bivalent metallic ions or PLP as cofactors, mimosine may have a role in plant defense, and based on its chemical composition, it may serve as a reservoir of carbon and nitrogen for survival and growth under nutrient-limiting conditions. But the utilization of mimosine as a source of carbon and nitrogen is possible only if the plant has specific enzymes to catabolize it. Interestingly, the
presence of such mimosine-degrading enzymes has been reported from seedling extracts of
leucaena and *Mimosa pudica*, another mimosine-containing plant (Suda, 1960; Smith and
Fowden, 1966). Smith and Fowden (1966) identified the mimosine-degrading enzyme from
leucaena seedling extracts as a C-N lyase that converted mimosine into 3,4-dihydroxypyridine
(3,4DHP), pyruvic acid, and ammonia (Fig. 1). Additionally, a mimosine-degrading enzyme
namely ‘mimosinase’ was purified from leucaena leaves (Tangendjaja et al., 1986) that was
found to degrade mimosine into 3-hydroxy-4-pyridone (3H4P) (Fig. 1). However, the genes
encoding the mimosine-degrading enzymes from leucaena have not been isolated and
characterized.

The goals of this study were to isolate cDNA for a mimosine-degrading enzyme from
leucaena, and to determine the biochemical and kinetic properties of the encoded enzyme. This
will help to understand roles of mimosine and mimosine-degrading enzymes in leucaena.
Additionally, it may be useful in developing transgenic leucaena with reduced mimosine content,
which will make this tree-legume suitable for use as a nutritious fodder for animals in the future.

**Results**

**Isolation of cDNA for mimosine-degrading enzyme from leucaena**

Previously, we isolated a set of 406 clones from the cDNA library of leucaena, made from
young shoots from ~8 weeks-old seedlings, through interspecies suppression subtractive
hybridization (iSSH) using cDNAs from leucaena and a related tree-legume *Acacia confusa*
(acacia) as the tester and driver, respectively (Negi et al., 2011). These cDNA clones represent
either leucaena-specific genes that are missing in acacia or genes that are highly expressed in
leucaena. Since mimosine-degrading enzyme activity is specific to leucaena, we expected that
one of these 406 cDNA clones from the iSSH library might encode the mimosine-degrading
enzyme. We also expected the mimosine-degrading enzyme from leucaena to be a C-N lyase for
two reasons: (i) Smith and Fowden (1966) showed that a C-N lyase from leucaena had
mimosine-degrading activity; and (ii) recently, we have found that the *midD* gene of the
leucaena symbiont *Rhizobium* sp. strain TAL1145 encodes a C-N lyase that degrades mimosine
into 3H4P, pyruvate and ammonia (Negi et al., 2013). Therefore, we analyzed the leucaena
iSSH library of 406 cDNA clones sequences to identify any sequence that shows homology to
lyases. blastp analysis of the deduced amino acid sequences of leucaena iSSH cDNA library against reference proteins sequence database identified one 764-bp cDNA clone, named ‘seq3’, which showed homology with cystathionine β-lyases from Glycine max (NP_001242026, 78% similarity), Ricinus communis (XP_002512818, 76% similarity), Vitis vinifera (XP_002274313, 76% similarity) and Arabidopsis thaliana (NP_850712, 75% similarity). Cystathionine β-lyase (CBL) catalyzes cystathionine degradation into homocysteine, ammonia and pyruvate. Two of the products of this reaction, pyruvate and ammonia, are the same as the products of mimosine degradation catalyzed by the enzymes from leucaena (Smith and Fowden, 1966) and Rhizobium (Negi et al., 2013) in which the third product is either 3,4DHP or its isomer 3H4P. Considering the similarity in the products from the degradation reactions catalyzed by CBL and the mimosine-degrading C-N lyases from leucaena and Rhizobium, and the homology of seq3 with CBL, we decided to obtain the full-length cDNA for this partial cDNA fragment, expecting that it might be the cDNA for the mimosine-degrading enzyme from leucaena. RLM-RACE of the seq3 cDNA fragment resulted in isolation of a 1520 bp cDNA fragment that consists of 1332 bp long open reading frame (ORF). The blastp analysis of the deduced amino acid sequence of the full-length ORF of seq3 using reference proteins database of NCBI showed its homology with various proteins that belong to aspartate aminotransferase (AAT) superfamily. The highest sequence similarity of seq3 sequence was observed to be 71% with chloroplastic-like CBL of Glycine max. Interestingly, the blastp analysis of the deduced amino acid sequence of the seq3 ORF against non-redundant protein (nr) database showed that seq3 had 100% sequence similarity with a sequence for mimosinase from leucaena (accession AB298597.1). This mimosinase sequence in the nr database (direct submission by M. Fukuta, T. Moriwaki, S. Kedashiro, and S. Tawata) has not been experimentally established to be the sequence for mimosinase, and the enzyme activity for the encoded protein has not been demonstrated. Therefore, we decided to test the enzymatic activity of the protein encoded by the 1332-bp seq3 ORF.

**Codon optimization of the seq3 ORF for expression in E. coli**

In order to establish if the seq3 encodes mimosinase, it was necessary to express the 1332-bp ORF in E. coli, purify the recombinant protein and determine its enzyme activity. Hydrophobic regions, if present, usually make protein purification difficult by forming inclusion bodies (Fink,
1998). Signal peptides of proteins are hydrophobic and are not essential for in vitro protein activity. Based on the homology of seq3 with chloroplastic-like CBL, we expected that seq3 might also have a chloroplast signal peptide. Therefore, for efficient expression of the seq3-encoded protein in *E. coli*, we decided to eliminate any possible signal peptide sequence and optimize the sequence based on the *E. coli* codon preferences. The deduced amino acid sequence of the seq3 ORF was subjected to TargetP 1.1 server using plant networks. The TargetP 1.1 server predicted a 43 amino acid long chloroplast transit peptide with a reliability class (RC) value of 2 at the N-terminus of the 443-amino acid sequence (Table S1, Supplemental data). The low RC value indicates strong prediction of the transit peptide, suggesting that the encoded protein may be localized in the chloroplast. The 126-bp sequence for the predicted chloroplast transit peptide from the 5’-end of the ORF, excluding the start codon, was eliminated and the remaining 1206-bp sequence was analyzed for presence of rare codons of *E. coli*, and a synthetic derivative of the ORF was obtained by replacing the rare codons with commonly used codons of *E. coli* (Fig. S1, Supplemental data). In the 1206-bp synthetic ORF, a total of 258 out of 402 codons were changed by replacing 301 nucleotides to obtain synthetic seq3 (syn-seq3).

**syn-seq3 expression in *E. coli* and purification of the encoded enzyme**

Recombinant protein encoded by the syn-seq3 was obtained by expressing it in *E. coli* under the control of an inducible T7 promoter. An expression plasmid pET-seq3 was constructed by inserting the syn-seq3 into pET14-b vector downstream from the polyhistidine-tag and T7 promoter (Fig. 2a) and expressed in *E. coli*. SDS-PAGE analysis of total soluble proteins from the isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced culture showed a major band of size ~45 kDa, which was absent from the uninduced culture. The polyhistidine-tagged protein purified from the induced culture appeared as a single band with a molecular mass of ~45 kDa in SDS-PAGE (Fig. 2b). The induction experiment showed that the optimum induction time for expression of the 45-kDa protein in *E. coli* was 6 h. Therefore, for subsequent purification of this protein, the *E. coli* cultures were induced for 6 h. The purified recombinant protein from *E. coli* was used for enzymatic assays.
Mimosine degradation assay using the recombinant protein

To determine if the purified 45-kDa recombinant protein has mimosine-degrading (mimosinase) activity, it was used in an in vitro enzyme assay with mimosine as the substrate. In the high performance liquid chromatography (HPLC) analysis, mimosine and synthetic 3H4P, used as standards, showed retention times of 3.2 and 5.2 min, respectively (Fig. 3a). HPLC chromatograms of control reactions that contain heat- inactivated enzyme did not show any decrease in the amount of mimosine. However, the reactions with purified recombinant protein exhibited a sharp decrease in the amount of mimosine and the product formed had identical retention time as that of 3H4P (Fig. 3b). This result establishes that the seq3-encoded enzyme has mimosine-degrading activity and the major degradation product may be 3H4P, an isomer of 3,4DHP, which was previously identified as a product of mimosine degradation catalyzed by a C-N lyase from leucaena (Smith and Fowden, 1966). However, it remains to be established if seq3 encodes the C-N lyase found in leucaena by Smith and Fowden (1966) or it encodes another mimosine-degrading enzyme.

In the previous section it was shown that the amino acid sequence of seq3 belongs to the AAT superfamily. However, it does not mean that it is an aminotransferase because all enzymes in the AAT superfamily are not aminotransferases. The seq3-encoded enzyme is homologous to CBL, which is a lyase but not an aminotransferase, although it belongs to the AAT superfamily. To determine if the purified enzyme was a lyase or an aminotransferase, we performed the reactions in the presence and absence of α-ketoglutarate (α-KG) in the reaction buffer, which is used as a co-substrate in the reactions catalyzed by aminotransferases. Since aminotransferases and lyases are both PLP-dependent enzymes we conducted the experiments in the presence and absence of PLP in the reaction buffer. The reactions with the purified protein resulted in the significant decrease in mimosine irrespective of the presence or absence α-KG in the reaction buffer (Fig. 3b). Since α-KG is not essential as the co-substrate for the reaction, we concluded that the enzyme is not an aminotransferase. Therefore, it is likely that the seq3-encoded enzyme is a lyase. However, the seq3-encoded enzyme degraded mimosine even in the absence of exogenously added PLP; we did not observe any detectable difference in the products formed in reaction mixtures with or without PLP, which is an essential cofactor for lyases. This suggests that the recombinant enzyme from E. coli does not require exogenously added PLP. However, it
may also be possible that the recombinant enzyme carried PLP from the *E. coli* host cell. To test this possibility we performed the mimosine degradation reaction in the presence and absence of hydroxylamine, which is a potent inhibitor of PLP-dependent enzymes. Hydroxylamine is known to react with PLP in the enzyme’s active site, in a reversible single-step reaction forming spectrally distinct oxime adducts, which result in loss of the enzyme activity due to the removal of the bound PLP. Hydroxylamine was added to the reaction at a final concentration of 0.01-50 mM, which at a final concentration of 50 µM completely inhibited the catalytic activity of the recombinant enzyme. However, supplementation of the hydroxylamine-added reaction with 0.1 µM PLP successfully overcame the inhibitory effects of hydroxylamine by restoring the catalytic activity of the enzyme (Fig. 3b). This confirms that the seq3 encodes for a PLP-dependent mimosine degrading enzyme.

**Biochemical properties of seq3-encoded mimosine-degrading enzyme**

The catalytic activity of the purified mimosine-degrading enzyme was calculated as the concentration of product formed, [P], in 1 h. The highest catalytic activity of the enzyme was observed at 37 °C, suggesting that the optimum temperature for the enzyme is 37 °C (Fig. 4a). The enzyme catalytic activity sharply decreased at temperature 45 °C or higher and below 22 °C. The enzyme had high catalytic activities in a pH range from 7.5 to 10.0, with maximum activity at a pH 8.5 (Fig. 4b). To determine the thermal stability of the enzyme, the percent enzyme activity for different pre-incubation temperatures was measured with reference to the enzyme activity at the pre-incubation temperature of 4 °C. The enzyme activity at 37 °C pre-incubation temperature was found to be 86% of that of the reference, and it was reduced to 74.5% when the enzyme was pre-incubated at 40 °C (Fig. 4c). However, at pre-incubation temperatures of 50 °C or higher, a sharp reduction in the enzyme activity was observed. This indicates that the enzyme is fairly stable up to 40 °C.

Kinetic properties of the seq3-encoded enzyme were determined at the optimum temperature (37 °C) and pH (8.0). The rate of reaction for different substrate concentrations ranging from 0.05 to 0.50 mM was found to be linear from 0 to 2 min (data not shown). Initial velocities of the enzyme for each substrate concentration were calculated as the slope of product formed at 0 and 2 min. The purified recombinant enzyme followed typical Michaelis-Menten
kinetic model of single substrate reaction. The experimental determination of the apparent \( K_m \) and \( V_{\text{max}} \) values for the recombinant enzyme was calculated from the linear regression of the Lineweaver-Burk plot (Fig. 4d). The \( K_m \) and \( V_{\text{max}} \) for the enzyme were found to be \( 1.16 \times 10^{-4} \) M and \( 5.05 \times 10^{-5} \) mole\( \cdot \)s\(^{-1} \)\( \cdot \)mg\(^{-1} \), respectively. Assuming one active site per enzyme molecule, the total enzyme concentration, \([E_t]\), was calculated to be \( 0.22 \times 10^{-7} \) mole\( \cdot \)mg\(^{-1} \). Turnover number (\( K_{\text{cat}} \)) of the seq3-encoded enzyme was estimated to be 2300 s\(^{-1} \).

The aromatic amino acids L-tyrosine, L-tryptophan, and L-phenylalanine, which are the structural analogs of mimosine, were tested as possible competitive inhibitors of mimosine. The amount of product formed in reaction where only mimosine was added as the substrate (control reaction) was found to be similar to that of the test reactions where the mimosine was supplemented with the one-, two-, or three-fold concentrations of structural analogs in separate reactions (Fig. S2, Supplemental data), indicating that these aromatic amino acids are not competitive inhibitors of mimosine catalysis by the seq3-encoded enzyme.

14 Characterization of seq3-catalyzed mimosine degradation product

The HPLC chromatograms for synthetic 3H4P and for one of the mimosine degradation products formed in the reaction catalyzed by the recombinant enzyme were identical, indicating that the 3H4P is the major degradation product of mimosine in the reaction. To verify this, synthetic 3H4P and the HPLC-purified product of mimosine degradation were subjected to fragmentation through MS/MS of the parent ion (\( m/z \) 112.2). The resulting fragmentation patterns of synthetic 3H4P (reference sample) and mimosine-degradation product (test sample) were found to be identical, generating common internal structural fragments of \( m/z \) 94.0 (\([\text{M-H}_2\text{O}]^+\)), 65.9 (\([\text{M-H}_2\text{O-CO}]^+\)), 56.0, and 39.1 (Figs. 5a and 5b). This confirms that 3H4P is the major product of the mimosine degradation reaction catalyzed by the recombinant enzyme.

24 The seq3-encoded enzyme is a C-N lyase

3H4P as the major degradation product of mimosine accounts for only 8 carbons and 2 nitrogens of mimosine among 11 carbons and 3 nitrogens in mimosine. This suggests that there must be other products formed in the degradation reaction that could account for the remaining three carbons and one nitrogen. Previously, an enzymes from Mimosa pudica was reported to degrade...
mimosine into serine and pyruvate (Suda, 1960), whereas enzyme from leucaena seedling extract was found to degrade mimosine into 3,4DHP, pyruvate and ammonia (Smith and Fowden, 1966). Therefore, we tested the reaction products catalyzed by the seq3-encoded recombinant enzyme for the presence of serine, pyruvate and ammonia. Considering that serine is structurally similar to alanine, we also tested the reaction products for the presence of alanine. Neither serine nor alanine appeared in the HPLC chromatograms of reactions catalyzed by the recombinant enzyme. However pyruvate and ammonia were detected spectrophotometrically in the reaction products. The amount of pyruvate, ammonia, and 3H4P produced in the mimosine degradation reaction were found to be 87.7%, 91.2%, and 83.2%, respectively of mimosine, indicating that these products are formed in equimolar quantities as that of the substrate (Fig. 6). Based on these observations, the completely balanced reaction of mimosine degradation catalyzed by the seq3-encoded enzyme can be as follows:

\[
\text{(Mimosine)} + \text{H}_2\text{O} \rightarrow \text{(3H4P)} + \text{(Pyruvate)} + \text{(Ammonium)}
\]

Thus, like C-N lyases, the seq3-encoded enzyme is PLP-dependent and the reaction products contain pyruvate and ammonia. Therefore, we conclude that the seq3-encoded enzyme is a C-N lyase and under treaties and by convention, as described by the International Union of Biochemistry and Molecular Biology, mimosinase can be classified in the enzyme category with the Enzyme Commission number EC4.3.1.

The seq3-encoded C-N lyase has conserved active site residues of *Arabidopsis* cystathionine β-lyase

Mimosinase in blastp analysis with protein databank reference database showed 69% similarity with cystathionine β-lyase (CBL) from *Arabidopsis thaliana* (Fig. S3, Supplemental data). A previous study on the three-dimensional structure of *Arabidopsis* CBL identified the key active site residues of the enzyme (Breitinger et al., 2001). Most of the known active site residues of CBL are also present in the mimosinase (Fig S3, Supplemental data). The high degree of
sequence similarity between *Arabidopsis* CBL and leucaena mimosinase and their conserved active site residues suggest that mimosinase may have similar catalytic mode of action as described by Breitinger, Clausen et al. (2001) for CBL.

**High temperature results in increased mimosine degradation without affecting the level of mimosinase**

Previously, Tangendjaja et al. (1984) isolated mimosinase from leucaena leaves and determined the mimosine contents of intact leaves at different temperatures. They observed that mimosine content decreased with increasing temperature above 45 °C, and recorded the lowest amounts of mimosine after exposure of the leaves to 70 °C for 5-30 min. These authors did not consider mimosinase to be a chloroplast-localized enzyme, although they mentioned that some tissue damage is required for mimosine to be in contact with the enzyme. The decrease in mimosine content in the leucaena leaves with increasing temperature may happen due to two possibilities: (i) increased expression of mimosinase at higher temperature, or (ii) partial breakdown of chloroplast at higher temperatures resulting in release of mimosinase and consequent decrease in mimosine content. We have tested these ideas by conducting two experiments. In the first experiment, we did a western analysis of mimosinase in intact young leaves from leucaena seedlings grown at 25 °C and exposed to 70 °C for 10 min. As seen in Fig. 7a, the mimosinase content appeared to be similar at both temperatures, suggesting that the expression of mimosinase did not increase at high temperature. Therefore, we considered the second possibility, which was based on the previous report that lesion occurred in the chloroplast envelope membrane of different plant species at temperatures between 53°C and 57°C (McCain et al., 1989). We hypothesized that due to lesions occurring in chloroplast membranes at high temperature, mimosinase would leak out of the chloroplast and degrade mimosine in the cytosol. As a result, the mimosine content should decrease and the content of its degradation product 3H4P should increase. As expected, we found that the mimosine content in the heat exposed leaves decreased but the 3H4P content increased. The mimosine content in control and heat-treated leaf samples were 85±7.5 µM and 30±5.36 µM, respectively. No 3H4P was detected in control samples compared to 64±10.93 µM 3H4P detected in heat-treated leaf (Fig. 7b).

**Discussion**
In this study, we isolated a cDNA from *L. leucocephala* encoding a mimosine-degrading enzyme, and expressed the coding region of the cDNA in *E. coli* as a recombinant enzyme. We have also demonstrated that the purified enzyme converts mimosine into 3H4P and produces pyruvate and ammonia as byproducts in the reaction. Previous studies reported either 3,4DHP (Smith and Fowden, 1966), or 3H4P (Tangendjaja et al., 1986) as the major degradation product of mimosine by a mimosine-degrading enzyme from leucaena seedlings and leaves, respectively. Both 3,4DHP and 3H4P are isomers of each other, and based on the experimental conditions of the mimosine-degradation reaction, different researchers might have found different isomers of the same compound. Since the above experiments were done with enzymes isolated from different leucaena tissues, it is not clear if the enzymes were the same or different in the two experiments. It is possible that leucaena has more than one isoform of mimosinase that may express at different developmental stages. The low $K_m$ and high $V_{max}$ and estimated turnover number of the recombinant enzyme show that it is highly efficient in cleaving the alanyl side chain of mimosine and thereby converting it to 3H4P.

The optimum pH of the recombinant enzyme was 8.0, which is same as found for mimosine-degrading enzyme from leucaena seedling extract (Smith and Fowden, 1966) and from leucaena leaves (Tangendjaja et al., 1986). The optimum temperature for the catalytic activity of the recombinant enzyme was determined to be 37 °C; which is significantly different from the reported optimum temperature, 45 °C for mimosine-degrading enzyme from leucaena leaves (Tangendjaja et al., 1984; Tangendjaja et al., 1986). The mimosinase enzyme in our study may be different from the enzyme described by Tangendjaja et al. (1986). It is likely that leucaena has more than one isoform of mimosinase that may express in different tissues or under different physiological conditions. Like other enzymes catalyzing single substrate reactions, mimosinase also followed typical Michaelis-Menten kinetics. Our results show that the recombinant mimosine-degrading enzyme is a C-N lyase that breaks the C-N bond of mimosine to produce 3H4P. This finding supports the Smith and Fowden’s report (1966) in which they identified the mimosine-degrading enzyme from leucaena seedling extracts as a mimosine C-N lyase. We have shown that like other lyases, the recombinant mimosinase is PLP-dependent and is inhibited by the presence of hydroxylamine in the reaction, and the enzyme activity in hydroxylamine-containing reaction can be restored by exogenously added PLP to the reaction.
Recently, we have characterized another mimosine degrading enzyme, rhizomimosinase (Negi et al., 2013) from *Rhizobium* sp. strain TAL1145, a leucaena root-nodulating bacterial strain. The leucaena enzyme mimosinase and the *Rhizobium* enzyme rhizomimosinase both are C-N lyases and have similar catalytic functions, biochemical properties, and kinetic properties (Table 1). However, the deduced amino acid sequence of mimosinase (BAF80449) and rhizomimosinase (AAG47972) in pairwise global alignment using EMBOSS needle web server exhibited only 26.9% similarity and 16.7% identity (Fig. S4, Supplemental data). Additionally, in the phylogenetic analysis, mimosinase and rhizomimosinase appeared in different clades (Fig. 8). Therefore, mimosinase and rhizomimosinase represent a good example of enzymes from two different systems that have similar catalytic roles but show only little sequence similarity.

The recombinant enzyme was predicted to have a chloroplast signal peptide indicating that it may be a chloroplast-localized protein. Tangendjaja et al. (1984) reported a decrease in mimosine content with increasing temperature, with the lowest amount of mimosine recorded at 70 °C. However, it was not established if the decrease in mimosine content was because of overexpression of the enzyme at higher temperature. Our results show that the decrease in mimosine content and increase in its major degradation product 3H4P at 70 °C were not associated with overexpression of mimosinase and therefore, it is possible that exposure of intact leaf to 70 °C might have caused damage to the chloroplast membrane, resulting in release of mimosinase to the cytoplasm, where it degraded mimosine into 3H4P. Leucaena leaves contains ample amount of mimosine, comprising 3-5% of plant dry weight (Soedarjo and Borthakur, 1998).

Production of such a high amount of mimosine is a significant investment of ‘C’ and ‘N’ resources by the plant. We estimate that if the energy resources invested for production of mimosine were diverted for growth, the leucaena plant would have grown at least 20% larger. The forage yield of leucaena is reported to be 6-18 tons of dry matter per ha (National Academy of Sciences, 1984). For our calculations, we assumed the forage yield of leucaena as 10 tons per hac and the mimosine content as 3%. Therefore, 10 tons of dry leucaena forage should contain approximately 42 kg of N due to mimosine (MW of mimosine is 198 and it has 2 N). Assuming that most plants can utilize up to 200 kg of N per ha for maximum growth, and considering that N is the only limiting factor for growth, and other nutrients, water and environmental conditions
are optimal, the 42 kg of N from mimosine, if diverted to growth, should result in 21% (42/200 x 100) additional growth.

The role of mimosine in leucaena is not well established. It is likely that as a nitrogen-rich compound, mimosine plays a role in maintaining homeostasis in the growth of leucaena under stress conditions, such as high temperature and drought. Under such stress conditions, when nutrient availability becomes limited, the stored mimosine may be used as a source of ‘C’ and ‘N’ to produce amino acids and other secondary metabolites. Thus, mimosinase may function as the first enzyme in channeling mimosine into other biochemical pathways, including amino acid synthesis during stress conditions. The ammonia released in the reaction may be used to convert glutamate to glutamine by glutamine synthase; pyruvate may enter the TCA cycle, and 3H4P may be degraded further by other plant enzymes. Although, plant enzymes involved in conversion of 3H4P to smaller compounds have not been identified, a dioxygenase and a hydrolase, encoded by pydA and pydB genes, respectively, have been isolated and characterized from *Rhizobium* sp. strain TAL1145 that forms nitrogen-fixing nodules on leucaena (Awaya et al., 2005). It was shown that these enzymes convert 3H4P into ammonia, pyruvate and formate. It is likely that leucaena leaves may also have similar enzymes for conversion of 3H4P to useable compounds during stress conditions. To explain the possible role of mimosine and mimosinase in leucaena we have proposed a compartmentalization model according to which, under favorable growth conditions, leucaena synthesizes mimsoine and stores it in the cytoplasm. To protect the stored mimosine from untimely degradation by mimosinase, leucaena might have adapted the strategy of keeping mimosine and mimosinase apart from each other by compartmentalizing mimosinase in the chloroplast. Based on this hypothesis we propose that mimosinase and other downstream enzymes provide a mechanism for utilization of mimosine under stress conditions (Fig. 9). This hypothesis also explains one possible way how leucaena manages to grow well under drought stress condition when the availability of carbon and nitrogen is scarce.

**Materials and methods**

**Identification and isolation of leucaena cDNA that encodes a mimosine-degrading enzyme**

For identification of leucaena cDNA that encodes a mimosine-degrading enzyme, a set of 406 cDNA sequences obtained from iSSH library of leucaena (Negi et al., 2011) were screened for
their homology with C-N lyase using blastx tool and reference protein database of NCBI. The cDNA fragment of iSSH clone that exhibited similarity with C-N lyase was amplified to full-length cDNA by using FirstChoice® RLM-RACE kit (Ambion, TX, USA) according to the manufacturer’s instructions. The primers used for RLM-RACE, seq3-F and seq3-R are listed in Supplemental data (Table S2). The resultant full-length cDNA sequence was cloned in pGEMT-easy vector and sequence verified.

**Sequence analyses for prediction of signal peptide and presence of rare codons**

The deduced amino acid sequence from the ORF of full-length cDNA was analyzed for the presence of any N-terminal signal peptide including chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (SP) using TargetP 1.1 server (Emanuelsson et al., 2000). For enhanced expression of plant gene in *E. coli*, the full-length ORF was evaluated for the presence of *E. coli* rare codons based on its codon usage bias using GenScript Rare Codon Analysis Tool (GenScript, NJ, USA) and a synthetic ORF, codon optimized for enhanced expression in *E. coli*, was obtained from GenScript. The *BamHI* restriction site was introduced at both the ends of the synthetic gene to facilitate its cloning in the expression vector.

**Cloning and construction of seq3-expression plasmid**

The codon-optimized synthetic ORF and the expression vector pET-14b (Novagen, WI, USA) were digested with *BamHI* (Promega, WI, USA) followed by dephosphorylation of 5’ phosphate from the vector using shrimp alkaline phosphatase (Promega). *BamHI*-digested synthetic ORF was cloned into *BamHI* site of the expression vector pET-14b behind the T7 promoter, and introduced into *E. coli* JM109 (Promega). The resultant plasmid was verified by sequencing and termed as pET-seq3 (Table S2, Supplemental data).

**Synthetic ORF expression in *E. coli* and purification of the encoded protein**

The expression construct, pET-seq3 was transformed into *E. coli* strain BL21 (DE3) pLysS (Promega). The transformed cells were grown in LB and at mid-log phase (OD\(_{600}\) = 0.6) IPTG was added to a final concentration of 1 mM to induce the expression of the recombinant protein.
Optimum induction time for protein expression was determined by inducing the cells for 0-10 h. Samples (200 µl) from the induced cells were collected at 0, 1, 2, 4, 6, 8 and 10 h of induction. The sample collected at 0 h served as the negative control. The collected samples were centrifuged at 4000 rpm for 10 min at 4 °C and the pellet was resuspended in SDS-PAGE loading buffer followed by heating at 95 °C for 5 min to extract total protein. The recombinant protein was purified from the induced culture by using MagneHis™ Protein Purification System (Promega) according to the manufacturer’s instructions. Total soluble proteins of samples and purified recombinant protein were analyzed through SDS-PAGE. The purified recombinant protein was sent to GenScript (Piscataway, NJ, USA) for the development of polyclonal antibody in rabbit.

**Enzymatic assay**

The purified recombinant enzyme was used in *in vitro* mimosine degradation assay using mimosine as the substrate. For each 1000 µl reaction, 0.016 mg of the purified enzyme and 1 mM mimosine were used in 0.1 M Tris-HCl buffer with a final pH of 7.5, and the reaction was incubated at 37 °C for 1 h unless otherwise stated. To determine whether the recombinant enzyme is an aminotransferase, the mimosine degradation assay was performed in both the presence and the absence of 1 mM α-KG and 20 µM PLP in the reaction buffer. Each reaction was carried out in three replications and the reaction was assayed by HPLC using a C18 column (4.6 x 250 mm; Dionex acclaim 120). For quantitative estimation of the product formed, different concentrations of chemically synthesized 3-hydroxy-4-pyridone (synthetic HP) were used as standards. Synthetic HP was obtained from Dr. Behrman, Ohio State University, OH, who previously described an improved method for synthesis of 3-hydroxy-4-pyridone (Behrman, 2009). The peak area of known concentrations of synthetic HP was used to plot the standard curve, which was then used to quantify the amount of product formed from the peak area of test samples. An isocratic solvent system of 0.02 M o-phosphoric acid with a flow rate of 1 mL/min, and an UV detection photodiode array (200 to 400 nm) was used for HPLC analysis. The enzyme was also tested for PLP dependence in enzymatic assays performed by supplementing 0.01-50 mM hydroxylamine in the reaction buffer containing enzyme but no substrate. The reaction mixture was incubated for 5 min before adding the substrate. In a separate assay, the reaction mixture that contained the least concentration of hydroxylamine required to inhibit the
enzyme was supplemented with 0.1-20 µM PLP after 5 min of incubation. The reaction mixture was again incubated for 5 min followed by the addition of the substrate. Each reaction was analyzed using HPLC as described above.

Characterization of the mimosine-degrading recombinant enzyme

The optimum temperature for the catalytic activity of the recombinant enzyme was determined by performing mimosine degradation assay at different temperatures including 4, 22, 30, 37, 45, 55, and 65 °C, but the pH of the reaction buffer in all the reactions was constant, pH 7.5. Similarly, for determining the pH optima, the reactions were set at different pHs ranging from pH 3 to pH 12 and incubated at a constant temperature of 37 °C. Enzymatic reactions were terminated by heating the reaction mixtures at 100 °C for 3 min. The subsequent assays were conducted at optimum temperature (37 °C) and optimum pH (8.0). The thermal stability of the recombinant enzyme was studied by pre-incubating the enzyme with reaction buffer lacking substrate at different temperatures (4, 37, 40, 50, 55, 60, 65, and 70 °C) for 30 min, followed by adding the substrate and incubating at 37 °C for 1 h. Mimosine degradation product in each enzymatic reaction was quantified by HPLC as mentioned in the previous section.

The enzyme was also tested for any possible competitive inhibition with aromatic amino acids including, L-tyrosine, L-tryptophan, and L-phenylalanine, which are structural analogs of mimosine. For this, the mixture of each amino acid with mimosine in the ratio of 1:1, 2:1, and 3:1 was used as the substrate in mimosine degradation assay. The reaction containing only mimosine as the substrate was used as the control. The product formed in each reaction was quantitated using HPLC.

Kinetic studies of the mimosine-degrading recombinant enzyme

The enzymatic assays to study kinetic properties of mimosinase were performed at optimum temperature and pH. To determine initial velocity, the rate of reaction for 0.05, 0.10, 0.15, 0.25, 0.35, 0.50 mM of mimosine was calculated at time points of 0, 2, 3, 5, 10, 20 and 30 min. The rate of reaction was linear between 0 and 2 min, therefore, the initial velocities of the recombinant enzyme-catalyzed reaction was calculated for different substrate concentrations as the slopes formed by the amount of product formed at 0 and 2 min of reaction time.
Lineweaver-Burk plot was plotted using the calculated values of initial velocities and substrate concentrations and the kinetic parameters including $V_{\text{max}}$ and $K_m$ for the encoded enzyme were determined from linear regression of the plot. The mole of enzyme active sites per mg of the enzyme ([Et]) was calculated assuming that there is only one active site per enzyme molecule and the turnover number ($K_{\text{cat}}$) of the enzyme was estimated using $V_{\text{max}}$ and [Et].

**Characterization of the mimosine degradation products**

The primary product of the mimosine degradation reaction catalyzed by the recombinant enzyme was characterized by MS/MS of the purified degradation product and the reference compound used was synthetic 3H4P. The product was purified via HPLC from the enzyme-catalyzed reaction. The HPLC-purified sample and synthetic 3H4P were freeze-dried and individually dissolved in carrier solvent (50/50% v/v of a 0.09% v/v aq. Formic acid and 90/10% v/v CH$_3$CN/0.09% v/v aq. Formic acid). The samples were delivered to the atmospheric pressure ionization (API) source of the AB/MDS-Sciex ESI-MS API 3000 triple quadrupole mass spectrometer (Ontario, Canada) using a continuous flow of 5 to 10 µL/min provided by a micro-syringe infusion pump (Harvard Apparatus, MA, USA). The ESI-MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve <5-ppm mass accuracy, as per manufacturer’s protocol before delivering the samples.

MS/MS of test and reference samples were performed with N$_2$ bombardment confined to quadrupole-2 (Q-2) with a collision cell gas thickness of 3x10$^{14}$ atoms.cm$^{-2}$ and a collision energy (Q-0 to Q-2 rod offset voltage) typically set at ~20 to 40 eV. The resulting MS/MS (daughter ion) spectra were obtained by scanning quadrupole-3 (Q-3) from $m/\zeta$ 10 to 150 in 0.6 s with a step size of 0.1 Da. ESI-MS data analysis was assisted with the use of Mac BioSpec v1.01 (PE Sciex, Ontario, Canada).

To identify the degradation products other than 3H4P in the mimosine degradation reaction so that all C and N of mimosine are accounted for, the reaction products were tested for the presence of alanine, serine, pyruvate, and ammonia. For alanine and serine detection, the reaction products were derivatized using o-phthalaldehyde (OPA) and β-mercaptoethanol. In a basic environment (pH 9 to 11) and in the presence of β-mercaptoethanol, OPA reacts with primary amines to form a fluorescent isoindole derivative. The reaction buffer used in previous
mimosine degradation assays contained 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), which is a primary amine that may also react with OPA. Therefore, we used 40 mM sodium phosphate as the buffer for enzymatic assay in this case. The reaction was set at optimum temperature and optimum pH for 1 h using 1 mM mimosine as the substrate. The reaction products in 40 mM sodium phosphate buffer were first analyzed for the presence of 3H4P using a C18 column as described in the previous section to verify if the sodium phosphate buffer does not interfere with the mimosine degradation reaction. 1 mM each of alanine and serine were prepared in the 40 mM sodium phosphate buffer for use as controls. The pH of the reactions and controls was adjusted to 10 before performing the OPA derivatization. The reactions and controls were applied to pre-column derivatization for 5 min at room temperature using OPA at a final concentration of 5 mM. The derivatized samples were then analyzed through HPLC using Kinetex C18 column (2.6 μ, 100 x 4.6 mm) in a gradient system in which mobile phase consists of 40 mM potassium phosphate, pH 7.8 (buffer A) and 50/50 methanol/acetonitrile (buffer B). The flow rate was 1.5 ml/min and the run time was 20 min with 3 to 60% buffer B. The sample detection was performed at 338 nm using UV detection photodiode array (200 to 400 nm). Pyruvate and ammonia in the reaction were detected by using a pyruvate assay kit (Biovision, CA, USA) and an ammonia assay kit (Sigma-Aldrich, MO, USA), respectively, according to the manufacturers’ instructions. In order to analyze mass balance between products and reactants, the reaction was performed to achieve the catalysis of the substrate to near completion by using excess of the enzyme (0.2 mg) and lower substrate concentration (500 µM mimosine) in 40 mM phosphate buffer. The reaction was performed at optimum temperature and optimum pH with a reaction time of 1 h and pyruvate, ammonia, and 3H4P were quantified using the line equation from respective standard curve.

**Prediction of conserved residues of the recombinant enzyme**

The deduced amino acid sequence of full-length seq3 ORF was used in blastp search against protein databank database in NCBI. The resulted sequence with highest similarity was aligned with the deduced amino acid sequence of seq3-encoded protein sequence using the ClustalW multiple sequence alignment program and the conserved residues were analyzed.

**Phylogenetic analysis**
The cystathione beta-lyase sequences from various plant species, which showed the highest similarities with the mimosinase sequence (without signal peptide) in the BlastP analysis were used for phylogenetic analysis. The top 15 sequences (excluding hypothetical proteins) and Rhizomimosinase (AAG47972), which showed 26.9% similarities and 16.7% identities with mimosinase (BAF80449) were included for the phylogenetic tree construction. The accession numbers of cystathionine $\beta$-lyases from various plant species are as follows: Solanum lycopersicum (XP_004249212), Theobroma cacao (EOY09022), Cucumis sativus (XP_004166746), Vitis vinifera (XP_002274313), Populus trichocarpa (ERP51507), Ricinus communis (XP_002512818), Glycine max (XP_003554134), Setaria italica (XP_004964713), Cicer arietinum (XP_004514725), Sorghum bicolor (XP_002436582), Zea mays (NP_001148100), Brachypodium distachyon (XP_003561024), Fragaria vesca (XP_004294046), Oryza sativa (BAA95830), and Aegilops tauschii (EMT05183). The signal peptides from these sequences were predicted using TargetP 1.1 server and removed prior to analysis. The web based phylogeny server http://www.phylogeny.fr/version2_cgi/advanced.cgi was used to construct phylogenetic tree and the statistical analysis for branch support was performed using the ‘approximate likelihood-ratio test’ (aLRT).

**Plant growth and heat-stress treatment**

*Leucaena leucocephala* cv. K-636 seeds were obtained from University of Hawaii research station, Waimanalo, Honolulu. Surface sterilization followed by scarification of mature seeds were done as described previously (Pal et al., 2012). The seeds were germinated on half-strength Murashige and Skoog (MS) medium for 2-3 days under sterile conditions at 28 °C in dark, and the resulting germinated seedlings were transferred to vermiculite. The seedlings were watered with Hoagland solution twice a week and were grown for eight weeks. The plants were maintained at 25±2 °C with 16/8 h dark photoperiod with an irradiance of 30 µmol s$^{-1}$m$^{-2}$. For heat stress treatment, the eight-week-old plants were incubated at 70°C for 10 min before collecting the intact leaf tissues, whereas for control the intact leaf tissues were directly collected. To avoid any enzyme catalyzed degradation of mimosine after collecting the intact leaf samples, the samples (0.3 g) from control and treatment were immediately homogenized in 3 ml of 0.1 N HCl mortar and pestle. The homogenized samples were centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatants
were filtered using 0.45 μm-pore-size filter (Corning Incorporated, New York, NY) and the filtrates were used for determination of mimosine concentration by HPLC as described in previous section of ‘Material and method’.

4 Western Blot Analysis

The effect of heat (70 °C) condition on the expression of mimosinase compared to control was studied using Western blot analysis using rabbit polyclonal antibody developed against purified recombinant mimosinase (rabbit anti-mimosinase). The rabbit anti-mimosinase antibody was used in 1:5000 dilution and the Western blotting was performed as described previously (Pal et al., 2012). For loading control, the PVDF blots were stripped off using ReBlot plus strong antibody stripping solution (Millipore, Billerica, MA, USA) and were blotted using mouse monoclonal anti-actin (plant) antibody (Sigma, St. Louis, MO, USA) as the primary antibody at 1:1000 dilution, and HRP-conjugated goat anti-mouse IgG antibody (Bio-Rad) at 1:3000 dilution as the secondary antibody to detect and quantify actin.

14 Acknowledgments

We are thankful to E. J. Behrman (Department of Chemistry and Biochemistry, Ohio State University) for generously providing us the synthetic 3H4P. We thank Nguyen Hue and Paul Singleton for useful discussions. VSN was supported by IFP fellowship from the Ford Foundation for three years.
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Table

Table 1: Comparison of rhizobial ‘rhizomimosinase’ and leucana ‘mimosinase’

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<th>Features</th>
<th>Rhizomimosinase</th>
<th>Mimosinase</th>
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<tr>
<td>Amino acid residues</td>
<td>406</td>
<td>400 (excluding 43 amino acid residues for predicted chloroplast signal peptide)</td>
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<td>Size</td>
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<td>PLP-dependence</td>
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<td>Yes</td>
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<td>Degrades mimosine into</td>
<td>3H4P, ammonia, and pyruvate</td>
<td>3H4P, ammonia, and pyruvate</td>
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<tr>
<td>Homology shown in blastp analysis</td>
<td>58% positive residues with cystathionine beta-lyase from <em>Bacillus bataviensis</em> (WP_007083900) in blast</td>
<td>70% positive residues with cystathionine beta-lyase, chloroplastic-like <em>Solanum lycopersicum</em> (XP_004249212) in blastp analysis</td>
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Figure legends

**Figure 1:** Chemical structures of (a) mimosine, (b) 3-hydroxy-4-pyridone, (c) 3,4-dihydroxypyridine, (d) pyruvate and (e) ammonium.

**Figure 2:** Cloning and expression of seq3-encoding protein in *E. coli*. (a) The plasmid for seq3 expression was constructed by cloning the codon-optimized synthetic seq3 ORF lacking coding region for chloroplast signal peptide, at the BamHI restriction site of pET-14b vector in the sense orientation. (b) SDS-PAGE of recombinant protein expressed in *E. coli*. Lane 1-7, total soluble protein from *E. coli* containing pET-seq3 induced for 0, 1, 2, 4, 6, 8 and 10 h, respectively; lane 8, purified recombinant protein from the 6 h induced *E. coli* containing pET-seq3.

**Figure 3:** HPLC chromatograms of standards and test samples in mimosine degradation assays. (a) The chromatograms represent mimosine and 3H4P that were used as standards. The chromatograms of mimosine and 3H4P had a retention time 3.2 and 5.2 min, respectively. (b) HPLC chromatograms of mimosine degradation assays: (1) reaction in which heat-inactivated recombinant enzyme was added exhibited a single peak of unused substrate, mimosine; (2) reaction catalyzed by functionally active recombinant enzyme in the absence of exogenously added α-KG and PLP exhibited a large peak of mimosine degradation product with the same retention time as that of 3H4P; (3) reaction catalyzed by functionally active recombinant enzyme in the presence of 50 μM hydroxylamine had only one peak of unutilized substrate; (4) functionally active recombinant enzyme catalyzed reaction in the presence of inhibitor, 50 μM hydroxylamine, restored the enzyme activity when supplemented with 0.1 μM PLP and showed large peak of mimosine degradation product with the same retention time as that of 3H4P.

**Figure 4:** Biochemical and kinetic properties of mimosine degrading enzyme. Catalytic activity of the enzyme was determined as the product formed [P] at (a) different temperatures; (b) different pHs; and (c) different pre-incubation temperatures. (d) Estimation of kinetic parameters of the enzyme obtained by plotting Lineweaver-Burk plot of initial velocities at different substrate concentrations.
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Figure 8: Cladogram of mimosinase and rhizomimosinase with cystathionine β-lyases from various plants. The web based phylogeny server www.phylogeny.fr/version2_cgi/advanced.cgi was used to construct phylogenetic tree and the statistical analysis for branch support was performed using the ‘approximate likelihood-ratio test’ (aLRT). The accession numbers of the sequence used are mentioned in the ‘Materials and methods’ section.

Figure 9: Compartmentalization model showing the possible role of mimosine and mimosinase in dealing with nutrient limiting stress conditions such as drought. (a) Under normal condition of growth, mimosine is synthesized and stored in the cell cytoplasm. The enzyme mimosinase is localized in the chloroplast and thus mimosine is inaccessible to mimosinase. (b) However, stress conditions may cause damage to the chloroplast membrane (1), resulting in the release of mimosinase from the chloroplast to the cytoplasm (2), where the substrate mimosine is accessible to mimosinase, which catalyzes first step of mimosine catabolism into 3H4P, pyruvate and ammonia (3). These degradation products of mimosine are rich in C and N and hence mimosine may serve as a source of stored C and N at the time of need.
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