Why does Anatabine, But not Nicotine, Accumulate in Jasmonate-Elicited Cultured Tobacco BY-2 Cells?

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Suspension-cultured cells of Nicotiana tabacum cv. Bright Yellow-2 (BY-2) grow rapidly in a highly homogenous population and still exhibit the general behavior of plant cells, and thus are often used as model systems in several areas of plant molecular and cellular biology, including secondary metabolism. While the parental tobacco variety synthesizes nicotine as a major alkaloid, the cultured tobacco cells mainly produce a related alkaloid anatabine, instead of nicotine, when elicited with jasmonates. We report here that cultured BY-2 cells scarcely express N-methylputrescine oxidase (MPO) genes even after jasmonate elicitation. MPO is the second enzyme in the biosynthetic pathway that supplies the pyrrolidine moiety of nicotine and nornicotine, but is predicted to be dispensable for the biosynthesis of anatabine, anabasine and anatalline, which do not contain the pyrrolidine moiety. When MPO was overexpressed in tobacco BY-2 cells, nicotine synthesis was dramatically enhanced while anatabine formation was effectively suppressed. As a complementary approach, we suppressed MPO expression by RNA interference in tobacco hairy roots that normally accumulate anatabine. In the MPO-suppressed roots, the contents of anatabine, anabasine and anatalline, as well as N-methylputrescine and putrescine, markedly increased to compensate for suppressed formation of nicotine and nornicotine. These results identify the transcriptional regulation of MPO as a critical rate-limiting step that restricts nicotine formation in cultured tobacco BY-2 cells.

Keywords: Alkaloids — Anatabine — Nicotine — N-methylputrescine — Tobacco.

Abbreviations: CaMV, cauliflower mosaic virus; MPO, N-methylputrescine oxidase; PCA, perchloric acid; PMT, putrescine N-methyltransferase; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; QPT, quinolinate phosphoribosyltransferase.

Introduction

The tobacco cell line BY-2 was originally induced from the pith of Nicotiana tabacum L. cv. Bright Yellow No. 2 by a Japanese researcher at Hatano Tobacco Experimental Station in 1968, and was distributed to many plant laboratories worldwide for basic research (Nagata et al. 1992, Nagata et al. 2004). Tobacco BY-2 cells grow very rapidly in culture; the typical doubling time is about 13 h. In cell suspension cultures, BY-2 cells are well separated from each other, and at most only form small aggregates of a few cells. The cell cycle of BY-2 cells can be synchronized easily and efficiently. Because of their high homogeneity and rapid growth, tobacco BY-2 cells have been used as model systems for higher plants, which are comparable with HeLa cells in human research, and are especially useful for studies of cell division, cytoskeletons, plant hormone signaling, intracellular trafficking, organelle differentiation, and primary and secondary metabolism.

Tobacco BY-2 cells retain their responses to exogenous application of fungal elicitors and jasmonates. Elicitation of BY-2 cells with methyl jasmonate induces large transcriptomic changes and initiates de novo synthesis of phenylpropanoids and alkaloids (Imanishi et al. 1998, Goossens et al. 2003, Gális et al. 2006). These jasmonate-inducible secondary metabolites are thought to be involved in chemical defenses of tobacco plants (Steppuhn et al. 2004). Interestingly, jasmonate-elicited BY-2 cells accumulate mainly anatabine, an analog of nicotine; nicotine and other tobacco alkaloids are produced in relatively minor amounts (Goossens et al. 2003). Since the original Bright Yellow variety of tobacco accumulates predominantly nicotine (Häkkinen et al. 2004), there should be one or more steps deficient in the biosynthetic pathway of nicotine in otherwise jasmonate-competent BY-2 cells.

The pyrrolidine moiety of nicotine is derived from a symmetric diamine putrescine (Fig. 1). Putrescine is first N-methylated by putrescine N-methyltransferase (PMT), and the product N-methylputrescine is then oxidatively deaminated by N-methylputrescine oxidase (MPO) to 4-aminobutanal, which spontaneously cyclizes to give N-methylpyrrolinium cation. MPO belongs to a subclass of diamine oxidases that require copper and topaquinone for activity (Heim et al. 2007, Katoh et al. 2007). The N-methylpyrrolinium cation is then coupled with a hypothetical nicotinic acid-derived intermediate to form nicotine (Leete 1983, Hashimoto and Yamada 1994).

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Nicotinic acid is formed in the salvage pathway of nicotinamide adenine dinucleotide (NAD), which is derived from aspartic acid in plants (Katoh et al. 2006). Quinolinate phosphoribosyltransferase (QPT) connects the aspartate-derived pathway to the pyridine nucleotide cycle (Sinclair et al. 2000). The structural genes for nicotine biosynthesis are generally under the control of the specific regulatory loci, NIC1 and NIC2 (Hibi et al. 1994, Reed and Jelesko 2004, Cane et al. 2005, Heim et al. 2007, Katoh et al. 2007; our unpublished results).

Other tobacco alkaloids are heterocyclic compounds composed of one nicotinic acid-derived pyridyl ring and one or two 5- or 6-membered N-containing ring(s) (Fig. 1). A main route to nornicotine is direct N-demethylation of nicotine, which is catalyzed by a particular subclass of P450 monooxygenases (Siminszky et al. 2005, Gavilano and Siminszky 2007). Feeding of labeled nicotinic acid to N. tabacum and N. glutinosa plants suggested that both rings of anatabine are derived from the dimerization of a metabolite of nicotinic acid (Leete and Slattery 1976). Anabasine, a major alkaloid in N. glauca, is mainly formed from lysine via a symmetric diamine cadaverine, as suggested by feeding studies (Watson et al. 1990, and references therein). Although recombinant tobacco MPOs show a weak but measurable activity that converts cadaverine to 5-aminopentanal (Heim et al. 2007, Katoh et al. 2007), it is not known whether MPO is involved in the anabasine biosynthesis. The biosynthetic route of anatalline, which contains two pyridyl rings and a central saturated heterocyclic ring, is still unresolved.

A very high ratio of anatabine to nicotine in jasmonate-treated BY-2 cells indicates that one or more step(s) in the pyrrolidine moiety formation of nicotine is blocked. Indeed, when PMT expression was down-regulated in tobacco hairy roots and regenerated plants, anatabine accumulated to high levels while nicotine levels decreased (Chintapakorn and Hamill 2003). In this study, we found that MPO, but not PMT, is responsible for the high anatabine to nicotine ratio in jasmonate-elicited BY-2 cells. Except for the identified misregulation of MPO genes, tobacco BY-2 cells apparently induce all other enzyme genes necessary for nicotine biosynthesis in response to methyl jasmonate treatment, and are useful for metabolic studies of tobacco alkaloids.

![Fig. 1](https://academic.oup.com/pcp/article-abstract/49/8/1209/1826181)  
Fig. 1  Biosynthesis of tobacco alkaloids. Nicotine and nornicotine are synthesized by condensation of the 1-methyl-Δ1-pyrrolinium cation and a nicotinic acid-derived precursor, whereas anabasine and possibly anatalline are derived from nicotinic acid only. Lysine-derived Δ1-piperidine is incorporated into the piperidine moiety of anabasine. PMT and MPO catalyze the initial two reactions leading to formation of the 1-methyl-Δ1-pyrrolinium cation from putrescine. QPT is involved in the synthesis of NAD, which is converted to nicotinic acid in the pyridine nucleotide cycle. MPO, N-methylputrescine oxidase; PMT, putrescine N-methyltransferase; QPT, quinolinate phosphoribosyltransferase. Broken lines indicate more than one reaction or undefined reactions.
Results

MPO is hardly expressed in anatabine-accumulating tobacco BY-2 cells

We first compared alkaloid profiles of tobacco BY-2 cells, which had been treated with methyl jasmonate at 100 μM for 3 d, with those of tobacco hairy roots that were derived from the cultivar SR1 (Fig. 2A). Methyl jasmonate treatment does not significantly change the alkaloid composition in tobacco hairy roots (Shoji et al. 2008). In this study, tobacco hairy roots were untreated with methyl jasmonate. The total amounts of tobacco alkaloids were comparable in two culture systems; the alkaloid levels far exceeded 1 mg g⁻¹ DW of cells. In tobacco hairy roots, nicotine was the major alkaloid (~69% of total alkaloids), followed by nornicotine (27%), anabasine (3%), anatabine (1%) and anatalline (0.1%). In contrast, jasmonate-treated BY-2 cells produced mostly anatabine (83%), with low levels of accumulation of nicotine (5%), anabasine (4%) and anatalline (8%). Nornicotine was not detected in our culture conditions. These alkaloid compositions were similar to and confirm those reported for tobacco hairy roots (e.g. Shoji et al. 2000) and for jasmonate-treated BY-2 cells (e.g. Goossens et al. 2003).

Since the tobacco BY-2 cell line was selected for its high growth rate and has been subcultured for several decades, it was of interest to determine whether the high anatabine to nicotine ratio is also found in other tobacco cell lines. We elicited with methyl jasmonate two other tobacco cell lines derived from cultivars Samsum NN and Xanthi NC under the same conditions. However, only trace amounts of tobacco alkaloids (~0.004 mg g⁻¹ DW of cells; mainly consisting of nicotine) were detected in these cultured tobacco cells (data not shown). Apparently, tobacco BY-2 cells are particularly responsive to jasmonate treatment, and are capable of metabolic reprogramming to synthesize tobacco alkaloids.

Next, expression levels of three structural genes for nicotine biosynthesis (MPO, PMT and QPT) were analyzed by RNA gel blots (Fig. 2B). PMT and QPT were expressed in high and comparable levels in hairy roots and jasmonate-treated BY-2 cells. In contrast, whereas considerable MPO expression was detectable in hairy roots, we could not detect significant accumulation of MPO mRNA in the elicited BY-2 cells. In addition, we found that MPO transcript levels were reduced by only 34% in nic1nic2 mutant roots, compared with wild-type roots, whereas the transcript levels of PMT and QPT were severely reduced by 88 and 66%, respectively, in the mutant roots (Fig. 2C). These results prompted us to explore whether MPO expression may be a critical factor that causes the high anatabine to nicotine ratio in jasmonate-treated BY-2 cells.

Overexpression of MPO restores nicotine formation in tobacco BY-2 cells

If very low expression of MPO is the primary cause for the failure to synthesize nicotine efficiently in tobacco BY-2 cells, overexpression of MPO should boost nicotine formation at the expense of anatabine accumulation. To test this possibility, tobacco MPO1 cDNA was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in tobacco BY-2 cells. By screening more than a dozen transgenic cell lines, we found three independent lines (OX4, OX6 and OX14) that showed highly increased MPO mRNA accumulation when examined in the jasmonate-elicited condition (Fig. 3A). Cultured tobacco

![Fig. 2](https://academic.oup.com/pcp/article-abstract/49/8/1209/1826181/fig2jssox.png?width=800&height=800&cell=link&anchor=fig2)  
**Fig. 2** Profiles of tobacco alkaloids and gene expression. Tobacco BY-2 cells were treated with 100 μM methyl jasmonate for 3 d for alkaloid analysis (A) or for 1 d for RNA gel blot analysis (B). Tobacco hairy roots of the cultivar SR1 (A, B) and the roots of wild-type and the nic1nic2 mutant tobacco plants of the cultivar Burley 21 (C) were not treated with methyl jasmonate. (A) Alkaloid contents in tobacco BY-2 cells (black bars) and tobacco hairy roots (white bars). Data are means ± SD of more than four replications. (B, C) RNA gel blot analyses.
cells of these cell lines also showed high enzymatic activity of MPO, in contrast to the negligible MPO activity in the control wild-type cells (Fig. 3B).

Alkaloids in tobacco BY-2 cells were analyzed after the treatment with methyl jasmonate. The three MPO-overexpressing cells accumulated nicotine at the level of 1.5–2.5 mg/g DW of cells, which was about a 14- to 24-fold increase compared with the wild-type BY-2 cells (Fig. 3C). Small amounts of nornicotine, which is undetectable in wild-type cells, were found in the OX4 and OX14 cell lines. In the MPO-overexpressing lines, the cellular levels of anatabine, anabasine and anatalline decreased by ≥50%, compared with the wild-type cells. Therefore, tobacco alkaloids containing the pyrrolidine ring (nicotine and nornicotine) increased while non-pyrrolidine alkaloids decreased by enhanced MPO activity, and the alkaloid profiles of MPO-overexpressing BY-2 cells resembled those of tobacco hairy roots (see Fig. 2A).

**Down-regulation of MPO in tobacco hairy roots**

As a complementary approach, we used an RNA interference (RNAi) technology to down-regulate the MPO genes in tobacco hairy roots. The tobacco genome contains two MPO genes, MPO1 and MPO2 (Katoh et al. 2007). Analysis of MPO transcripts by reverse transcription–PCR (RT–PCR), followed by discriminative digestion with restriction enzymes, showed that MPO1 was expressed much more strongly than MPO2 in tobacco hairy roots (Supplementary Fig. S1). Therefore, we used an MPO1 cDNA fragment to suppress expression of both MPO1 and MPO2. After screening 11 independent hairy root lines, we identified three lines (R10, R11 and R39) in which the transcript levels of MPO genes were significantly decreased (Fig. 4A). Individual RT–PCR amplification of MPO1 and MPO2 transcripts by using specific primer sets showed that expression of both MPO genes was effectively suppressed. In contrast, expression levels of two other non-targeted nicotine biosynthetic genes (PMT and QPT) and of tubulin genes were indistinguishable among wild-type hairy roots, a vector-transformed line and MPO RNAi lines. Consistent with the reduced RNA level, the MPO enzyme activities were significantly lower in these three lines than those in the control roots (Fig. 4B).

We analyzed alkaloid contents in the MPO down-regulated lines. In all three lines, the contents of nicotine and nornicotine markedly decreased to 1–7 and 3–7%, respectively, of the controls, while the respective contents of anatabine, anabasine and anatalline increased 5- to 10-fold, 1.5- to 6-fold and 5- to 7-fold compared with the controls, respectively (Fig. 4C). Accordingly, anatabine now constituted the major alkaloid (80% of total alkaloids) accumulating in the hairy roots, and the alkaloid profiles in the MPO-suppressed hairy roots became similar to those of BY-2 cells (see Fig. 2A).
We extended our metabolic analysis to cellular polyamines that are related to the MPO reaction (Fig. 1). Polyamines were first separated into two fractions based on their solubility in perchloric acid (PCA), and then polyamines conjugated to phenylpropanoids (Gális et al. 2006) and other compounds were analyzed after acid hydrolysis, along with non-conjugated free polyamines (Fig. 5). In tobacco hairy roots, diamines (putrescine and N-methylputrescine) were more abundant than higher polyamines (spermidine and spermine), and the conjugated forms constituted considerable proportions of total polyamines. In the MPO-suppressed lines R11 and R39, the contents of free and conjugated forms of diamines increased; the increase was higher in N-methylputrescine (2.4- to 3.8-fold of the control) than putrescine (1.4- to 1.9-fold). In contrast, the contents of spermidine and spermine were not markedly affected. Overall, the metabolic impact of suppressed MPO activity in tobacco hairy roots was restricted to its substrate and, to a lesser extent, to the immediate precursors, among the compounds tested.

**Discussion**

*MPO is the rate-limiting enzyme for nicotine biosynthesis in tobacco BY-2 cells*

A high anatabine to nicotine ratio in elicited tobacco BY-2 cells indicates that one or more steps in the subpathway that supplies the pyrrolidine moiety of nicotine is blocked. PMT and MPO are two known enzymes that are specifically involved in this subpathway. Indeed, when either *PMT* (Chintapakorn and Hamill 2003) or *MPO* (this study) was suppressed in hairy roots or whole plants of tobacco, anatabine formation greatly increased while nicotine formation was reduced to very low levels. These studies suggest that when the cellular supply of 1-methyl-Δ1-pyrrollinium cation is limited, an activated derivative of nicotinic acid condenses with another molecule of the nicotinate metabolite to form anatabine. Anatabine contents vary from 2 to ≥20% of the total alkaloids in the roots of *Nicotiana* species (Saito et al. 1985, Sisson and Severson 1990). The natural variation of anatabine might be caused...
when gene expression or the catalytic activity of PMT or MPO is uncoordinated with other enzymes in the nicotine biosynthetic pathway.

Jasmonate-elicted BY-2 cells expressed very low levels of MPO transcripts, compared with tobacco hairy roots, whereas PMT was expressed strongly in both culture systems. Accumulation of N-methylputrescine in the elicited BY-2 cells (Goossens et al. 2003) is consistent with a much lower MPO enzymatic activity than the PMT activity. In this study, we demonstrated that overexpression of MPO is sufficient to restore efficient formation of nicotine to compensate for suppressed formation of anatabine. Therefore, in jasmonate-treated BY-2 cells, MPO is the major rate-limiting enzyme for biosynthesis of the pyrrolidine moiety of nicotine, and the expression level of MPO determines the ratio of pyrrolidine-type and other tobacco alkaloids.

The piperidine ring of anabasine is derived from lysine by way of cadaverine (Watson et al. 1990). Cadaverine is converted to 5-aminobutanal by a diamine oxidase (Walton et al. 1988), followed by a spontaneous cyclization to form Δ1-piperidine (Fig. 1). The diamine oxidase MPO prefers N-methylated diamines for substrates and is much less active toward non-N-methylated amines, such as cadaverine (Heim et al. 2007, Katoh et al. 2007). In our transgenic studies with overexpression and suppression of MPO, anabasine contents changed in concert with anatabine contents and in an inverse way to nicotine contents, offering in vivo evidence that MPO is not involved in the biosynthesis of anabasine.

Regulation of MPO expression

Structural genes encoding biosynthetic enzymes of a particular secondary metabolic pathway are often coordinately regulated (Koes et al. 2005, Saito et al. 2007). In nicotine biosynthesis, tobacco genes for PMT, QPT, and A622 oxidoreductase are preferentially expressed in the root, up-regulated by jasmonate treatment or wounding, and are under the control of NIC regulatory loci (Hibi et al. 1994, Shoji et al. 2000, Shoji et al. 2002, Goossens et al. 2003, Reed and Jelesko 2004, Sinclair et al. 2004, Cane et al. 2005; our unpublished results). Although MPO is regulated similarly by these factors (Heim et al. 2007, Katoh et al. 2007), the Nic loci regulate MPO expression less tightly than expression of PMT and QPT (Fig. 2C). The enzymatic activity of MPO is not reduced as much in the roots of nic mutants, compared with the PMT activity (Saunders and Bush 1979). Apparently MPO expression is not strictly coordinated with expression of other structural genes for nicotine biosynthesis. Poor jasmonate-induced activation of MPO in cultured BY-2 cells might be mechanistically linked to its loose control by the NIC regulatory genes. Promoter analysis of tobacco MPO genes may provide further information on their unique control patterns.

Materials and Methods

Plant materials and transformation

Binary vectors were introduced into Agrobacterium rhizogenes strain ATCC15834 and A. tumefaciens strain EHA105 by electroporation. Sterile plants of the N. tabacum cv. Petit Havana line SR1 were grown on half-strength B5 medium (Gambarog et al. 1968) containing 1.5% sucrose and 0.3% gellan gum under continuous illumination at 26°C. To generate the transgenic hairy roots, leaf discs were co-inoculated with A. rhizogenes harboring pBI-MPO-R1 as described (Kaneke et al. 1994). After selection and disinfection on solid MS medium containing 50 mg l⁻¹ kanamicin and 250 mg l⁻¹ carbenicillin, the hairy roots were subcultured in liquid MS medium (Murashige and Skoog 1962) with 3% sucrose every 2 weeks.

Tobacco BY-2 cells (N. tabacum cv. Bright Yellow-2) were subcultured in liquid MS medium supplemented with 20 mg l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MES and 0.2 mg l⁻¹ 2,4-D, every week. Transgenic tobacco BY-2 cells were generated with A. tumefaciens strain EHA105 with the pBI-MPO as described (An 1985). After 4-day-old BY-2 cells were first rinsed with and transferred to fresh auxin-free MS medium with methyl jasmonate at 100 μM, they were cultured for an additional 1 d for the RNA and protein analyses, or for 3 d for alkaloid measurement.

Tobacco cell lines derived from cv. Samsam NN and Xanthi NC were cultured in liquid MS medium with 0.2 mg l⁻¹ 2,4-D, and liquid B5 medium with 0.22 mg l⁻¹ 2,4-D and 8.6 μg l⁻¹ kinetin, respectively. One-week-old cells were treated with 100 μM methyl jasmonate for 3 d in the auxin- and cytokine-free media.
Plasmid construction

For RNAi experiments, a portion of MPO1 cDNA (+1,530 to +2,050 bp) was amplified by PCR, subcloned into pHANNIBAL (Wesley et al. 2001), and then inserted into pBI121 (Clontech, Mountain View, CA, USA) to provide pBI-MPO-RI. For MPO overexpression, the full-length MPO1 ORF was amplified from MPO1 cDNA with a pair of appropriate primers and placed between the BamHI and SalI sites of pBI121. High-fidelity KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used for all PCR amplifications and the PCR products were sequenced to verify that no unintentional mutations were introduced.

RT-PCR and RNA gel blot analyses

Total RNA was isolated by using an RNeasy Kit (Qiagen) from plant tissues frozen by liquid nitrogen, and first-strand cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Aliquots of the first-strand cDNA were amplified by PCR with appropriate primers (both MPO1 and MPO2, 5’-CCTTGGACCTTTATCTGTCG-3’ and 5’-GGTCTTGCATATCCATTTCATTTG-3’; MPO1, 5’-CAGCTTCTTCTTAGTAAGC-3’ and 5’-CTCTCTGTCTGAAATACAAAGTG-3’; MPO2, 5’-TGAAGTGTGCTTGTTGTCG-3’ and 5’-AAAGCTGTACCTGTCCAAACTG-3’; PMT, 5’-CAGCTTCTTCTCAGCCAAACGG-3’ and 5’-GCTTGTTGTCATACAACTCC-3’; OPT, 5’-TCATCTGACAGTGACTCCT-3’ and 5’-TCTATGGGTCCGCCACACCT-3’; α-TUBLIN, 5’-AGTGGAGGAGGTGAGTGTAGTATG-3’ and 5’-TATGGTTGGCTGACTGAATGGTC-3’). To avoid saturated amplification, template amounts and cycle numbers were adjusted in individual experiments.

Total RNA (10 μg) was separated by electrophoresis on a 1.2% formaldehyde agarose gel, and transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Equal loading was confirmed by ethidium bromide staining. The blots were hybridized in 50% (v/v) formamide, 5% ssPE and 0.1% (w/v) SDS at 65 °C for 2 h and dissolved in 100 μl aliquot was mixed with 100 μl of saturated sodium carbonate and 400 μl of 7.5% (w/v) dansylchloride acetate solution, and kept at 60 °C for 1 h. To the reaction mixture, 100 μl of 10% (w/v) proline was added and incubated at room temperature for 30 min in the dark. The sample was extracted with 500 μl of toluene and dried at 37 °C for 20 min in an evaporator. The dried residues were dissolved in acetonitrile and analyzed by HPLC (LC-10 system; Shimadzu) equipped with a YMC-Pack ODC-AM column (YMC, Kyoto, Japan). Dansylated polyamines were eluted from the column with a programmed solvent gradient of 2% phosphate triethylamine buffer (pH 5.2); acetonitrile (v/v) from 60 to 90% in 30 min at a flow rate of 1.0 ml min⁻¹ at 40 °C, and were detected at the excitation wavelength of 365 nm and the emission wavelength of 510 nm.

Enzyme assay

Fresh tissues were homogenized in 200 mM potassium phosphate buffer (pH 7.4) by using a Polytron (Kinematica, Lucerne, Switzerland). After the homogenate was centrifuged at 15,000 r.p.m. at 4 °C, the cleared supernatant was desalted by passing it through a NAP-5 gel filtration column (Amersham Pharmacia Biotech). Protein concentration was determined by a Coomassie Protein Assay Reagent Kit (Pierce). The enzyme reaction was carried out in 200 mM potassium phosphate buffer (pH 7.4) containing 50 mM N-methylputrescine, 24 kU l⁻¹ glutamate dehydrogenase from beef liver (Oriental Yeast, Tokyo, Japan), 5.7 mM 2-oxoglutarate and 0.19 mM NADH, at 30 °C. Ammonia formed by the MPO reaction was measured enzymatically with glutamate dehydrogenase by monitoring the decrease in NADH at 339 nm (Kusche and Lorenz 1983).

Supplementary data

Supplementary data mentioned in the article is available at Plant and Cell Physiology online.

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References


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