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THE SPECIFICITY OF METHIONINE SULFOXIMINE AND AZASERINE INHIBITION IN PLANT TISSUES

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Abstract—According to the current paradigm, an inhibition of nitrate and ammonium assimilation by methionine sulfoximine (MSX) and azaserine indicates the importance of glutamine synthetase (GS) and glutamate synthase (GOGAT) in the assimilation of ammonia and the production of glutamate. Inhibitors of GS and GOGAT also result in altered levels of asparagine and glutamine. These amides have been shown to inhibit the induction of nitrate uptake and its reduction to ammonium ions. In the current experiments, the expected reduction in glutamine levels were found when we used MSX; with azaserine, however, levels of both amides were higher. In roots, MSX additions resulted in enhanced levels of nitrate reductase (NR) as expected. In shoot tissues, on the other hand, MSX additions resulted in an inhibition of the NADH dependent NR and a seven-fold enhancement of the NAD(P)H dependent NR. With azaserine there was a severe inhibition of NR as would be predicted with an increase in the levels of either of the amides. Thus although MSX and azaserine do have direct effects respectively on GS and GOGAT, there are other fundamental effects on the assimilation of nitrate caused by 1) altered levels of nitrogen metabolites, and 2) effect of the inhibitors themselves at other sites in the nitrate assimilation pathway.

INTRODUCTION

In maize seedlings, asparagine and glutamine inhibit the induction of nitrate reductase (NR). The accumulation of these amides in seedling tissues is most apparent 3 days after imbibition, a time when (NR) is maximally repressed. By 5 days post imbibition, the level of both amides is markedly reduced and there is a good induction of NR [1]. The amides could be supplied by protein turnover or by *de novo* synthesis. In order to determine whether endogenous levels of glutamine or asparagine were responsible for the repression of NR at day 3, we decided to use two inhibitors which might be expected to reduce the bio-synthesis of either glutamine or asparagine. We chose a time of 5 days post imbibition for our experiments as at this time there is a maximal induction of NR.

Methionine sulfoximine (MSX) is a potent inhibitor of glutamine synthetase (GS) and azaserine of glutamate synthase (GOGAT). These two enzymes are implicated in the proper functioning of the photorespiratory cycle [2–4]. In other systems, GS and GOGAT also appear to mediate the assimilation of ammonium ions and the net synthesis of glutamate [5]. Thus when MSX is added to plant systems, glutamine levels should decline and glutamate levels should rise (see Fig. 1, reaction 3) where glutamine synthesis is involved and where GS is the catalyst active in the synthesis of glutamine. With azaserine, one would predict a rise in glutamine and a fall in glutamate where GOGAT is the active catalyst (Figure 1; reaction 4). Where glutamine is the nitrogen-donor in the synthesis of asparagine [6, 7], one would also expect an inhibition of asparagine synthetase (AS) by azaserine. In this case, the inhibitor should lead to an accumulation of glutamine and aspartic acid and a decline in levels of asparagine (Figure 1; reaction 4 in brackets). These predictions are only valid where there is an actual *de novo* synthesis of the amides.

Recently results from several laboratories [1, 8–11] indicate that asparagine or glutamine could have a negative effect on the nitrate uptake system or on the reduction of nitrate itself. In this communication we have examined the influence of these two inhibitors on levels of glutamine, asparagine and on nitrate

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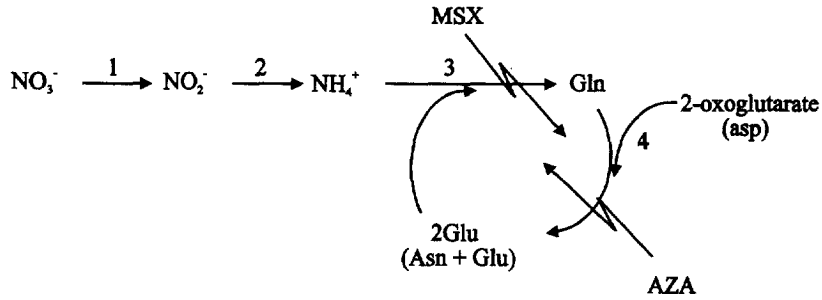

Fig. 1. Specificity of inhibitors in the nitrate assimilation pathway in plants. 1. nitrate reductase; 2. nitrite reductase; 3. Glutamine synthetase; 4. glutamate synthase or asparagine synthetase.

reductase activities (NRA) in root and shoot tissues of *Zea mays* in order to determine whether the endogenous levels of glutamine and asparagine influence the induction potential of NR.

RESULTS

Effect of MSX and azaserine on amide levels and NRA in root and shoot tissues

Since either asparagine or glutamine could result in the inhibitions of NO_3^- uptake or nitrate reductase activity (NRA), we used the metabolic inhibitors MSX and azaserine in an attempt to perturb the endogenous levels of either glutamine or asparagine. We predicted that glutamine levels should be reduced with addition of MSX and that as a result we should see an increase in NRA. With azaserine we expected to see an increase in glutamine and a decrease in asparagine Fig. 1. We hoped this would help to distinguish the importance of the amides in the regulation of NR.

To our surprise root and shoot tissues responded differently to the additions of the inhibitors (Table 1). Azaserine resulted in an increase in both amides in the shoot and root tissue, and to an inhibition in the induction of NR. Root tissues were more responsive to this treatment. Root tissue also had, as expected, less glutamate after this treatment. With MSX there was a modest decline in levels of glutamine in root tissues, and as expected with an inhibition of GS, a significant enhancement in levels of both NADH- and NAD(P)H bispecific NRA. In the leaves there was an inhibition in the induction of the NADH-NR but a 7-fold higher level of the NAD(P)H bispecific NRA.

The results demonstrate, as would be predicted, that the induction of NR in root and shoot tissues is severely inhibited in the presence of azaserine. In agreement with earlier results [12], we found that MSX results in an enhanced induction of both forms of NR in maize roots as would be expected, but contrary to expectations there was an inhibition of the induction of the NADH-NR in leaves. In addition, in our experiments, there was a dramatic increase of the NAD(P)H-bispecific NR in the leaf tissues with the MSX treatment, an observation that suggests that we had triggered the derepression of this isoform of NR.

DISCUSSION

Additions of MSX and azaserine which interfere respectively with GS and GOGAT activities resulted

Table 1. Effect of azaserine and methionine sulfoximine on amide levels and NR activity in maize root and shoot tissues

Treatment	ASP	GLU	ASN	GLN	NR activity (NRA) $\mu mol \cdot gfw^{-1} h^{-1}$ NADH	NADPH
		$\mu mol \cdot gfw^{-1}$				
SHOOT						
Control	1.34	2.12	0.67	Trace	8.41	1.10
Aza	1.72	3.03	5.45	0.90	0.44	0.01
MSX	2.10	2.61	1.82	Trace	5.60	7.13
Aza + MSX	0.26	0.98	0.66	Trace	1.83	1.14
ROOT						
Control	0.66	1.15	0.64	0.44	5.59	4.66
Aza	0.07	0.39	3.93	7.47	0.14	0.20
MSX	0.85	2.02	0.77	0.38	9.38	5.01
Aza + MSX	1.06	1.30	2.62	Trace	0.06	0.50[1]

Details are as described in the Experimental section. The experiment was repeated twice and the trends were similar.

in the expected alterations in the levels of the amides. However, there were two unique and unexpected responses:

1. An increase in asparagine levels in both root and shoot tissue in response to azaserine. This observation suggests that there is an alternative route for the synthesis of asparagine in both tissues. In 1975, Rognes [7] established that there was a glutamine dependent asparagine synthetase in soybean cotyledons. Work in our laboratory presented confirmatory evidence with regards to soybean cotyledons [13], and recent research from Coruzzi's laboratory also indicates the importance of a glutamine dependent AS [6]. However, our results with root AS, quite clearly demonstrated that the AS from this source could use either ammonium ions or glutamine [13]. Our interpretation of the current observations is that azaserine somehow derepresses the synthesis of an alternative AS, possibly an ammonium-dependent AS which could be activated when the glutamine-dependent AS is inhibited.
2. With MSX there was, in roots, the expected enhancement of NR activity, but in leaves a minor inhibition. Since in previous experiments we did not see this effect at the level of transcription [12], there appears to be a post-transcriptional event that is sensitive to MSX. More important however, is the striking increase in the NAD(P)H-bispecific NR activity. Causes of the derepression/activation of the second NR in leaf tissue remain to be examined.

Our results clearly show that there are secondary effects with both azaserine and MSX treatments that have potentially profound influences on nitrate and ammonium assimilation in higher plants. They also indicate that a *de novo* synthesis of the amides is involved in seedlings 5 days post imbibition.

EXPERIMENTAL

Seed germination and plant growth

Maize kernels (*Zea mays* cv. Pioneer hybrid no 3475) were soaked for 5–6 h in a modified 1/10 strength Hoagland's solution that contained no combined nitrogen, and then were allowed to germinate on paper towels soaked in the same Hoagland's solution. After 48 h in the dark at 28°C, the seedlings were transferred to a hydroponic "dry" system as described by Sivasankar and Oaks [10]. The seedlings were grown in growth chambers maintained at 28/26°C day/night temperatures, with a 16 h light/8 h dark cycle and a light intensity of 225 μmol $m^{-2} s^{-1}$ at canopy level. Control plants received nitrate (5 mM) added five days after imbibition and two hours into the light period. When MSX (0.5 mM) and azaserine (0.5 mM) were used they were added to the medium at the same time as the nitrate. Plants were harvested 20 h after the addition of nitrate. Shoots above the second node and roots below the first node were harvested, frozen in liquid nitrogen, ground to a fine powder, and stored at −70°C. A minimum of 15 plant parts were harvested per sample, and the experiment was repeated twice. Tissues were extracted the next day for determinations of enzyme activity or within one week for the amide determinations.

Tissue amide levels

Amides in tissue extracts were separated and quantified by HPLC (Model 421 chromatograph, Beckman). The amides and amino acids were derivatized with *o*-phthaldialdehyde (OPA) before injection into the column (reverse phase Ultrasphere, ODS, column with 5 μm particles, Beckman) and were measured by fluorescence with a fluorometer manufactured by Gilson (Middletown, WI) as described previously [1].

Assay for nitrate reductase

Tissue samples were extracted for NRA and activities were determined as described previously [10].

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