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Deamination role of inducible glutamate dehydrogenase isoenzyme 7 in *Brassica napus* leaf protoplasts

Masami Watanabe [a,*], Ohnishi Yumi [a], Yasuhiro Itoh [a], Kaori Yasuda [a], Kazunari Kamachi [b], R. George Ratcliffe [c]

[a] Laboratory of Plant Nutrition, Faculty of Horticulture, Chiba University, 648 Matsudo, Chiba 271-8510, Japan
[b] Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashimurayama, Tokyo 208-0011, Japan
[c] Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

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ABSTRACT

Glutamate dehydrogenase (GDH) is a ubiquitous enzyme that catalyzes the reversible amination of 2-oxoglutarate to glutamate. In *Brassica napus*, GDH isoenzymes 1 and 7 are hexamers of β and α subunits, respectively and the isoenzyme profile in leaves is known to change on wounding. Here, parallels were sought between the effects of wounding and protoplast isolation because of the possible relevance of changes in GDH activity to the perturbed metabolism in recalcitrant *B. napus* protoplasts. When leaf protoplasts of *B. napus* were isolated, GDH7 isoforms predominated. Transcription of *GDH2*, which encodes the GDH α subunit, was activated and translation of the *GDH2* mRNA was also activated to synthesize α subunit polypeptides. When detached leaves absorbed either acidic 5 mM jasmonic acid or salicylic acid solutions via petioles, GDH7 isoenzymes were activated and the GDH isoenzyme patterns were similar to those of protoplasts. Salicylic acid β-glycosides were generated soon after treatment with the pectinase–cellulase enzyme solution and peaked at 1 h. NMR spectroscopic analysis of protoplasts and unstressed leaves incubated with 5 mM $^{15}NH_4Cl$ showed that the change in GDH isoenzyme profile had no effect on ammonium assimilation. Protoplast isolation changed the redox state with NAD(P)H and oxidized glutathione levels increasing, and ascorbate, dehydroascorbate, NAD(P) and glutathione decreasing. ATP content in protoplasts declined to 2.6% of that in leaves, while that in wounded leaves increased by two-fold. It is concluded that GDH7 does not support net amination *in vivo* and it is suggested that the increase in GDH7 activity is a response to oxidative stress during protoplast isolation.

1. Introduction

Glutamate dehydrogenase (GDH; EC 1.4.1.2) catalyzes the amination of 2-oxoglutarate with ammonium and the deamination of glutamate. The hexameric enzyme can form seven isoforms via the random association of two subunits, α and β, and plants typically exhibit complex isoenzyme profiles (Cammaerts and Jacobs, 1985; Itagaki et al., 1988; Loulakakis and Roubelakis-Angelakis, 1990; Osuji et al., 2003). While it is clear that assimilation of ammonium ion is largely due to operation of the glutamine synthetase (GS; EC 6.3.1.2)/glutamate synthase (GOGAT; EC 1.4.7.1) cycle (Bernard and Habash, 2009; Miflin and Habash, 2002), with GDH supporting deamination of glutamate (Lea and Miflin, 2003; Aubert et al., 2001; Fox et al., 1995; Robinson et al., 1991, 1992), analysis of the role of GDH is complicated by the possibility that different isoenzymes function in different directions *in vivo*. Moreover, since the isoenzyme pattern is sensitive to a wide range of abiotic factors the possibility arises of GDH functioning in different ways under different conditions.

Mechanical slicing of leaves, and isolation of leaf protoplasts, from *Brassica napus* altered the GDH isoenzyme profile and induced synthesis of the GDH7 isoenzyme, leading to an increase in GDH-specific activity (Watanabe et al., 1998, 1994). Native PAGE and tetrazolium staining showed that the higher specific activity of NAD–GDH (deamination) in leaves correlated with the most cationic isoenzyme (GDH1), and that the higher specific activity of NADH–GDH (amination) in leaf protoplasts correlated with the most anionic isoenzyme (GDH7) (Watanabe et al., 1992). This study suggested that GDH isoenzymes can have different anabolic and catabolic functions, with GDH1 playing a catabolic role in non-stressed leaf tissue, and GDH7 functioning anabolically in leaf protoplasts or wounded leaves (Loulakakis and Roubelakis-Angelakis, 1990; Watanabe et al., 1992).

More recently treatment of tobacco (*Nicotiana tabacum*) BY-2 suspension cells with sodium chloride generated reactive oxygen species (ROS), increased intracellular ammonium and induced the transcription of the *gdh-NAD:A1* gene encoding the α subunit

* Corresponding author. Tel./fax: +81 47 308 8820.
E-mail address: masamiwata@faculty.chiba-u.jp (M. Watanabe).

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of GDH (Skopelitis et al., 2006). This led to an increase in the level of the α subunit, a shift towards the anionic polypeptides in the isoenzyme profile, and increased aminating activity measured using GC/MS and ^{15}N labeling in the presence of inhibitors of the GS/GOGAT pathway. It was concluded that the induced anionic GDH assimilated excess ammonia, producing glutamate for proline synthesis (Skopelitis et al., 2006). However, the aminating role for the anionic polypeptides was not borne out in later work since it was shown that α_6 (isoenzyme 7, GDH7) actually had a high deaminating activity and only a low aminating activity in tobacco *in vivo* under normal growth conditions (Skopelitis et al., 2007).

Evidence is also accumulating that cationic isoenzymes of GDH function catabolically under normal growth conditions. For example, when tobacco plants were transformed with either antisense or sense copies of the gene encoding the β subunit, the changes in the level of the β subunit had little effect on leaf ammonium or free amino acid content, implying that the cationic isoenzymes are not important for ammonium assimilation (Purnell and Botella, 2007).

Thus it seems increasingly likely that deamination is the predominant role for all the isoenzymes of GDH, in agreement with the small differences in the kinetic properties of the purified α_6 and β_6 isoenzymes (Loulakakis and Roubelakis-Angelakis, 1996; Watanabe et al., 1999). Purification of GDH1 (β_6) and GDH7 (α_6) from *B. napus* leaf tissue, and characterization of their kinetic properties showed only minor differences in the apparent Michaelis constants (K_m) of GDH1 and GDH7 for ammonium, 2-oxoglutarate, NADH, NAD, and glutamate (Watanabe et al., 1999).

An earlier paper addressed the mechanism by which wounding caused changes in the GDH isoenzyme profile in *B. napus* leaves (Watanabe et al., 2007). Wounding released oligosaccharides (OGs) and then jasmonic acid (JA) from leaf tissue, and changes in the GDH isoenzyme profile could be induced by OGs, JA, salicylic acid (SA), and ethylene. Wounding stress activated transcription of the *GDH2* gene, but repressed the translation of α subunit polypeptides. Despite this, the activity of the α_6 isoform increased as a result of the reduction of disulfide bonds in the α subunits.

Wounding is inevitable during protoplast isolation and it is possible that changes in the GDH isoenzyme profile contribute to the complex metabolic modifications that occur during the isolation and culture of protoplasts. Earlier studies of protoplasts from *Petunia hybrida* and *B. napus* leaves have highlighted differences in amino acid metabolism that may ultimately be relevant to the difficulty of initiating cell division in *B. napus* protoplasts (Watanabe et al., 1998, 2002b). Here, it is shown that protoplast preparation activates both transcription of the *GDH2* gene and translation of the α subunit polypeptides. However, although this resulted in a strong increase in the activity of the α_6 isoform, ^{15}N NMR spectra showed that inhibiting ammonium assimilation by GS had exactly the same effect in unstressed leaves and protoplasts, providing further evidence that the α_6 isoform does not support amination *in vivo*. In contrast deamination *in vivo* would generate NADH for the mitochondrial electron transport chain, and it is argued that this may be a response to ROS-induced damage to enzymes of the tricarboxylic acid (TCA) cycle arising from oxidative stress during the preparation of the protoplasts.

2. Results

2.1. Changes in GDH isoenzyme profiles and GDH2 gene expression

The GDH isoenzyme profile was examined in control leaves, wounded leaves and protoplasts using native-PAGE (Fig. 1). Wounding induced formation of GDH5–7, and GDH7 was the dominant isoenzyme in protoplasts (Fig. 1a). In parallel, based on the deaminating activity and the subunit composition of the isoenzymes, the proportion of the α subunit increased from 13% in non-stressed leaves, to 28% in wounded leaves, and 92% in protoplasts.

The GDH α and β subunits were separated using 2D electrophoresis and visualized by Western blotting (Fig. 1b). On this basis the percentage level of the α subunit was 78% in non-stressed leaves, 76% in wounded leaves and 98% in protoplasts.

The expression level of *GDH2* mRNA was measured using semi-quantitative RT–PCR (Fig. 2). The amount of *GDH2* mRNA was four times higher in wounded leaves than in control leaves, and 10 times higher in protoplasts.

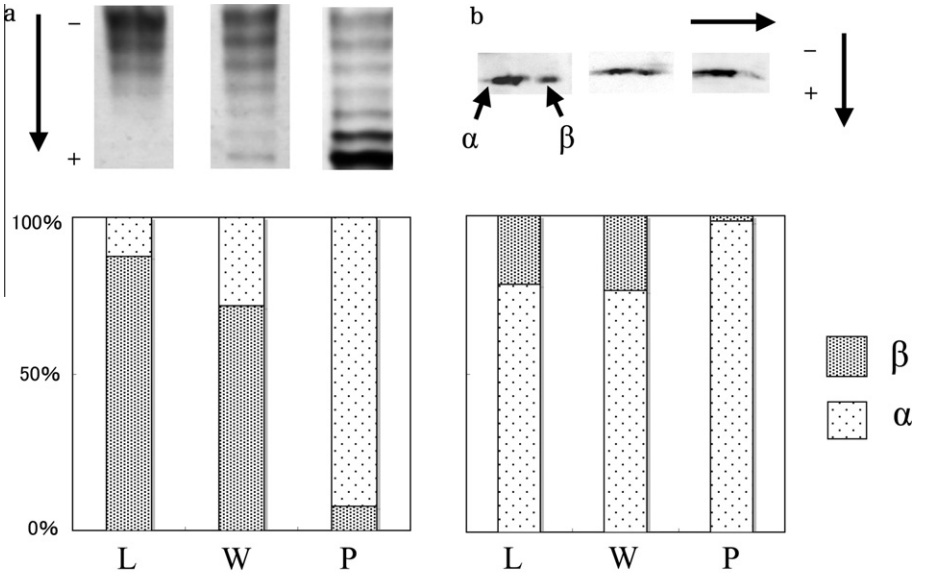

Fig. 1. GDH activity, and the levels of the α and β subunits of GDH, in leaves (L), wounded leaves (W) and leaf protoplasts (P) of *B. napus*. (a) GDH isoforms were separated by native PAGE and the deaminating activity of GDH was visualized using a tetrazolium assay. (b) GDH α and β subunits were separated using 2D electrophoresis and detected using anti-GDH antibody. Lumina Vision software (Mitani Corp., Chiba, Japan) was used for the densitometric analysis.