FUNCTIONAL GENOMICS OF GABA METABOLISM IN YEAST

THERMOTOLERANCE

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FUNCTIONAL GENOMICS OF GABA METABOLISM IN YEAST THERMOTOLERANCE

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FUNCTIONAL GENOMICS OF GABA METABOLISM IN YEAST

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Juxiang Cao, daughter of Zhenbin, Cao and Yinxiu, Xiao, was born in Septerber 18, 1972 in Hubei, China. She graduated from number 1 high school in Gong'an, Hubei, China in 1992. In July of 1992, she entered Hubei Agricultural University and graduated in 1995 with a Bachelor's degree in the department of Plant Protection. She worked in industry for three years and in September 1998, she entered the department of Plant Pathology, college of natural resources and environmental sciences of South China Agricultural University and received her Master's degree in July 2001. In January of 2002, she joined the department of Biological Sciences to pursue her PhD degree in biochemistry and molecular biology. She is married to Shaoqiang, Dong in 2003.

DISSERTATION ABSTRACT

FUNCTIONAL GENOMICS OF GABA METABOLISM IN YEAST THERMOTOLERANCE

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γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid which accumulates rapidly in response to diverse environmental stresses. The GABA shunt is a pathway involving three enzymes, glutamate decarboxylase (GAD, encoded by *GAD1*), GABA aminotransferase (GABA-T, encoded by *UGA1*), and succinate semialdehyde dehydrogenase (SSADH, encoded by *UGA2*). These three enzymes acting in concert convert glutamate to succinate. GABA specific permease (encoded by *UGA4*) mediates the transportation of GABA into cells. The GABA specific transcription factor (encoded by *UGA3*) regulates the expression of GABA genes (*UGA1, UGA2* and *UGA4*). We have constructed deletion mutants of each of these genes in yeast and have found that

mutants of GAD, GABA-T and SSADH are more susceptible to stress induced by lethal temperature (45°C) than wild type yeast cells. Additionally, set of the combinations of double and triple mutants were examined. With a pretreatment at 40°C (a non-lethal temperature) for 30 min, the mutants retained susceptibility to stress at 50°C compared to the wild type. The levels of accumulated ROS were correlated to the susceptibility of heat stress. In addition, in the *uga1* and *uga2* mutants, GABA and α-ketoglutarate accumulated markedly higher compared to the wild-type, while glutamate accumulated at higher levels in *gad1* mutant. Deletion mutations of *UGA3* and *UGA4* grown showed heat tolerant to 45ºC with overexpression of antioxidant genes superoxide dismutase compared to the wild-type. However, *Δuga3* mutant strain grown in minimal-GABA medium showed heat sensitive phenotype while *Δuga4* maintained heat tolerance. RT-PCR analysis showed that the expression of all GABA shunt genes and *UGA3* and *UGA4* genes were GABA inducible and were also up-regulated by lethal heat at 45ºC. In addition, acidic pH in the growth medium induced the expression of *UGA1*, *UGA2* and *UGA4* but not that of *GAD1* and *UGA3*. Under heat stress, deletion of *UGA3* suppressed the expression of *UGA1* and *UGA4* but not on *GAD1* and *UGA2*, while deletion of *UGA4* did not affect the expression of all GABA shunt genes and *UGA3*. Additionally, the antioxidant genes superoxide dismutase (encoded by *SOD1* and *SOD2*) were found to be gradually induced by heat in the wild-type strain but overexpressed in the *Δuga3* and *Δuga4* mutant strains.

Bioinformatic programme "TargetP and pSORT' predicts that GABA transaminase from *Arabidopsis* is localized in mitochondria with a 54 nucleotide mitochondrial transit peptide sequence, yeast GABA transaminase is localized in cytosol. We constructed vectors expressing ScGABA-TKG and AtGABA-TP in both yeast cytosol and

mitochondria to complement yeast GABA transaminase mutant *Δuga1* and succinate semialdehyde dehydrogenase mutant *Δuga2* phenotypes: GABA growth defect, thermo sensitivity and heat induced production of reactive oxygen species (ROS). Our studies revealed that plant AtGABA-TP is functionally interchangeable with yeast ScGABA-TKG for GABA growth, thermotolerance and limiting production of ROS whether located in mitochondria or cytosol in yeast. However, yeast GABA-TKG, whether located in mitochondria or in the cytosol, displayed much stronger effect.

The yeast succinic semialdehyde dehydrogenase gene (SSADH; EC 1.2.1.16) was cloned and overexpressed in *E. coli*., and kinetically characterized. It has a molecular mass of subunit around 54 kDa, the purified enzyme has a tetramer molecular mass of 200 kDa. The recombinant protein was highly specific for succinate semialdehyde, can use both NAD^+ and $NADP^+$ as a cofactor but with higher affinity to NAD^+. The enzyme activity can be inhibited by substrate SSA, product NADH and adenine nucleotides AMP, ADP and ATP.

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I. LITERATURE REVIEW

GABA is a ubiquitous non protein amino acid which is widely found from prokaryotic to eukaryotic organisms. It was first discovered by Steward *et al*. (1949). in potato tubers . Later on, it was found in rat brain and many parts of vertebrates, invertebrates, plants, bacteria, and fungi (Santosh *et al*., 1997). Interest in GABA shifted to animals when it was found in large quantity in brain. It is well known that GABA functions as a negative neurotransmitter in animals (Erdo and Kiss, 1986). In plants, GABA accumulates in response to various biotic and abiotic stresses including cold shock, heat shock, mechanical damage, and water stress (Streeter and Thompson, 1972; Wallace *et al*., 1984; Reggiani *et al*., 1988; Craw ford *et al*., 1994). However, in most organisms, the role of GABA is not well known.

GABA is produced in the cytosol through the decarboxylation of L-glutamate by glutamate decarboxylase (GAD). GABA is then transaminated to α-ketoglutarate or pyruvate by GABA transaminase (GABA-T) producing glutamate or alanine and succinate semialdehyde (SSA), which is an irreversible reaction. Finally, SSA is converted to succinate (SUCC), a metabolite of the tricarboxylic acid cycle (TCA cycle) by succinate semialdehyde dehydrogenase (SSADH). These three enzymes (GAD, GABA-T and SSADH) work in concert to convert glutamate to succinate, and along with glutamate dehydrogenase or several α-ketoglutarate-dependent aminotransferases to

bypass 2 enzymes in the TCA cycle (Appendix). Therefore, these reactions constitute the GABA shunt.

In plants, Bouché, N. *et al*. (2003) demonstrated that the disfunction of SSADH leads to hyper sensitivity to environmental stresses, and accumulation of higher levels of reactive oxygen species (ROS). In addition, in yeast, Coleman *et al*. (2001) found that the GABA shunt is involved in oxidative stress. However, the role of the GABA shunt in response to other environmental stress conditions is uncertain. Of particular interest in this dissertation, is defining the role of GABA shunt in thermotolerance.

This review will discuss the role of GABA in different organisms, the role of the three enzymes of GABA shunt, and the role of these enzymes and the GABA shunt in heat stress and its relation to the production of ROS, and finally the expression of the three enzymes and the related regulatory proteins under heat stress.

GABA Shunt enzymes

The GABA shunt is a metabolic pathway that links nitrogen and carbon metabolism using glutamate as a substrate in the first catalytic step while producing succinate as its end product. The reactions of the GABA shunt are catalyzed by three enzymes, glutamate decarboxylase, GABA transaminase and succinate semialdehyde dehydrogenase. Understanding the properties of each individual enzyme in the metabolic pathway helps to understand the specific regulation of GABA shunt under stress conditions and the possible physiological roles of GABA in different organisms.

Glutamate decarboxylase

In mammals, the cDNAs of GAD have been isolated and sequenced, it is encoded by two different genes of *GAD1* and GAD2 which are located on different chromosomes (Erlander *et al*., 1991), the two isoforms are GAD67 and GAD65 which have molecular weights of 67 and 65 kDa respectively. *GAD1* is only expressed in brain tissue, and GAD2 is expressed in brain but also in pancreas (Erlander *et al*., 1991). It is widely thought that the two GAD isoforms in brain are localized within different subcellular compartments; GAD67 is related mainly to cytoplasmic pools of GABA, while GAD65 is associated with vesicular pools of GABA (Soghomonian and Martin, 1998). It is possible that GAD67 regulates GABA synthesis for metabolic functions of the cell, while GAD65 regulates GABA synthesis for synaptic release (Soghomonian and Martin, 1998). Mammalian GAD plays an important role in the regulation of GABA synthesis through interaction with its co-factor pyridoxal-phosphate (pyridoxal-P) (Martin and Rimvall, 1993).The two GAD isoforms in brain differ in binding with the co-factor, recombinant GAD data showed that GAD65 is more preferable to co-factor pyridoxal-P than GAD67 (Erlander *et al*., 1991), but characterization of the differences is not yet available. In most regions of the rat brain, GAD65 appears to be the major GAD isotype, however, research from knockout mice and rats treated with vigabatrin, an irreversible inhibitor of GABA transaminase, indicated that most of GABA in the brain was synthesized by GAD67 (Soghomonia and Martin, 1998). In neuronal cell cultures or brain slices, it was found that GABA can be released through both Ca2+-dependent and -independent mechanisms (Pin and Brockaert, 1989, Attwell *et al*., 1993, Belhage *et al*., 1993). The mammalian GADs have a pH optimum of approximately 7 (Wu *et al*., 1974).

In plants, GAD genes from various sources such as Petunia (Baum *et al*., 1993), tomato (Gallego *et al*., 1995), tobacco (Yu and Oh, 1998), and Arabidopsis (Turano and Fang, 1998, Zik *et al*., 1998) have been identified. The activity of plant GAD was found to be associated with senescence, seed germination and ripening (Gallego *et al*., 1995). Plant GAD utilizes L-glutamate as a substrate and has pyridoxal 5'-phosphate as a cofactor (Satya and Nair, 1986). Plant GADs demonstrate Km's between 3 and 25 mM depending on the plant species, and tissue sources (Satya and Nair, 1985; Snedden *et al*., 1995; 1996) and from 22 to 25 mM respectively (Chen *et al*., 1960). GAD appears to be localized in cytosol of plant cells and possesses a calmodulin-binding domain (Breitkreuz and Shelp, 1995). Unlike mammalian GAD, plant GADs have sharp acidic pH optima around pH 5.8 (Satya and Nair, 1985; Snedden *et al*., 1995). The in vitro activity assay of recombinant Petunia GAD shows that it has little activity in pH 7.0 in the absence of calcium ion and calmodulin (Snedden *et al*., 1996).

Environmental stresses such as oxygen deficiency or heat shock ((Roberts *et al*., 1984; 1992; Locy *et al*., 2000) and acid or ammonium treatments (L.A. Crawford *et al*., 1994) induce GABA synthesis resulting from a reduced cytosolic pH, presumably as a result of stimulated GAD activity at lowered cytosolic pH. Other environmental stresses such as cold shock or touch are known to increase cytosolic Ca^{2+} levels (Knight *et al*., 1991). GAD activity in vitro in a lot of species and plant tissues is shown to be stimulated by Ca^{2+}/calmodulin at neutral pH 7.0 ~ 7.5, instead of acidic pH lower than 6.5 (Snedden *et al*., 1995; 1996; Ling *et al*., 1994).

In vivo experiments with 1 hour pretreatment by Ca^{2+} channel-blockers and calmodulin antagonists in aerobic conditions for Ca^{2+}/calmodulin activation of GAD in

the rice roots did not result in GABA accumulation under 3 hour of anoxia (Aurisano *et al*., 1995). Hence, both in vivo and in vitro experiments suggest that the stimulation of GABA synthesis results from the stimulated GAD which is regulated by increased Ca^{2+} levels and calmodulin. Furthermore, in isolated Asparagus mesophyll cells, Chung *et al*. (1992) found that GAD activity is also stimulated by elevated glutamate levels. The catalytic activity of plant GAD is inhibited by reagents which can react with sulfhydryl groups. GAD activity is both transcriptionally and translationally regulated in different Petunia organs (Chen *et al*., 1994). In *Arabidopsis*, there are at least two GAD isoforms: *GAD1* is root specific and GAD2 is distributed in all organs (Zik *et al*., 1998; Turano and Fang, 1998). Additionally, with the whole genome sequenced in Arabidopsis, three more putative GAD isoforms have been revealed by sequence comparisons, GADs 2 – 5 are 75 ~ 82% identical to *GAD1* for their protein sequences (Shelp *et al*., 1999).

In fungi, the GAD enzyme was first reported in yeast in 1953 (Krishnaswamy and Giri, 1953; 1956). Fungal GAD has been purified and characterized in several species such as *R. glutinis* (Krishnaswamy and Giri, 1953), *N. crassa* (Schmit and Brody, 1975), *A. niger* ((Kubicek *et al*., 1979), and best studied in *A. bisporus* (Baldy, 1975). Like mammalian and plant GADs, fungal GAD is exclusively localized in the cytosol , the optimum pH is between 4.0 an 6.0, the Km for L-glutamate is in the lower millimolar range and requires pyridoxal phosphate as a cofactor. Partially purified *R. glutinis* GAD is inhibited by hydroxylamine and the addition of excess amount of pyridoxal phosphate can reverse the inhibition (Krishnaswamy and Giri, 1953; 1956). *N. crassa* GAD has been purified from conidia, it is a 30 to 33 kDa monomer and the Km for pyridoxal phosphate of 40 nM (Schmit and Brody, 1975). *A. bisporus* GAD is similar to *N. crassa* GAD. In *A.*

niger, only the conidiating mycelia, but not vegetative cells, show GAD activity (Baldy, 1975). Blast searches show that GAD from yeast shares sequences with GADs from *Petunia hybrida* and *A. thaliana* with identities of 38% and 39% respectively. Recombinant GAD studies demonstrated that yeast GAD can also bind to CaM like plant GAD does, but no activities were found with the CaM bound form, the functional GAD inside cells is required for oxidative stress tolerance (Coleman *et al*., 2001).

In bacteria, GAD is best characterized in *Escherichia coli*, it is easily induced in the growth media by glutamate (Fonda, 1985). Two isoforms of *gadA* and *gadB* were cloned in *E. coli*, (Smith *et al*., 1992), both isozymes showed identical kinetic and physico-chemical properties (Biase *et al*., 1996). Purified GAD from *E. coli* is a 53 kDa hexamer, with one pyridoxal 5'-phosphate (PLP) site on each subunit (Strausbauch and Fischer, 1970). The optimum pH is between 4.0 and 4.5, and it has broad ranges of substrates such as γ-methylene -glutamate, threo-β-hydroxy- glutamate, and homocysteine sulfinate, but -glutamate was shown to be the best substrate (Fonda, 1972). The activity of Escherichia coli GAD was inhibited by suicide substrates such as serine-O-sulfate, R-(−)-4-aminohex-5-ynoic acid, and α-fluoromethyl-glutamate (Kuo and Rando, 1981; Sukhareva and Braunshtein, 1971; Likos *et al*., 1982), the inhibition mechanism was found to be the same as aspartate aminotransferase (Likos *et al*., 1982). *GAD* has also been purified and characterized from other bacteria such as *Streptococcus pneumoniae* which resembles mammalian *GAD* (Garcia and Lopez, 1995) and *Lactobacillus brevis* which resembles *E. coli GAD* (Ueno *et al*., 1997).

GABA transaminase

In mammals, GABA is present in many mammalian tissues and is a major inhibitory neurotransmitter in the central nervous system (Fonnum, 1987). The concentration of GABA is controlled by GAD and GABA transaminase (GABA-T). After its synthesis by GAD, GABA is catabolized by GABA-T to succinic semialdehyde for subsequent conversion into succinate. In mammals, GABA-T has been purified and characterized mostly from brain tissues such as mouse, rat, rabbit and human brains (Maitre *et al*., 1975; John and Fower, 1976; White and Sato, 1978). The basal level of GABA-T activity is low. Recombinant human GABA-T has an apparent Km values for substrates α-ketoglutarate and GABA of 0.11 mm and 1.27 mm respectively (Seong *et al*., 2000). These values are similar to those from bovine and pig brains (Choi *et al*., 1993). The activity of GABA-T is inhibited by vigabatrin. Mammalian GABA-T requires pyridoxal-5-phosphate as co-factor. It catalyzes the transamination of GABA with a Km of 1.1 mM and catalyzes the transamination of β-alanine to the same extent of GABA (Schousboe *et al*.,1973; Buzenet *et al*., 1978).

In plants, the catabolism of GABA takes places in mitochondria (Breitkreuz and Shelp, 1995). It was shown that tobacco crude extracts apparently contain two GABA transaminases. One enzyme utilizes GABA, together with pyruvate while the other enzyme utilizes GABA and α-ketoglutarate. The two enzymes form succinate semialdehyde and alanine or glutamate respectively (Shelp *et al*., 1999; Satya and Nair, 1990; Van Cauwenberghe *et al*., 1999). However, the α-ketoglutarate-dependent GABA-T remains to be further elucidated, the activity can only be detected in tobacco crude extract and is undetectable subsequently during purification (Van Cauwenberghe *et al*.,

1999). Additionally, it is noteworthy that there are no α-ketoglutarate-dependent GABA-T sequences found in any plant genome utilizing the BLAST search engine at NCBI to search the GenBank database.

Plant GABA-T has not been purified from any plant. However, biochemical studies from plant crude preparations show the optimum pH for plant GABA-T is between 8.6 to 9.0 (Streeter and Thompson, 1972; Satya and Nair, 1986), and requires pyridoxal-phosphate (PLP) as a cofactor (Satya and Nair,1986; 1990, Van Cauwenberghe *et al*., 1999). Plant pyruvate dependent GABA-T has been cloned from *Arabidopsis* (Van Cauwenberghe *et al*., 2002) and rice (Ansari *et al*., 2005). These enzymes show putative mitochondrial targeting sequences and conserved PLP binding domains. In addition, the GABA-T knockout in Arabidopsis provides evidence that GABA accumulates greatly in flowers up to 113- fold compared with wild type suggesting GABA-T functions in vivo (Palanivelu *et al*., 2003).

In yeast and other fungi, Roberts *et al*. (1953) discovered that α-ketoglutarate-dependent GABA-T is involved in nitrogen utilization. Subsequently, Yonaha *et al*. (1983) found that GABA-T exists in a variety of micro-organisms including yeast and other molds. GABA-T was induced in yeast species like *Saccharomyces, Hansenula* and *Candida* on medium with GABA but not on β-alanine, while in molds and other genera such as *Rhizopus, Aspergillus, Penicillium* and *Neurospora*, GABA-T was detected on both GABA and β-alanine-containing media (Yonaha *et al*., 1983). Characterization of GABA-Ts purified from *Candida* (Der *et al*., 1986) or *A. bisporus carpophore* (Baldy, 1976) indicated that they share some catalytic features: pyridoxal phosphate-dependent, optimum pH of 8.0, α-ketoglutarate specificity and inhibition by short chain fatty acids

such as propionate and butyrate. GABA-T has been cloned from yeast *Saccharomyces cerevisiae* (Andre and Jauniaux, 1990), and it is encoded by UGA1 gene. Previous studies from UGA1 knockout demonstrated that yeast GABA-T, like other genes in GABA shunt pathway, plays an essential role in oxidative stress tolerance (Coleman *et al*., 2001). Bacterial GABA-T, is most similar to yeast GABA-T, but different from the GABA-T of other fungi and mammals. It is specific for GABA, does not utilize β-alanine, and requires α-ketoglutarate as an amino acceptor (Yonaha *et al*., 1983). GABA-T was found to be abundantly distributed in most strains of bacteria grown on media with β-alanine or GABA as nitrogen sources (Yonaha *et al*., 1983). Studies from cowpea *Rhizobium* also show that GABA-T requires α-ketoglutarate. The activity of GABA-T is induced on media containing GABA as the sole carbon and nitrogen source (Jin *et al*., 1990). Recently, a GABA-T gene (gabT) from *Rhizobium leguminosarum* bv. *Viciae* was identified, cloned, and characterized (Prell *et al*., 2002). It is induced by GABA and highly expressed in bacteroids. Mutants of gabT lost the α-ketoglutarate-dependent GABA-T activity, but they are still able to grow on GABA as the sole carbon and nitrogen source, suggesting the existence of multiple GAB-Ts which have different substrate specificities in this organism. According to Tunnicliff (1993), the activity of GABA-T from *Pseudomonas fluorescens* is inhibited by ATP, and its analogues ADP, CTP and XTP in a competitive manner, the inhibition effect can be antagonized by GABA, indicating ATP competes with GABA for the enzyme binding site. Voellym and Leisinger (1976) purified GABA-T from *Pseudomonas aeruginosa*. In addition to GABA, the purified enzyme catalyzed the transamination of N2-acetyl-L-ornithine, L-ornithine,

putrescine, L-lysine, and cadaverine in order of decreasing activity. The enzyme is induced by GABA, guanidinobutyrate, or putrescine as well.

Succinate semialdehyde dehydrogenase

In mammals, SSADH has been purified to apparent homogeneity from rat, pig and human brains (Chambliss and Gibson, 1992; Ryzlak and Pietruszko, 1988; Lee *et al*., 1995). The purified human brain SSADH is localized in mitochondria, has SSA as the best substrate, but also has activities for the substrates of glutaric semialdehyde, nitrobenzaldehyde, and short chain aliphatic aldehydes. The enzyme can only use NAD^+ as a cofactor (Ryzlak and Pietruszko, 1988). The enzyme from pig brain is substrate inhibited by SSA and by the product, NADH (Duncan and Tipton, 1971).

In plants, SSADH has been purified from wheat embryos (Galleschi *et al*., l983), barley seeds (Yamura *et al*., 1988) and potato tubers (Satya and Nair, 1989). The first cloned SSADH was from *Arabidopsis* (Bu *et al*., 1999). Biochemical analysis from previous studies show plant SSADH is localized in mitochondria (Breitkreuz and Shelp, 1995). Mitochondrial localization is common among most organisms except yeast. The plant SSADH has an optimum pH of 9.0, is highly specific for substrate SSA, and can only use NAD^+ as a cofactor, the catalytic activity is inhibited by the substrate SSA at higher concentrations, the product NADH and also adenine nucleotides such as AMP, ADP and ATP. A disruption of the unique SSADH gene in *Arabidopsis* has been shown to cause necrotic lesions and programmed cell death during periods of heat stress, UV light stress, or hydrogen peroxide stress as result of accumulation of toxic reactive oxygen species (Bouché *et al*., 2003).

In yeast and other fungi, SSADH has received relatively little attention. Most studies have been limited to enzyme activities from crude cell preparations (Pietruszko & Fowden, 196; Ramos *et al*., 1985). Ramos *et al*. (1985) found that SSADH from *Saccharomyces* has a pH optimum of 8.4. It is highly specific for the substrate succinate semialdehyde with a Km in the μM range. It can use both NAD^+ and $NADP^+$ as a cofactor but has higher affinity for NAD^+. Its activity is substrate inhibited by succinate semialdehyde. These kinetic properties are also shared by SSADH from niger mycelia and plants (Kumar and Punekar, 1994; Pietruszko and Fowden, 1961; Rating *et al*., 1984; Satya and Nair, 1989). Ramos *et al*. (1985) found a thio requirement is not necessary for yeast SSADH activity. Some exception was also observed in fungi, for example, the *T. utilis* extracts were shown to catalyze the reverse reaction by reducing SSA to 4-hydroxybutyric acid (Pietruszko and Fowden, 1961).

In *Saccharomyces cerevisiae*, SSADH is encoded by the *UGA2* gene (Coleman *et al*., 2001). It is localized in the cytosol (Huh *et al*., 2003). Together with UGA1, *UGA2* is involved in the degradation of GABA to succinate. Mutation of either of UGA1 or *UGA2*, makes yeast cells more sensitive to oxidative stress (Coleman *et al*., 2001), and prevent them from growing on GABA as the sole nitrogen source cells (Ramos *et al*., 1985). Like *GAD1* and *UGA1*, *UGA2* expression is also induced by GABA (Ramos *et al*., 1985). The expression levels of *UGA1* and *UGA2* are under the control of transcription activator UGA3p (Ramos *et al*., 1985; Vissers *et al*., 1989; Talibi, *et al*., 1995; Coleman *et al*., 2001). Under oxidative stress, levels of *UGA2* transcript were found to be up-regulated (Coleman *et al*., 2001). Detailed regulation of the expression of GABA shunt genes and the genes which control their expression will be reviewed later.

In bacteria, two proteins are involved in the activity of succinate semialdehyde dehydrogenase: NAD^+-dependent and $NADP^+$-dependent succinate semialdehyde dehydrogenases. Cozzani *et al*. (1980) separated these two proteins from *E. coli*. The partially purified enzymes differ in their co-factors (NAD^+ or $NADP^+$). The $NADP^+$-dependent SSADH is very specific for the substrate SSA while the NAD^+-dependent SSADH can utilize n-butyraldehyde in addition to SSA. In *E. coli*. B, both NAD^+-and $NADP^+$-dependent SSADH were induced by GABA, but the $NADP^+$-dependent enzyme has higher activity of GABA-T (Donnelly and Cooper,1981).

SSADH has also been cloned from *E. coli*.. It is part of the gene cluster which encodes the enzymes for GABA degradation. (Metzer and Halpern, 1990; Bartsch *et al*., 1990; Niegemann *et al*., 1993). In *E. coli*, a second sad-encoded SSADH was identified in addition to the gabD-encoded SSADH (Donnelly and Cooper, 1981; Marek and Hensen,1988), and in *Pseudomonas sp.*, *Klebsiella pneumoniae,* and *Ralstonia eutropha* also an NADP+- and/ or an NAD+-dependent SSA-DH were detected (Nirenberg and Jacoby, 1960; Sanchez *et al*., 1989)

The role of GABA in different organisms

In animal systems, GABA has been well known as an inhibitory neurotransmitter mainly found in the central nervous systems (CNS). In vertebrates, GABA acts at inhibitory synapses in the CNS by binding to specific transmembrane receptors, which causes the opening of ion channels to allow negatively-charged chloride ions to flow into the cell or positively-charged potassium ions to flow out of the cell (Roth *et al*., 2003). Three general classes of GABA receptor have been found: GABAA and GABAC

ionotropic receptors, which are ion channels themselves, and GABAB metabotropic receptors, which are G protein-coupled receptors. (Dzitoyeva *et al*., 2003; Mihic *et al*., 1997; Boehm *et al*., 2006; Dimitrijevic *et al*., 2005).

In plants, for many years, GABA has been shown from numerous reports to accumulate rapidly upon various stress factors including heat, cold, drought, acidosis, anoxia, mechanical damage. For example, in heat-stressed cowpea cells, GABA accumulated 1,800-fold in 24 hours as compared to the non stressed control (Mayer *et al*., 1990). In soybeans subjected to cold stress and mechanical damage, GABA level in soybean leaves increased 2,000- and 2,700- fold respectively in 5 min (Wallace *et al*., 1984). However, the physiological role GABA plays in plants is not well understood yet. Early studies suggested different roles of GABA in response to stress conditions. These roles are generally related to: contributing to the C: N balance (Rolin *et al*., 2000, Breitkreuz *et al*., 1999, Snedden and Fromm, 1999), regulation of cytosolic pH (Snedden *et al*., 1995, 1996), protection against oxidative stress (Bouché *et al*., 2003), defense against insects (Ramputh and Brown, 1996), or GABA as an osmoregulator (Rentsch *et al*., 1996)

More recently, scientists are trying to find evidences to show that GABA is a signaling molecule by analogy to such a clearly defined role in animals. In *Arabidopsis*, the pollen–pistil-interaction2 (pop2) gene encodes GABA-T. The pop2 mutant accumulated GABA in flowers, and the guidance and growth of the pollen tube was disturbed. In the wild type, a GABA gradient was created in the pistil, that was required for proper pollen tube guidance to the ovule (Palanivelu *et al*., 2003; Bouché *et al*., 2004). Thus, this finding indicates GABA may play an important role in signaling. However,

there is a strong need in plants to elucidate the GABA signaling pathway by showing the presence of GABA receptors. Genes which share high sequence similarities with animal ionotropic glutamate receptors have been identified in *Arabidopsis* as putative glutamate receptors (designated as ATGLRs) (Lacombe *et al*., 2001), these ATGLRs posses domains which are structurally highly homologous to GABAB receptors, thus, it is reasonable to assume GABA interacts with ATGLRs to play its signaling role by binding to these domains.

In bacteria, GABA was first found to play a role in carbon and nitrogen metabolism, wild type laboratory strains of *E. coli* K-12 are unable to utilize γ-aminobutyrate (GABA) as the sole carbon and nitrogen source. However, Dover and Halpern (1972) found UV induced mutants are able to grow on GABA as a sole carbon and nitrogen source, and also the activities of GABA aminotransferase and SSADH were both increased six- to nine-fold. Foester and Foester (1973) reported that GABA plays a role in the germination of the *Bacillus megaterium* spores. GAD activity increased dramatically during germination leading to accumulation of GABA. In addition, Castaniethere *et al*. (1999) reported that the production of GABA was involved in acidic pH resistance in *E. coli*. The GAD enzyme protects the cell from extreme acid conditions during transit through the host stomach since GAD consumes protons by catalyzing decarboxylation of glutamate. The product GABA is finally exported from the cells.

In yeast and other fungi, GABA was first isolated from acid treated yeast extracts (Reed, 1950). It was subsequently found in various other fungi. GABA accumulated unusually in *A. niger* during acidogenesis coupled with the increase of citric acid (Kubicek *et al*., 1979). In understanding the role of GABA in fungi, the following have

been summarized from previous studies: Firstly, GABA metabolism is involved in the catabolism of nitrogen compounds. In *S. cerevisiae* (Ramos *et al*., 1985) and *A. nidulans* (Yonaha *et al*., 1983), GABA in the growth media as a sole nitrogen source induces the expression of the enzymes which catabolize GABA such as GABA-T and SSADH, the GABA transporter gene (*UGA4*) is also induced (Ramos *et al*., 1985).

GABA metabolism is involved in conidiation and germination of conidia. This role was established from the work of Schmit *et al* (Schmit and Brody, 1975; Hao and Schmit, 1991; 1993), Schmit and Brody (1975) who found GAD can only be detected at conidiation stage but GAD is not found in mycelia. The levels of GAD increase at conidia as they mature and decline in the early phase of conidial germination. Therefore, this work enabled Schmit *et al*. (1975) to first clone GAD as a conidiation stage-specific marker enzyme.

Furthermore, GABA metabolism seems to be involved in oxidative stress tolerance. Coleman and co-workers (Coleman *et al*., 2001) showed the three genes including *GAD1*, *UGA1* and *UGA2* in the GABA metabolic pathway are all required to tolerate oxidative stress. Strains bearing single mutations in these genes are more sensitive to oxidative stress compared to wild type, and the single mutants of gaba-t and ssadh can no longer grow on GABA as their sole nitrogen source. However, a definitive physiological role of GABA in yeast, other fungi and most of other organisms remains understood for other stress conditions.

Heat shock and yeast thermotolerance

Induced thermotolerance, or the increased resistance of cells and tissues to severe

(or lethal) heat shock following a prior exposure to mild (sublethal) heat shock has been described in all living organisms from prokaryotes (Morozov *et al*., 1997) to eukaryotes (Mager and Moradas, 1993). Direct exposure to elevated lethal temperature can damage major components of cells such as proteins and membranes. Prior exposure to a mild heat shock can induce heat shock proteins (HSP) and other cellular changes, thereby making cells more resistant to a subsequent, even more severe heat shock. For yeast *Saccharomyces cerevisiae*, the optimal growth temperature is within the range of 25 to 35°C, at temperature of 35 to 37°C, yeast cells continue to grow, but are moderately stressed, developing a protective tolerance against elevated lethal temperature. At temperatures above 45°C, yeast cells are severely stressed, and 99% of growing non-adapted aerobic yeast cells die after a 5 min exposure at 50°C (Davidson *et al*., 2001a, 2001b). Thermotolerant yeasts are considered to have an optimum growth temperature above 40°C (Walker, 1998).

Heat-shock is one of the best studied stress-inducible responses, not only of yeast but also of virtually all living organisms. The heat shock response system is understood very well at the DNA level (Craig, 1986; Lindquist and Craig, 1988; Parsell and Lindquist, 1993). For yeasts, the induced thermotolerance acquired from a pre-exposure to a mild heat treatment is related to heat shock factors and stress response element pathways that regulate the synthesis of heat shock proteins (HSPs) (Mager and Moradas, 1993). Miller *et al*. (1982) have shown a temperature shift from 23 to 37°C in *Saccharomyces cerevisiae* transiently induces approximately 80 proteins. Twenty of these induced proteins belong to major HSP families. These proteins play important roles in helping cells cope with the toxic effects of high temperatures, but they have different

expression patterns and molecular functions. Some of the HSPs have been functionally characterized, but the function of most of the HSPs remains to be understood (Mager and Moradas, 1993).

In the yeast *Saccharomyces cerevisiae*, heat shock proteins of Hsp90 and Hsp70 are important at all temperatures and are required for growth at higher temperature (Borkovich *et al*., 1989). Sanchez and Lindquist (1990) have shown Hspl04 is induced by heat but expressed at a low level under non stressful temperatures. Hsp104 apparently plays a crucial role in helping cells tolerate short exposures to extreme temperatures. Sanchez and Lindquist (1990) show that with a preheat treatment (30 min at 37°C) followed by stress at 50°C, both wild type and cells bearing a deletion in Hsp 104 acquired thermotolerance, but the hsp104 deletion mutant died 100-1000 fold faster than the wild type cells.

In general, heat shock proteins are molecular chaperones required for survival under stress conditions (Brosnan *et al*., 2000). HSPs confer thermotolerance in organisms by preventing the denaturation of proteins during the heat stress and by facilitating the refolding of already damaged proteins. HSPs are induced in yeast cells under stresses other than heat shock. Parsell *et al*. (1994) have shown that Hspl04 functions to promote the resolubilization and reactivation of proteins that have unfolded and aggregated after exposure to high temperatures. Hsp70 and Hsp90 prevent aggregation by binding to unfolded proteins, maintaining them in a native, functional state (Parsell and Lindquist, 1993). Other Hsps help to usher unfolded proteins along the degradation pathway.
In addition to HSPs, it has been suggested that acquired thermotolerance is mediated by other factors. For example, in heat shock factor (HSF) mutant *hsfl-m3* yeast cells, the

induction of thermotolerance was not impaired (Smith and Yaffe, 1991). Furthermore, recent work by De Virgilio *et al*. (1994) has shown that trehalose plays an important role in acquired thermotolerance in *S. cerevisiae,* and the mutations that block trehalose synthesis sharply reduce thermotolerance. Trehalose is a disaccharide that changes the solvent properties of the fluid phase and reduces the denaturation of proteins at high temperatures (Yancy *et al*., 1982). Trehalose acts synergistically with HSP104 to protect cells of *S. cerevisiae* from heat damage (Estruch, 2000).

Heat shock and reactive oxygen species

The production of reactive oxygen species (ROS) is an unavoidable consequence of life in an aerobic environment. ROS are characterized by their high chemical reactivity and include both free radicals (that is, species with one or more unpaired electrons, such as superoxide (O_2 .−) and hydroxyl radicals (OH.)), and non-radical species such as hydrogen peroxide (H_2O_2) (Shah and Channon, 2004). Indeed, organisms keep a balance between ROS generation and antioxidant systems that scavenge or reduce ROS concentrations. The imbalance caused by over production of ROS and / or reduced antioxidant capabilities generates oxidative stress.

However, during episodes of environmental stresses such as high temperature or during the fermentation process, ROS levels increase dramatically, which can result in significant damage to cellular components including DNA fragmentation, protein or enzyme inactivation, the modification of carbohydrate compounds, and changes in membrane fluidity via lipid peroxidation (Elisa *et al*., 2000). Cells are normally able to defend themselves against ROS damage through the use of detoxifying enzymes such as

superoxide dismutase, catalase, and glutathione peroxidase, small molecule antioxidants such as ascorbic acid (vitamin C), uric acid, and glutathione, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals (Arrigo *et al*., 2002). Heat stress has been documented to produce oxidative stress (Davidson *et al*., 1996; Davidson and R. H. Schiestl, 2001a; 2001b), and to some extent, heat stress is equivalent to oxidative stress (Sugiya *et al*., 2000). For example, Collinson and Dawes (1992) have shown heat stress induces HSPs as well as a set of antioxidant enzyme genes such as catalase (CTT1), thioredoxin peroxidase (TPX1 and TPX2), and cytochrome c peroxidase (CCP1). Mutant cells with deletion of protein antioxidant enzymes including catalase, cytochrome c peroxidase, superoxide dismutase, and thioredoxin peroxidase are more sensitive to heat stress compared to the wild type (Davidson *et al*., 1996).

Bouché *et al*. (2003) show disfunction of mitochondrial succinate semialdehyde dehydrogenase in plants caused cells to be very sensitive to heat stress and over-accumulation of ROS, suggesting a role of GABA shunt in thermotolerance by restricting the production of ROS. Krahe *et al*. (1996) found aerobic exposure of yeast cells to higher temperatures increases the concentration of intracellular oxygen, consequently increasing the production and reactivity of ROS (Issels *et al*., 1986). In addition, it has been found that H_2O_2 pretreatment strongly induces many of HSPs (Godon *et al*., 1998). Thus, some overlap must exist between mechanisms of heat and oxidative stress supporting the concept that both HSPs and antioxidants contribute to the thermotolerance of yeast cells (Jamieson, 1995).

In respiring yeast cells, the main source of ROS production is the mitochondrial electron transport chain, the principal site being proximal to the cytochrome c oxidase

complex (Guidot *et al*., 1993). In the cytosol, ROS such as H_2O_2 and superoxide may be formed by P450 cytochromes and dioxygenases (Dalton *et al*., 1999), or generated during glycolytic fermentative metabolism (Hamm-Kunzelmann *et al*., 1997). In mammalian cells, Halliwell and Gutteridge (1989) indicated the major source of ROS is mitochondria, other potential ROS sources include, xanthine oxidase, cytochrome P450 based enzymes, NADPH oxidases, dysfunctional NO synthases, peroxisomes and infiltrating inflammatory cells. However, a major ROS source for redox signalling is NADPH oxidases (Shah and Channon, 2004). In plant cells, ROS are generated at a number of cellular sites, predominantly in mitochondria, chloroplasts, and peroxisomes. Another source includes the extracellular side of the plasma membrane (Laoli *et al*., 2004).

Heat shock and the expression of yeast GABA shunt genes

The three genes *GAD1*, *UGA1* and *UGA2* in the GABA shunt pathway play important role in the metabolism of GABA. To cope with the damaging effects from the environmental and physiological stresses such as heat stress, osmotic stress, or oxidative stress, yeast cells have developed rapid molecular responses by changing their gene expression patterns (Estruch, F.. 2000). In response to heat stress, heat shock proteins may not be the major factor regulating thermotolerance. Expression analysis upon environmental changes in yeast has been investigated by several scientists using the technique of DNA microarray. Gasch *et al*. (2000) show in response to mild heat stress 37°C shifting from various temperatures from 17°C to 33°C, all the three enzymes in the GABA shunt pathway were similarly up-regulated at least 3 fold upon heat stress at 37°C in a time dependent manner. A genome wide gene profiling conducted by Sakaki *et al*.

(2003) indicated that 104 genes were up-regulated and 287 genes were down-regulated under mild heat stress by shifting cells from 25°C to 37°C, among the down-regulated or repressed genes are mitochondrial related, among the up-regulated genes are heat shock proteins and antioxidant genes, the three stress responsive genes (*GAD1*, *UGA1* and *UGA2*) in GABA shunt pathway, and also genes involved in production of oxaloacetic acid (OAA) and acetyl-coenzyme A (CoA) were induced, therefore an increased synthesis of α-ketoglutarate may occur through TCA cycle leading to an elevated level of glutamate, which may subsequently stimulate GABA synthesis. These results further indicated that cells adapted to heat stress by down-regulation of mitochondrial genes to avoid heat induced ROS and also by up-regulation of genes to activate metabolic pathways. However, the effect of lethal temperature on the gene expression pattern and the specific response of GABA shunt remain to be investigated. Under other environmental stress conditions, Coleman *et al* (2003) demonstrated UGA1p and UGA2p together with GAD1p are oxidative stress responsive proteins. By screening among different nitrogen sources, Ramos *et al*. (1985), Andre *et al*. (1993) Talibi *et al*.(1995) and Patrice Godard *et al*. (2007) found the expression of *UGA1* and *UGA2* genes were induced 30 - 240 fold by the presence of GABA as sole nitrogen source instead of ammonium sulfate; According to Coleman *et al*. (2001), the expression of *UGA2* was induced 3- fold when yeast cells were exposed to 1 mM H2O2 stress, however, information obtained on the expression of these three genes (*GAD1*, *UGA1* and *UGA2*) under stress conditions is limited.

The expression of genes regulating GABA metabolism related genes

In addition to the environmental changes, the expression of GABA shunt genes is also under the control of two other regulatory genes: *UGA3* and *UGA4*.

UGA3 gene is a transcription factor which regulates the expression of GABA metabolism related UGA genes such as *UGA1*, *UGA2* and *UGA4* (Coornaert *et al*., 1991), *UGA35* or *DAL81* gene is a general positive regulator of genes involved in nitrogen utilization related to metabolisms of GABA, urea, arginine and allatoin (Vissers *et al*., 1990). Together with UGA35p/DAL81p, UGA3p controls the gene expression by recognizing the promoter elements centered around a GATAA sequence involving in the metabolism of poor nitrogen source such as GABA (Cunningham *et al*., 1994). In yeast, UGA3p is required for the transcriptional activation of *UGA1* and *UGA4* (Vissers *et al*., 1989), deletion of either *UGA3* or *UGA35* impairs the expression of *UGA1* and *UGA4* (Vissers *et al*., 1990). Gasch *et al*. (2000) has found the expression of *UGA3* was strongly induced by nitrogen depletion but unaffected by mild heat stress at 37ºC.

In yeast, GABA is imported into cells by three proteins: the general amino acid permease (GAP1), proline specific permease (PUT4p) and the GABA permease (UGA4p) (Grenson *et al*., 1987). The expression of the *UGA4* gene was induced by GABA as sole nitrogen source (Ramos *et al*., 1985), but also dependent on the cell growth conditions (Moretti *et al*., 1998). As reviewed above, the constitutive expression of *UGA4* requires two positive-acting proteins, the specific UGA3p and the pleiotropic UGA35p/DAL81p (Andre *et al*., 1995; García *et al*., 2000). By measuring β-galactosidase activity in the cells carrying a *UGA4*::lacZ fusion gene, Moretti *et al*. (2001) have shown the expression of *UGA4* was induced in response to acid pH (4.0) medium in the condition without the

repression by *UGA43* repressor factor or induction by GABA. Previous studies have demonstrated cells with the mutation of *UGA1* or *UGA2* are unable to grow on GABA as the sole nitrogen source (Ramos *et al*., 1985; Coleman *et al*., 2001), Andre *et al*. (1993) found mutation of *UGA4* has the same phenotype if with the mutation of the other GABA transporter genes GAP1 and PUT4. From Gash *et al*.'s (2000) expression investigation, in response to mild heat stress at 37ºC, slight induction (~1.5 fold) was observed after 5 min, but this induction was only temporary. In contrast, the expression of *UGA4* was induced after the temperature was shifted from 37ºC to 25ºC for 45 min.

Yeast is powerful genetic tool

"Yeast" is often taken as a synonym for *S. cerevisiae* (Kurtzman, 1994). It is unicellular, and is a proven model eukaryote for molecular and cellular biology studies. It is well known that yeast has a small genome, grows fast, and is easy to manipulate which makes *S. cerevisiae* a popular tool for genome wide studies. The genome has been sequenced, and the corresponding databases are available online. The most comprehensive database about yeast genes and research references is provided by Saccharomyces Genome Database. By the strategy of homologous recombination, knockouts of almost every yeast gene have been constructed (as heterozygous diploids for essential genes; as homozygous diploids and haploids for the others), and are available from American Type Culture Collection (ATCC). These collections have been widely used for numerous genome wide studies, or for competition studies using the specific “bar codes” of each disruption for identification (Fröhlich *et al*., 2007).

Yeast has also been widely used for protein-protein interactions by two-hybrid or

synthetic lethality studies. Because of its simple genome, yeast provides a "clean background" for expressing genes from other eukaryotic systems such as humans or plants. Yeast also provides a convenience for knocking out genes which have only single copies in yeast but exist in homologues of multiple isoforms in other organisms such as plants, which is still lack of an effective gene knockout strategy for functional characterization under stress conditions. Because of these advantageous features, yeast has become the model organism for medical, applied, and fundamental research (Mager and Winderickx, 2005).

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II. THE GABA SHUNT MEDIATES BASAL THERMOTOLERANCE IN *SACCHAROMYCES CEREVISIAE* BY REDUCING THE PRODUCTION OF REACTIVE OXYGEN SPECIES

Abstract

The GABA shunt pathway involves three enzymes: glutamate decarboxylase (GAD), GABA aminotransferase (GABA-TA), and succinate semialdehyde dehydrogenase (SSADH). These enzymes act in concert to convert α-ketoglutarate (through glutamate) to succinate. Deletion mutations in each of these genes in *Saccharomyces cerevisiae* resulted in growth defects at 45°C or at 50°C following an induction period at 40°C. Double and triple mutation constructs were compared for thermotolerance with the wild type and single mutant strains. Although wild type and all mutant strains were highly susceptible to even brief heat stress at 50°C, a 30 min at 40°C (a non-lethal temperature) induced tolerance of wild type and all of the mutants to the 50°C (lethal temperature). The mutant strains collectively exhibited similar susceptibility at both 45°C and the induced 50°C treatments. Intracellular reactive oxygen species (ROS) accumulation was measured in wild type and each of the mutant strains. ROS accumulation in each of the mutants and under various stress conditions was correlated to heat susceptibility of the mutant strains. The addition of ROS scavenger *N-tert*-butyl-α-phenylnitrone (PBN) enhanced the mutant growth defect and strongly inhibited the

accumulation of ROS, but did not have significant effect on the wild-type. Measurement of intracellular GABA, glutamate and α-ketoglutarate during lethal heat exposure at 45°C showed higher level of accumulation of GABA and α-ketoglutarate in the *uga1* and *uga2* mutants, while glutamate accumulated at higher level in *gad1* mutant. These results suggest that GABA shunt pathway plays a crucial role in protecting yeast cells from heat damage by restricting reactive oxygen species production involving the flux of carbon from α-ketoglutarate in yeast cells to succinate during heat stress in mutant cells lacking a functional GABA shunt pathway.

Introduction

Virtually all living organisms contain the GABA shunt pathway consisting of 3 enzymes: glutamate decarboxylase (GAD) catalyzing the conversion of glutamate (Glu) to GABA, GABA aminotransferase (GABA-AT) catalyzing the conversion of GABA and α-ketoglutarate (α-KG) into succinate semialdehyde (SSA) and Glu, and succinate semialdehyde dehydrogenase (SSADH) catalyzing the NAD(P)-dependent conversion of SSA into succinate. The pathway serves to move α-KG to succinate bypassing two reactions of the tricarboxylic acid cycle.

The biological function of the GABA shunt is varied depending on the organism in which it exists. In bacteria and fungi, this pathway is responsible for the assimilation of exogenously supplied and excess glutamate (Jacob, 1962; Piquemal *et al*., 1961). In animals, it is well established that GABA and associated metabolism is associated with inhibitory neurotransmission (Petroff, 2002; Wang and Joseph, 1999). The disfunction of each enzyme in the pathway is related to human neurological genetic disorders (Jakobs *et al*., 1993). In plants, the GABA shunt appears to contribute to the control of cytosolic pH, balance between carbon and nitrogen metabolism, and adaptation to stress (Bouche and Fromm, 2004). GABA has been shown to play a potential signaling role in pollen tube guidance (Palanivelu *et al*., 2003). Disruption of the *SSADH* gene in *Arabidopsis* results in the accumulation of reactive oxygen species (ROS), necrotic leaf lesions, dwarfism, and hypersensitivity to environmental stresses (Bouche *et al*., 2003). In baker's yeast, *GAD1* is required for tolerance to oxidative stress and the loss of *UGA1* and *UGA2* reduced the oxidative stress tolerance (Coleman *et al*., 2001). These studies suggest that the GABA shunt pathway or at least an enzyme or enzymes in the pathway play a role in

limiting the production or lethality of ROS produced during environmental stress episodes. However, the molecular mechanism via which such actions are mediated remains largely unknown.

In fungi and higher plants, GAD is known to contain a calmodulin binding domain, and the activity of the GABA shunt as well as GABA accumulation appears to be regulated by the binding of calcium and calmodulin to the GAD enzyme (Baum *et al*., 1993; Coleman *et al*., 2001). Thus, the accumulation of GABA during episodes of environmental stresses including heat stress in plants and fungi appears to be modulated by calmodulin and stress modulated intracellular calcium pools (Baum *et al*., 1996, Bouche *et al*., 2005; Coleman *et al*., 2001).

Although the mechanism of modulating GABA shunt activity appears to be elucidated, the role of the enzymes of the GABA shunt in abating the deleterious effects of stress in fungi and plants remains to be elucidated. Here, we report the role of the three GABA shunt enzymes during heat stress in *Saccharomyces cerevisiae.* Deletion mutants of Δ*gad1*, Δ*uga1*, and Δ*uga2* and the double and triple mutation constructs of these three genes were made. Viability and thermotolerance of cells, intracellular levels of ROS, GABA shunt metabolite levels were measured in the wild type and various deletion strains during the mid-log phase of growth during heat stress.

Materials and Methods

Yeast strains and growth media. Yeast strains used in this study were all derived from stain W303-1A (*Mat a leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*) and the precise genotypes of the mutants are listed in Table 1.

In various experiments as detailed in text, table and figure legends, the strains were grown on either YPD medium or YNB medium. YPD medium contains: 2% glucose, 1% yeast extract, 2% yeast bactopeptone, and was used for growth and cell survival assay.YNB medium contains: 0.67% [wt/vol] yeast nitrogen base, 2% glucose, supplemented with essential amino acids (Sherman *et al*., 1979).

Disruption of *GAD1, UGA1 and UGA2* genes. The *GAD1* disruption was generated by PCR amplification / homologous recombination strategy (1) that replaced the entire open reading frame of *GAD1* with *TRP1* gene. The *TRP1* gene was amplified from plasmid pRS414 template DNA using a forward primer (named GAD1UP45TRP1A, Table 1) that consisted of the 45 nucleotides immediately upstream of the *GAD1* gene fastened upstream of the 5'most portion of the gene. The reverse primer (named GAD1DN45TRP1D, Table 2) consisted of the 45 nucleotides immediately downstream of the *GAD1* gene ligated to the 22 nucleotides at the 3' end of the TRP1 gene contained in pRS414. The PCR product was then transformed into yeast strain W303-1A by lithium acetate as previously described (Geitz and Woods, 2003), and TRP+ colonies were selected for genomic DNA isolation. The correct integration was verified by isolating genomic DNA (Hoffman and Winston, 1987) from each selected strain and conducting a series of PCR reactions using primers that land 300-500 nucleotides upstream and downstream of *GAD1* open reading frame and within the *TRP1* gene (Table 2).

The *UGA1* gene was disrupted by replacing a fragment of the gene between bases +293 to +911 with the *HIS3* gene. The *UGA1* coding sequence was amplified by PCR

from yeast genomic DNA utilizing PCR primers UGA1HIS3FOR and UGA1HIS3REV (Table 2). The amplified *UGA1*-orf was digested using *Kpn*I and *Sac*I according to manufacturer's procedures and ligated into pBluescript, which had been linearized by restriction with the same two enzymes to create pBS-*UGA1*-orf. This pBluescript -*UGA1*-orf construct was then double digested with *Sal*I and *Age*I according to manufacturer's procedures generating a 618 nucleotide deletion from the *UGA1*-orf. The *HIS3* gene was PCR amplified from the pRS413 plasmid using forward and reverse primers that had a *Sal*I restriction site added 5' to the 5'-25 nucleotides of the *HIS3* gene and an *Age*I restriction site added 3' to the 3' end of the *HIS3* gene sequence. This PCR product was then ligated into pGEM-T easy vector, and the plasmid was amplified in *E. coli* strain DH5-α, the insert was recovered from this amplified plasmid by digestion with *Sal*I and *Age*I restriction endonucleases. The *Sal*I / *Age*I insert was subsequently ligated into the linearized, digested pBSΔ*UGA1*-orf to replace the deleted 618 base-pair sequence of *UGA1*-orf generating pBSΔ*uga1*-orf::*HIS3.* The disrupted *uga1*-orf::*HIS3* sequence was PCR amplified from pBSΔ*uga1*-orf::*HIS3* and this PCR product (*uga1*::*HIS3*) was used as the disruption cassette to transform and eventually disrupt the endogenous W303-1A *UGA1* gene.

The *UGA2* disruption was generated by PCR amplification / homologous recombination strategy (Bauldin *et al*., 1993) that replaced the entire open reading frame of *UGA2* with *URA3* gene. The *URA3* gene was PCR amplified from plasmid pRS416 template DNA using a forward primer (named UGA2UP45URA3A, Table2) that consisted of the 45 nucleotides immediately upstream of the *UGA2* gene fastened upstream of the 5'most portion of the gene. The reverse primer (named

UGA2DN45URA3D, Table 2) consisted of the 45 nucleotides immediately downstream of the *UGA2* gene ligated to the 22 nucleotides at the 3' end of the *URA3* gene contained in pRS416. The PCR product was then transformed into yeast strain W303-1A (Geitz and Woods, 2003), and URA3+ colonies were selected for genomic DNA isolation. The correct integration was verified by isolating genomic DNA from each selected strain (Hoffman and Winston, 1987) and conducting a series of PCR reactions using primers that land 300-500 nucleotides upstream and downstream of *UGA2* open reading frame and within the URA3 gene (Table 2).

To make the double deletion construct, *gad1::TRP1* cassette (see above) was used to transform the single *uga1::HIS3* deletion strain and the *uga2::URA3* deletion strain to make Δ*gad1*Δ*uga1* and Δ*gad1*Δ*uga2* strains respectively. The *uga1::HIS3* cassette was also transformed into the *uga2::URA3* deletion strain to make Δ*uga1*Δ*uga2* deletion strain, and finally Δ*uga1*Δ*uga2* double deletion was transformed by *gad1::TRP1* cassette to make the triple deletion construct of Δ*gad1*Δ*uga1*Δ*uga2*. In addition to the chromosomal PCR verification, all disruptions of *GAD1*, *UGA1*, *UGA2* in the single, double and triple deletion strains were verified by appropriate PCR for each of the single mutants as described above, and by failure to growth on YNB minimal medium containing 0.1% GABA or 0.2% ammonium sulfate as the sole nitrogen source. All deletion mutants except Δ*gad1* failed to grow on GABA-containing plates.

Plasmid Construction. The *S. cerevisiae* glutamate decarboxylase gene (*GAD1*) was PCR amplified from genomic DNA isolated from wild type strain W303-1A using a forward primer GAD1FOR (Table 2) and a reverse primer GAD1REV (Table 2). To facilitate plasmid construction, two restriction sites for *Sma*I and *Xho*I (underlined, Table

2) were introduced at each end of the GAD1 specific primers. Following PCR amplification, the 1.778-kb SmaI / XhoI fragment containing the entire gene was digested from the PCR product by SmaI and XhoI (Biolabs, New England) according to manufacturer's procedures and ligated into the unique SmaI / XhoI sites of yeast shuttle vector p425 GPD (American Type Culture Collection) using DNA ligase (Promega) according to manufacturer's procedure to create plasmid p425 GPD-GAD1. The *S. cerevisiae* GABA aminotransferase gene (*UGA1*) was PCR amplified as described above using forward primer UGA1FOR (Table 2) and reverse primer UGA1REV (Table 2). The 1.436-kb *Bam*HI / *Hind*III fragment (sites underlined in primers in Table 2) containing the entire gene digested from the PCR product and ligated into the unique BamHI / HindIII sites of p425 GPD as described above to create plasmid p425 GPD-UGA1.The *S. cerevisiae* succinate semialdehyde dehydrogenase gene (*UGA2*) was similarly PCR amplified using forward primer UGA2FOR (Table 2) and reverse primer UGA2REV (Table 2). The 1.504-kb *Sma*I / *Xho*I fragment (restriction sites underlined in the primers above) containing the entire gene was digested from the PCR product and ligated into unique *Sma*I / *Xho*I restriction sites of plasmid p425 GPD to create a plasmid p425 GPD-UGA2. The constructed plasmids p425 GPD-GAD1, p425 GPD-UGA1 and p425 GPD-UGA2 were used for overexpression in wild-type and single deletion strains of *Δgad1, Δuga1* and *Δuga2* by yeast transformation as described (Geitz and Woods, 2002).

Lethal heat stress survival assays. A modification of the procedure of Davidson and Schiestl (2001) was used. Briefly, yeast cells were grown to mid log phase (5×10^6 cells/ml) in YPD medium. To assess a possible protective effect of free spin trap reagent

N-tert-butyl-α-phenylnitrone (PBN, Sigma Chemical Co.), a final concentration of 5 mM PBN was added directly to the YPD medium for 4 – 5 h as described (Ren *et al*., 2005) before heat stress. Cells were harvested by centrifugation (15,000 g), washed in 0.87% NaCl, and concentrated to 2×10^8 cells/ml in fresh YPD medium. Aliquots of 100 µl were taken into 0.6-ml PCR tubes for each time point and heated at 45^{o}C in a thermocycler. At set time points, tubes were removed and placed on ice for 1 minute. Cells were then diluted so that colonies were countable and spread onto YPD plates. Colonies were counted after plates were incubated at 30^{o}C for 2 days, the survival rate was calculated against unheated cells. The effect of PBN on cell survival was determined by 60 min lethal heating, cell survival without PBN treatment served as control.

Induced thermotolerance assays. Exponentially growing cultures with and without PBN pretreatment were washed and concentrated to 2×10^8 cells/ml as described above. Aliquots of 100 µl in 0.6ml PCR tubes were preheated at 40°C in thermocycler for 30 min followed by quickly raising temperature to 50°C. Aliquots of cells were taken out at different time and placed on ice for 1 minute. Suitable dilutions of cells were plated on YPD plates and incubated at 30°C for two days to determine percent of survival as described earlier. The effect of PBN on induced cell survival was determined by 45 min heating under 50°C after preheating at 40°C, cell survival without PBN treatment served as control.

Measurement of ROS production. Intracellular ROS generated during heat stress were measured using 2', 7'-dichlorofluoroscin diacetate (DCFH-DA) essentially as described by Davidson (2001). With uptake of the probe, the intracellular esterase

removes the acetate groups, resulting in the formation of the non fluorescent substrate 2', 7'-dichlorofluoroscin (DCFH). Subsequent oxidation of DCFH produces highly fluorescent 2', 7'-dichlorofluoroscein (DCF) which absorbs at a wavelength of 504 nm and emits at a wavelength of 524 nm (Wang and Joseph, 1999).

Fluorescence was measured for cells both with and without PBN pretreatment, under lethal heat directly, and cells heated after preheat treatment. Overnight culture growing in SC or selective media were diluted into 10 ml of fresh YPD media to 0.2 OD_{600} and allowed to grow at 30°C on a shaker until an OD_{600} of 1.0 was achieved. Cells were then washed twice and concentrated to 2×10^8 cells/ml in phosphate-buffered saline (pH7.4) (PBS). A 5 mM stock solution of dichlorofluorescin diacetate dissolved in ethanol was added to each culture to reach a final concentration of 10 μM and incubated at 30°C for 15 min to allow entry of the probe into the cells. Five hundred μl of each culture with DCFH-DA included was taken for heating under different temperature conditions as described in the text, table and figure legends. Immediately after heating, cells were cooled on ice for 1 min, washed twice in ice-cold distilled water and resuspended in 100 μl of ice cold distilled water. Cells were disrupted by agitation on a vortex mixer with an equal volume of glass beads at maximum speed five times for 1 min each. Between agitation, samples were cooled on ice for 1 min. The clear supernatant was collected after 2.5 min centrifugation in a microcentrifuge at maximum speed in a cold room (4°C) and kept on ice. Thirty μl of each supernatant was mixed with 2.5 ml of water and the fluorescence was recorded at 25°C at an excitation wavelength (Ex) of 504 nm and an emission (Em) wavelength of 524 nm, using a Hitachi F-2000

spectrofluorimeter. Background fluorescence was estimated using cells heated without DCFH-DA and using DCFH-DA without any cells.

Measurement of intracellular GABA, glutamate, α-ketoglutarate and succinate semialdehyde. Exponentially growing cells were harvested, washed and suspended in fresh YPD to 2×10^8 cells/ml. Aliquots of 500 µl were submitted to lethal heat stress at 45°C for varying periods of time as indicated in text, table and figure legends. Immediately after stress, cells were cooled on ice for 1 minute, washed twice with distilled water, and quickly flash frozen in liquid nitrogen and resuspended in 500 µl distilled water. Cell dry weight was determined by incubation at 105°C overnight until there is no change of weight.

Intracellular glutamate and GABA were extracted by boiling cells for 10 min. Intracellular succinate semialdehyde was extracted by breaking the cells with glass beads. After centrifugation for 5 min (15,000 g), the supernatant was collected for measurement of intracellular amino acids. Cell extract for α-ketoglutarate was prepared by boiling ethanol method as described in Gonzalez *et al*. (1997).

The amount of GABA was determined in a 200 µl reaction mixture which contained 86 mM potassium phosphate buffer (pH 8.6), 3.3 mM 2-mercaptoethanol, 1.2 mM β-NADP, and 0.004 unit of GABAase (Sigma-Aldrich Fine chemicals, St. Louis, MO), and an appropriate amount of the cell free extract prepared as above. The reaction was initiated by the addition of 5 mM α-ketoglutarate after pre-incubation of components at 30^oC for 5 min.

Glutamate assays were performed in a 200 μl reaction mixture which contained 90 mM triethanolamine hydrochloride (pH 7.3), 60 μM NAD^+, 250 μM EDTA, 0.06 units of L-glutamic dehydrogenase (sigma-Aldrich Fine chemicals, St. Louis, MO) and an appropriate amount of the cell free extract prepared as above.

α-ketoglutarate was determined in a 200 μl reaction mixture which contained 90 mM triethanolamine hydrochloride (pH 7.3), 53 mM ammonium acetate, 60 μM NADH, 250 μM EDTA, 0.06 units of L-glutamic dehydrogenase, and an appropriate amount of cell free extract prepared as described above. Succinate semialdehyde was determined 200 μl reaction mixture which contained 87 mM potassium phosphate buffer (pH8.4), 3 mM 2-mercaptoethanol, 1.3 mM β-NAD, 0.83% glycerol and 0.025-0.05 unit of yeast succinate semialdehyde dehydrogenase (Ramos *et al*., 1985).

The enzymatic determination of GABA, glutamate and succinate semialdehyde is based on the change in absorbance at 340 nm caused by the reduction of $NAD(P)^+$ to NAD(P)H and the enzymatic determination of α-ketoglutarate is based on the change in absorbance at 340 nm caused by the oxidation of NADH to NAD^+. All enzymatic reactions are performed at 30^{o}C for 60 min and the change of absorbance at 340 nm was monitored using microplate spectrophotometer (Power Wave™ XS, BioTek instruments, Inc, Winooski, VT, USA). Concentrations of glutamate, α-ketoglutarate, GABA and succinate semiladehyde were expressed as μmol/g cell dry weight and calculated from the calibration curve of the standard solutions. All assays were repeated at least three times and data represented the mean ± SD of the results from at least three experiments.

Results

Gene disruption of GABA shunt enzymes and heat sensitivity.

A *GAD1*, *UGA1* and *UGA2* wild type strain, and the isogenic deletion strains Δ*gad1*, Δ*uga1*, and Δ*uga2* were subjected to lethal heat stress at 45^{o}C for 15, 30, 45 and 60 min (Fig. 1). After 60 min, the wild type cells showed 65% survival compared to wild- type without heat treatment. The Δ*gad1*, Δ*uga1, and* Δ*uga2* deletions showed 30%, 19%, and 25% survival respectively. The double deletions of Δ*gad1*Δ*uga1*, Δ*gad1*Δ*uga2* and Δ*uga1*Δ*uga2* showed an enhanced heat sensitivity and survival of 10%, 18% and 1%, respectively after 60 min at 45^{o}C. While the viability of the triple deletion strain (Δ*gad1*Δ*uga1*Δ*uga2)* was 0% .

Induced thermotolerance in GABA shunt mutant strains.

Induction of thermotolerance by pretreatment at a sublethal temperature of 40^{o}C for 30 min before exposure to a lethal temperature of 50^{o}C in wild-type and the isogenic deletion mutants of GABA shunt enzymes was investigated. The survival rate at 50^{o}C for 5 min without pretreatment in wild-type was 14% and below 10% in all deletion strains (Fig. 3A and Fig. 3B). Survival of all mutant and wild-type strains increased at 50^{o}C with preheating at sublethal temperature. Wild-type showed survival above 50% and mutant strains showed survival up to 40% after 15 min at 50^{o}C. After longer exposure at 50^{o}C, the survival rate of the deletion strains declined more rapidly than in the wild-type.

ROS production during heat stress in wild-type and GABA shunt mutants

It has been shown that lethal heat stress damages cells by production of reactive oxygen species (ROS) including hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2) and

the super oxide anion ($O_2\bullet^-$), which can damage macromolecules and other cellular components (Cryer *et al*., 1975; Davidson, and Sehiestl, 2001; Wang and Joseph, 1999). The level of intracellular oxidants produced during lethal heating at both 45°C, and 50°C after preheating at 40°C for 30 min was measured by monitoring the oxidation of DCFH-DA.

Fig. 4 shows when cells of the deletion strains were exposed to lethal heating at 45°C, DCFH oxidation increased 2- to 5-fold compared to unheated controls, while in wild-type, the increase was less than 1-fold. Generation of higher level of intracellular ROS in mutants is consistent with survival of the yeast cells at higher temperature. With longer exposure to heat, significantly higher levels of DCFH oxidation were observed in single, double and triple mutants than in wild-type (Fig. 4). After preheating at 40^{o}C for 30 min (Fig. 6), the level of ROS increased 3- to 9- fold in deletion strains after exposure at 50^{o}C, while in wild-type this increase was about 1.2- fold. These findings are consistent with the results of the survival experiments. The viability of deletion mutant cells was correlated with the level of DCFH oxidation observed.

This correlation of heat stress tolerance with oxidation status of DCFH is further substantiated by results summarized in Fig. 5 (compared to Fig. 2) where overexpression of the three enzymes of the GABA shunt in each of the three respective single mutant strains led to levels of DCFH oxidation similar to wild type. Overexpression of *GAD1, UGA1, or UGA2* in wild type resulted in either wild type levels of DCFH oxidation (*GAD1*), or reduction in ROS production below the levels found in heat treated wild type cells (*UGA1* or *UGA2*). These data further support the hypothesis that all the three

enzymes in the GABA shunt pathway are involved in restricting the intracellular levels of ROS during lethal heat stress.

To determine whether the increased level of ROS is essential for heat induced cell death, or ROS is just a byproduct of the heat stress, oxygen radicals were scavenged with free radical spin trap, PBN. Wild-type and all mutant strains were cultured at 30°C. The cultures were pretreated with PBN at a final concentration of 5 mM for 5 h. Cells were then submitted to heat stress for the determination of cell survival and production of ROS. As shown in Fig. 7, under 45 °C lethal heating for 60 min, PBN treatment had no significant effect on cell survival for wild-type, however, the cell survival was greatly enhanced for all mutants from 1-fold (*Δgad1* mutant) up to more than 10-fold (*Δgad1Δuga1Δuga2*), comparatively, in Fig. 8, the accumulation of ROS in the control decreased from 1- to 2-fold for mutant strains by the pretreatment of PBN, while the accumulation of ROS had no significant change. Similar effects had been found for the cells stressed under 50°C with preheating treatment (Fig. 9 and Fig. 10). These results suggest by scavenging ROS production under heat stress, cells were more able to tolerate heat stress, the ROS accumulation played an essential role in heat induced cell death.

Effect of GABA shunt deficiency on intracellular metabolite levels.

GABA is produced from decarboxylation of L-glutamate by glutamate decarboxylase (*GAD1*). GABA and α-ketoglutarate is then converted to succinate semialdehyde and glutamate by GABA transaminase (*UGA1*) which is then used to make GABA. Changes in the levels of GABA, glutamate, α-ketoglutarate and succinate semialdehyde during lethal heat stress in wild-type and mutant strains were investigated.

Under normal growth conditions, GABA level was very low in wild type and all strains bearing deletion mutants. Under lethal heat stress at 45^{o}C, an 80-fold increase in GABA occurred in the wild-type in 15 min and remained at that level for up to 60 minutes of stress (Fig. 11). However, in the Δ*gad1* strain and in all double and triple mutant strains containing Δ*gad1*, GABA levels increased less than 3-fold during 15 minutes of heat stress at 45^{o}C and remained comparatively lower at all time points up to 60 minutes (Fig. 11). In strains bearing a *uga1* deletion (Δ*uga1 and* Δ*uga1*Δ*uga2*) the intracellular GABA pool increased over 160-fold during 15 minutes of heat stress at 45^{o}C. This value increased to over 200-fold after 60 minutes of heat stress. Slightly lower increases in GABA levels were observed in the *uga2* deletion mutants at all time points examined. These results suggest GABA accumulation during heat stress requires a functional *GAD* gene, and that the highest levels of GABA accumulation occur in strains with impaired GABA degradation (Δ*uga1*, Δ*uga2*, *and* Δ*uga1*Δ*uga2*).

In unstressed wild type cells glutamate levels were substantially higher than GABA levels (39.2 μmole/g dry weight versus 0.065 μmole/g dry weight), and glutamate levels increased 3.2-fold and 4.6- fold during 15 minutes and 60 minutes respectively at 45^{o}C (Fig. 12). The same general pattern of glutamate accumulation was observed in all mutant strains examined, but the absolute levels accumulated in all strains bearing a *gad1* mutation (Δ*gad1*, Δ*gad1*Δ*uga1*, Δ*gad1*Δ*uga2*, and Δ*gad1*Δ*uga1*Δ*uga2*) were approximately doubled at each time point compared to the wild-type strain. Glutamate levels in Δ*uga1*, Δ*uga2*, Δ*uga1*Δ*uga2* were approximately the same as or slightly lower than wild type. These results confirm that GAD1p is the crucial enzyme involved in glutamate consumption in yeast cells.

In unstressed wild type W303-1A cells, α-ketoglutarate concentrations were approximately 1/3 the glutamate levels (13 versus 39 μmole/g dry weight). Following heat stress at 45^{o}C, α-ketoglutarate accumulated linearly for at least 60 minutes reaching 61 μmole/g dry weight by 60 minutes of heat stress (Fig. 13). Similar linear patterns of α-ketoglutarate accumulation were observed for all deletion strains, but accumulation rates were significantly higher in Δ*uga1* and Δ*uga2* strains (6.6-fold and 6.1-fold respectively after 60 minutes) compared to wild type (Fig. 13). The Δ*uga1*Δ*uga2* double mutant strain demonstrated the higher rate of α-ketoglutarate accumulation (7.3-fold after 60 minutes) compared to wild-type (Fig. 9). In all strains carrying a *gad1* deletion (Δ*gad1*, Δ*gad1*Δ*uga1*, Δ*gad1* Δ*uga2* and Δ*gad1*Δ*uga1*Δ*uga2*), the accumulation of α-ketoglutarate showed a slow linear pattern of accumulation reaching only 2-fold after 60 minutes of heating at 45^{o}C. Since α-ketoglutarate is not a product of the reaction catalyzed by GAD1p, it is not clear why strains bearing a *gad1* mutation accumulate α-ketoglutarate. By comparison,α-ketoglutarate is a substrate consumed in the reaction catalyzed by UGA1p. Thus, strains bearing a *uga1* mutation would logically accumulate greater levels of α-ketoglutarate.

Since succinate semialdehyde is a metabolite unique to the GABA shunt, accumulation of this metabolite was investigated. Unstressed wild type cells produced 2.95 μmol/g dry weight SSA while the Δ*uga2* strain produced 4.37 μmol/g dry weight SSA. All of the other mutants produced less than 2.5 μmol/g dry weight SSA. Heat stress at 45^{o}C produced a linear accumulation of SSA only in wild type and in the Δ*uga2* strain while no SSA accumulation occurred in any of the other deletion mutants (Fig. 14). SSA accumulation levels in Δ*uga*2 were nearly double the levels accumulated in wild type.

Mutation of *gad1* or *uga1* reduces accumulation of SSA while mutation of *uga2* leads to high levels of SSA accumulation.

Discussion

Mutation of each of the 3 genes of the GABA shunt resulted in a reduction in tolerance of yeast cells to a lethal heat stress at 45ºC and to heat stress at 50ºC following an inductive period at 40ºC (Fig. 1 and Fig. 3). Deletion of *GAD1* resulted in the least reduction in stress tolerance, while deletion of *UGA1* resulted in the greatest effect. All double mutants were at least as sensitive to heat stress as Δ*uga1* (e.g. Δ*gad1* Δ*uga2*) or more heat sensitive (Δ*gad1* Δ*uga1 and* Δu*ga1* Δ*uga2*) than the single mutants, and the triple mutant was most sensitive. Essentially identical results were obtained whether the cells were grown on YPD medium (Fig. 1) or YNB minimal medium with ammonium sulfate as nitrogen source (data not shown) indicating that the rich source of amino acids present in YPD medium does not contribute to the heat stress tolerance phenotype of wild type or the mutants. Tolerance of lethal heat stress essentially reverted to wild type levels when each of the single genes was transformed into the respectively comparable mutant driven by a strong promoter (Fig. 2), and overexpression of each gene in wild type resulted in only small increases in heat tolerance. This further demonstrates a role for the enzymes of the GABA shunt in abating some aspect of heat stress. Tolerance to even more severe heat stress (50^{o}C) can be induced by pretreatment of cells at sublethal stress of 35-40^{o}C (Davidson and Schiestl, 2001). The sensitivity of the GABA shunt mutants used above to 50^{o}C heat stress following a 40^{o}C adaptation period was proportionately similar to the 45^{o}C lethal heat stress above (Fig. 3 compared to Fig. 1). Thus, the basic

mechanism(s) involved in abating heat stress by function of the enzymes of the GABA shunt are critical to basal thermotolerance but appear not to be involved in induced thermotolerance (Lindquist and Kim, 1996).

The enzymes of the GABA shunt have been shown to play a role in protecting yeast cells against damage from exogenous oxidative stress generated by H_2O_2 (Coleman *et al*., 2001). Similarly in *Arabidopsis thaliana* SSADH appears to play a role in heat and UV stress tolerance by restricting ROS accumulation during episodes of stress (Bouche *et al*., 2003). Thus, the production of ROS was investigated in mutants of the GABA shunt. Either during lethal heat stress at 45^{o}C (Fig. 4 and Fig. 5) or at 50^{o}C following an inductive period at 40^{o}C (Fig. 6) in all single, double, and triple mutants and in cells expressing various genes on plasmids, the level of heat-stress-induced ROS produced relative to wild type was proportional to the lethality of the mutant(s) relative to wild type. The presence of 5 mM free radical scavenger PBN strongly inhibited the ROS accumulation in the mutant strains (Fig. 8 and Fig. 10) at both stress conditions and enhanced the cell survival. Furthermore, in the single mutants, the heat-stress-induced ROS production was reversed when the respective gene was overexpressed on a plasmid. These observations are consistent with previously published results that the production of ROS is a critical component of basal heat-stress-induced damage to yeast cells (Davidson and Schiestl, 2001) and with the conclusion that heat-stress-induced-damage to yeast cells is prevented by the function of the enzymes of the GABA shunt through a mechanism that involves abatement of the accumulation of ROS during heat stress.

However, the role of the enzymes of the GABA shunt pathway in protecting cells from heat stress damage involving heat-induced ROS accumulation is not clear. In yeast

the GABA shunt pathway is known to be involved in the assimilation of supplied glutamate, and to feed the carbon from this metabolite into the respiratory chain within the mitochondria with succinate (Pietruszko and Fowden, 1961; Ramos *et al*., 1985). As expected, all strains bearing a *GAD1* deletion demonstrated a reduction in the intracellular pool of GABA produced during heat stress (Fig. 11), while strains bearing a deletion of *UGA1* accumulated the highest heat-stress-induced levels of GABA. *UGA2* deletion strains produced intermediate GABA levels during heat stress. The effect of deletion of *GAD1* was epistatic to deletion of *UGA1* or *UGA2* which is consistent with the hypothesis that GAD1p is the primary if not exclusive source of GABA derived from glutamate. Since the Δ*gad1* strain, the least thermosensitive strain and the strain producing lowest ROS levels during heat stress, produced essentially identical heat-stress-induced levels of GABA with the triple mutant strain, the most thermosensitive and highest heat-stress-induced ROS producing strain, yet neither strain produced even wild type levels of GABA, it would appear that GABA itself is not a metabolite directly or indirectly involved in the protection of yeast cells from thermo-damage. Specifically, the accumulation of GABA in these mutants does not support a role for GABA as a signaling molecule during heat stress even though GABA is known to be a powerful signaling molecule in animal systems (Calver *et al*., 2000; Gamel-Didelon *et al*., 2003, Geigerseder *et al*., 2003).

The highest levels of heat-stress-induced glutamate were observed in the triple mutant strain, but all other strains bearing a *GAD1* deletion produced accumulated glutamate pools that were larger than wild type. Strains bearing a *UGA1* or *UGA2* deletion (without a *GAD1* deletion) produced lower levels of glutamate during heat stress

relative to wild type. The opposite patterns were observed for α-ketoglutarate, i.e. in strains bearing *GAD1* deletions, α-ketoglutarate levels were lower than wild type during heat stress while in strains bearing *UGA1* or *UGA2* deletions, α-ketoglutarate was elevated relative to wild type. The fact that glutamate and α-ketoglutarate levels are altered dramatically during heat stress in GABA shunt mutants is consistent with the hypothesis that the GABA shunt is a major pathway for the assimilation of glutamate during episodes of stress. However, the specific pattern of glutamate and α-ketoglutarate accumulation in the mutants does not suggest a specific mechanism for limiting ROS production, but rather that flux through the pathway is the critical feature for heat-stress tolerance and limiting ROS production.

This conclusion is further substantiated by examination of succinate semialdehyde (SSA) accumulation (Fig. 14). All of the mutant strains except Δ*uga2* accumulated little or no SSA during heat stress beyond the level found in unstressed cells. However, unexpectedly, wild type accumulated substantial SSA during heat stress, and the Δ*uga2* strain accumulated even higher levels of SSA. This accumulation was dependent on functional *GAD1* and *UGA1* genes, and thus the function of the complete pathway is required for the production of SSA. However, the accumulation of SSA in the various mutants does not suggest that this metabolite plays an exclusive role in mediating ROS production during heat stress.

Taken together, it appears that carbon flux through the entire GABA shunt is required to abate ROS production during heat stress. This suggests that moving carbon from α-ketoglutarate to succinate may be the function most critical to abating ROS production and consequent damage during heat stress. Clearly, this function is only

efficiently accomplished by the 3 enzymes of the GABA shunt working in concert, and disturbance of any one of these enzymes can have a variable effect on the pathway based on alternative competing mechanisms for moving carbon past various steps in the pathway. Because it is likely that the greatest flux of carbon from α-ketoglutarate to succinate occurs inside the mitochondria as part of the tricarboxylic acid cycle and UGA1p and UGA2p both appear to be cytosolic proteins in *Saccharomyces cerevisiae* (Chapter IV), it is a reasonable working hypothesis that the compartmentation of metabolites into the cytosol and/or the vacuole may be a critical aspect of the function of the GABA shunt. Such a movement of these metabolites out of the mitochondria would clearly have an effect on the redox status of the mitochondria during stress and thus could affect ROS accumulation.

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Table 1. Yeast strains used in this study

Strain	Genotype	Source and description
W303-1A	Mat a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*	ATCC[a], WT
Δ*gad1*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met gad1::TRP1*	This study, *gad1* disruptant
Δ*uga1*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga1::HIS3*	This study, *uga1* disruptant
Δ*uga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga2::URA3*	This study, *uga2* disruptant
Δ*gad1Δuga1*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met gad1::TRP1 uga1::HIS3*	This study, *gad1 uga1* double disruptant
Δ*gadΔ1uga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met gad1::TRP1 uga2::URA3*	This study, *gad1 uga2* double disruptant
Δ*uga1Δuga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga1::HIS3 uga2::URA3*	This study, *uga1 uga2* double disruptant
Δ*gad1Δuga1Δuga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met gad1::TRP1 uga1::HIS3 uga2::URA3*	This study, *gad1 uga1 uga2* triple disruptant

[a]ATCC, American Type Culture Collection

Table 2. Primers used for *GAD1*, *UGA1* and *UGA2* gene cloning, deletions and verifications

Primer Name	Sequence (5' to 3')
GAD1FOR	GAGACCCGGGATGTTACACAGGCACGGTTCTAAG
GAD1REV	GTGTCTCGAGTCAACATGTTCCTCTATAGTTTCTCG
UGA1FOR	GAGAGGATCCATGTCTATTTGTGAACAATACTACCCA
UGA1REV	GTGTAAGCTTTCATAATTCATTAACTGATTTGGCTAA
UGA2FOR	GAGACCCGGGATGACTTTGAGTAAGTATTCTAAACCAAC
UGA2REV	GTGTCTCGAG TTAAATGCTGTTTGGCAAATTCC
	CACGTCGCTCTTAACAATCCAGGCTGAACAAAACAAGGA
GAD1UP45TRP1A	ATAATGGGAAGCATTTAATAGACAGCATCGT
	TACATACATATAGGGGGCGGTATATTGGATGACCTTTTC
GAD1DN45TRP1D	AACTCAAGGCAAGTGCACAAACAATACT
GAD1/TRP1KO_A	TGCGTTTATAAATAATCTTTCTGGC
GAD1/TRP1KO_B	CATCTTTACCAGCATTCTTCATTCT
GAD1/TRP1KO_C	ATTCAGGGTATAGAACACAATTCCA
GAD1/TRP1KO_D	TATTCCCGCATAACTTTTCTATCAC
UGA1HIS3FOR	GGGGTACCCCAGAACAGACAAGAAACCGTCA
UGA1HIS3REV	GAT GAG CTC GCG GCC TCG CTA ATA TAC AAT
	CCAGCTACATTAAAAGCAAATTTTACAAACTACTATTTCA
UGA2UP45URA3A	ACATGCGGTTTCTTTGAAATTTTTTTGA
	ACATGAAACCATACCAGTTTCCAAAGCTTCAGACACAGT
UGA2DN45URA3D	GTATTAGGGTAATAACTGATATAATTAAATTGAAGC.
UGA2/URA3KO_A	CGGTCGTTGAAGTGCTATAGTTTAT
UGA2/URA3KO_B	AGGTTGCCTAATTGTGAATACTCTG
UGA2/URA3KO_C	ACTTTTACTGGTTCTACAAACGTCG
UGA2/URA3KO_D	GTGAAAAACTTCAAAACTCCGTAAA

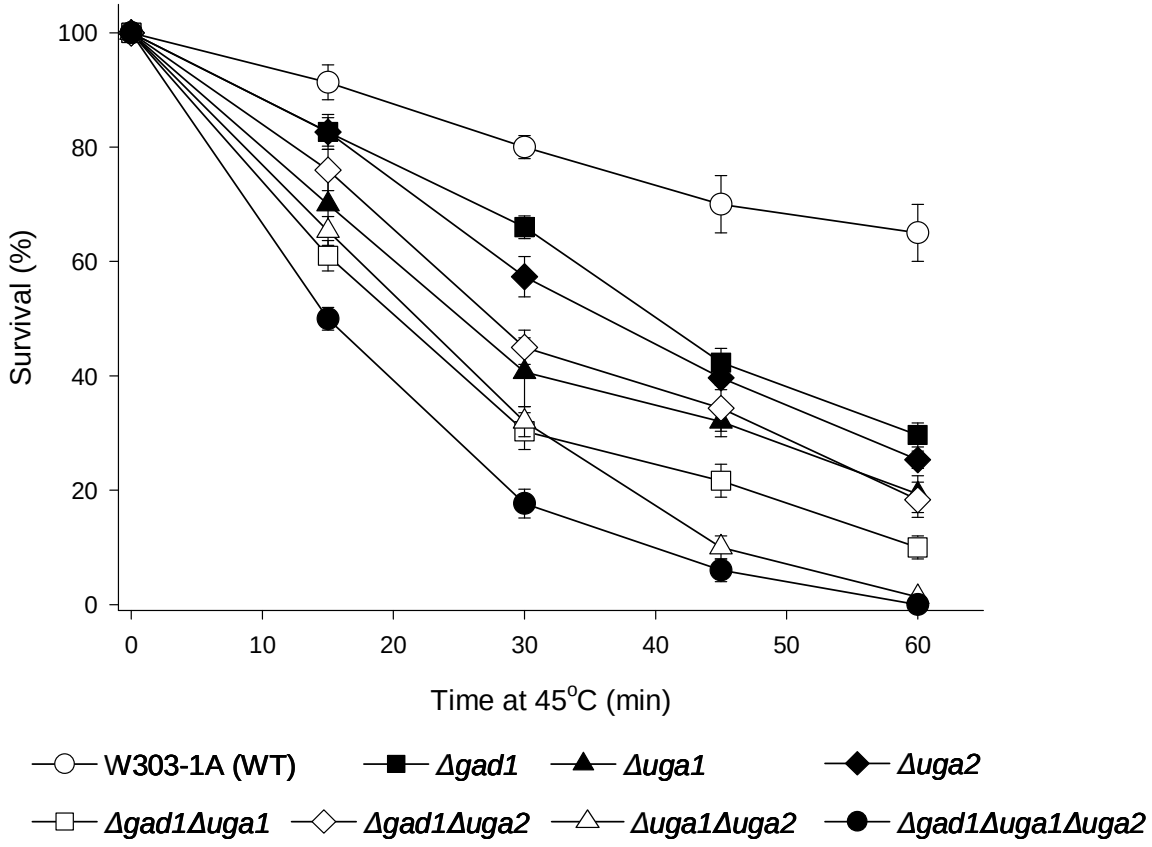

Fig. 1. Viability of wild-type and mutant strains after lethal heat treatment at 45°C. Strains were grown to mid log phase (5×10^6 cells/ml) in YPD, washed in 0.87% NaCl, and concentrated to 2×10^8 cells/ml in fresh YPD. Aliquots of 100 µl were heated at 45°C in a thermocycler for 0, 15, 30, 45 and 60 min. Viability was determined as described. Each point represents the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

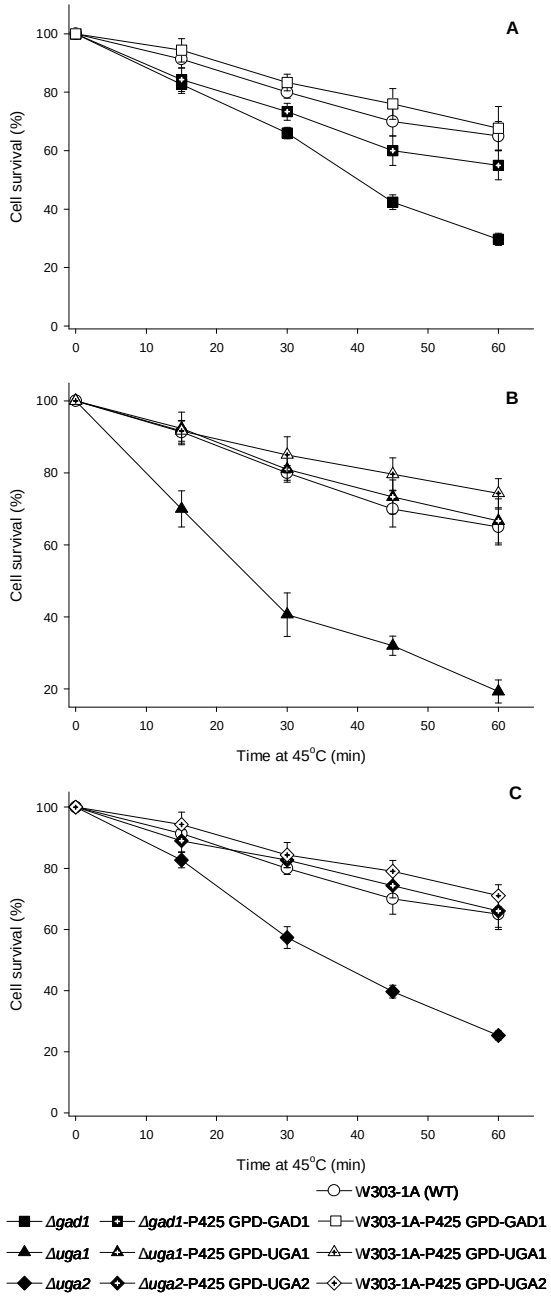

Fig. 2. Viability of wild-type and *Δgad1*mutant with overexpression of yeast GAD1 (A), *Δuga1* mutant with overexpression of yeast *UGA1* (B) and *Δuga2* mutant with overexpression of yeast *UGA2* (C) after lethal heat stress at 45°C. Heat stress was performed and viability was determined as the same in Fig. 1. Each point represents the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes. Data for empty vector were not shown.

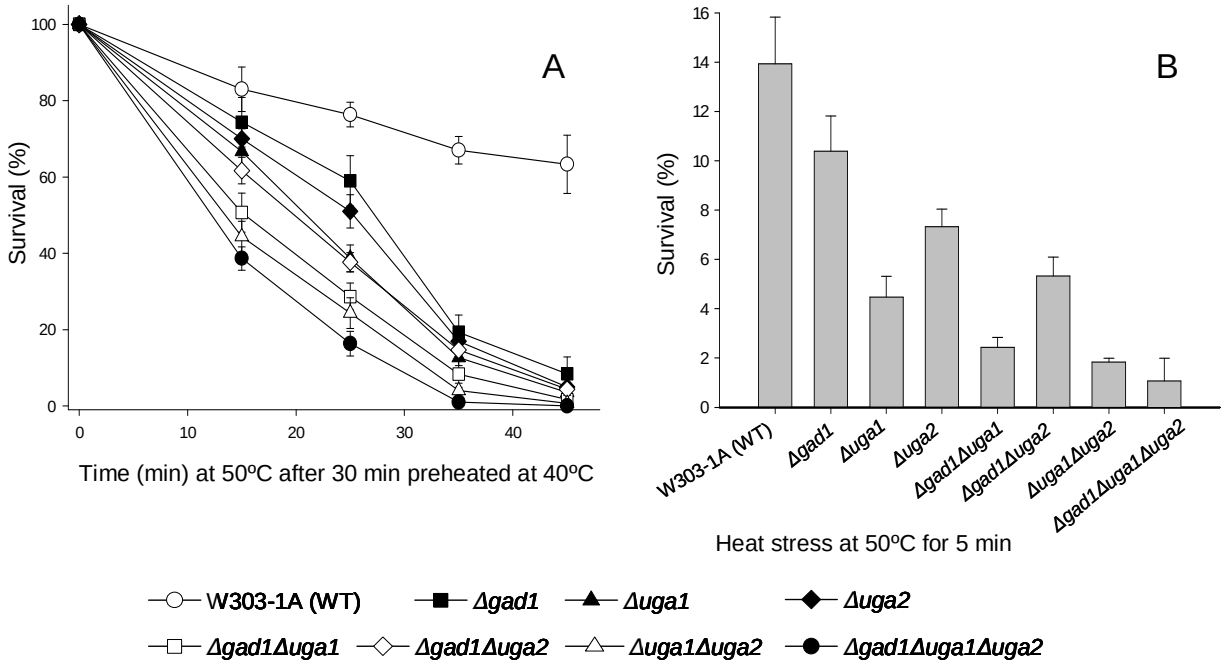

Fig. 3. Viability of wild-type and mutants at lethal heat stress 50°C. Mid log phase cells grown in YPD were collected and washed in 0.87% NaCl, aliquots of 100 µl concentrate in fresh YPD were heated at 50^{o}C preceded by sub-lethal heat stress at 40°C for 30 minutes (A), as a control, cells were heated at 50°C for 5 min without preheat treatment (B). Cell survival was determined as described. Each point represents the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

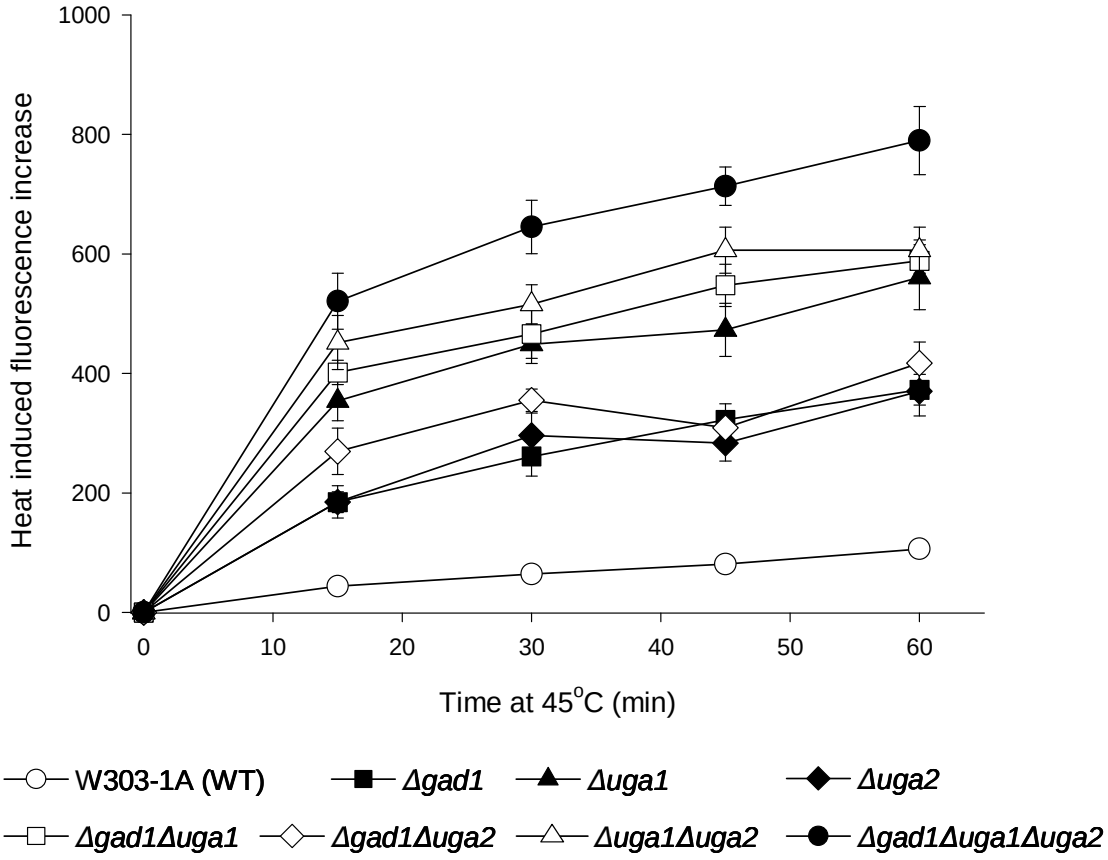

Fig. 4. Intracellular ROS levels induced by lethal heat stress at 45ºC in wild-type and mutant strains. Fluorescence was measured at an excitation wavelength of 504nm and an emission wavelength of 524nm in crude cell extracts as described. As a control, fluorescence was recorded during heat stress without cells and cells without DCFH-DA (date not shown). Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

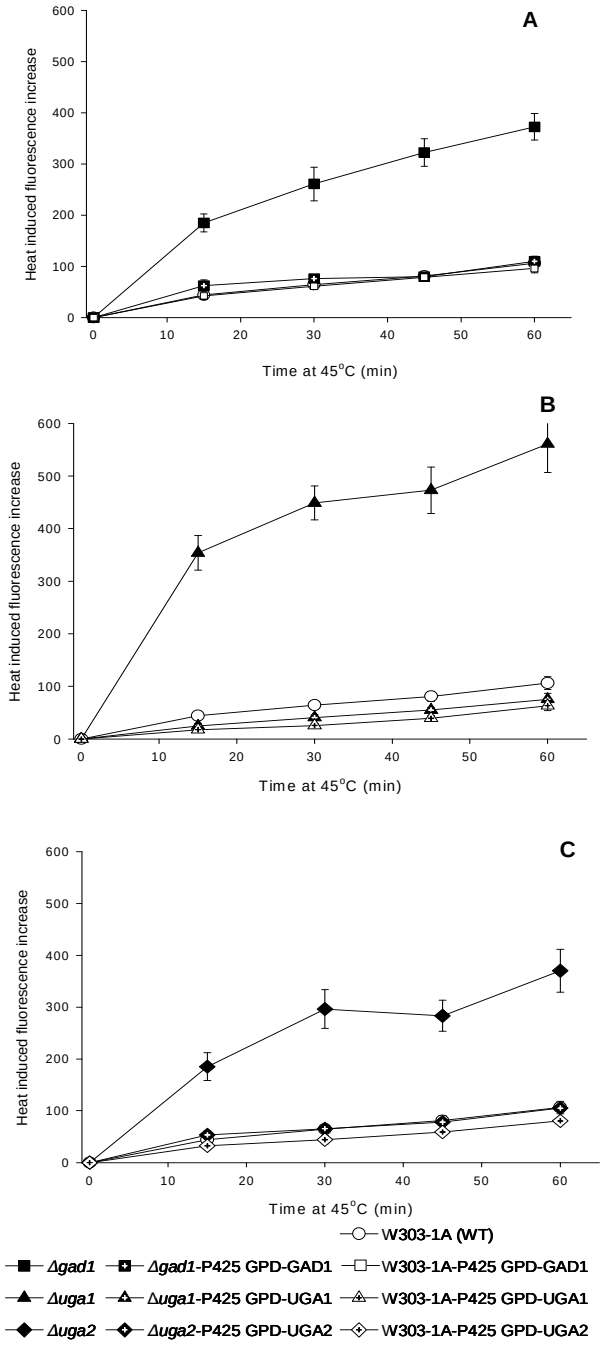

Fig. 5. Intracellular ROS levels induced by lethal heat stress at 45ºC in wild-type and Δ*gad1*mutant transformed with plasmid P425 GPD- GAD1 (A), Δ*uga1* mutant transformed with plasmid P425 GPD- UGA1 (B), Δ*uga2* mutant transformed with plasmid P425 GPD- UGA2 (C). Fluorescence was recorded the same as described in Fig. 4. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

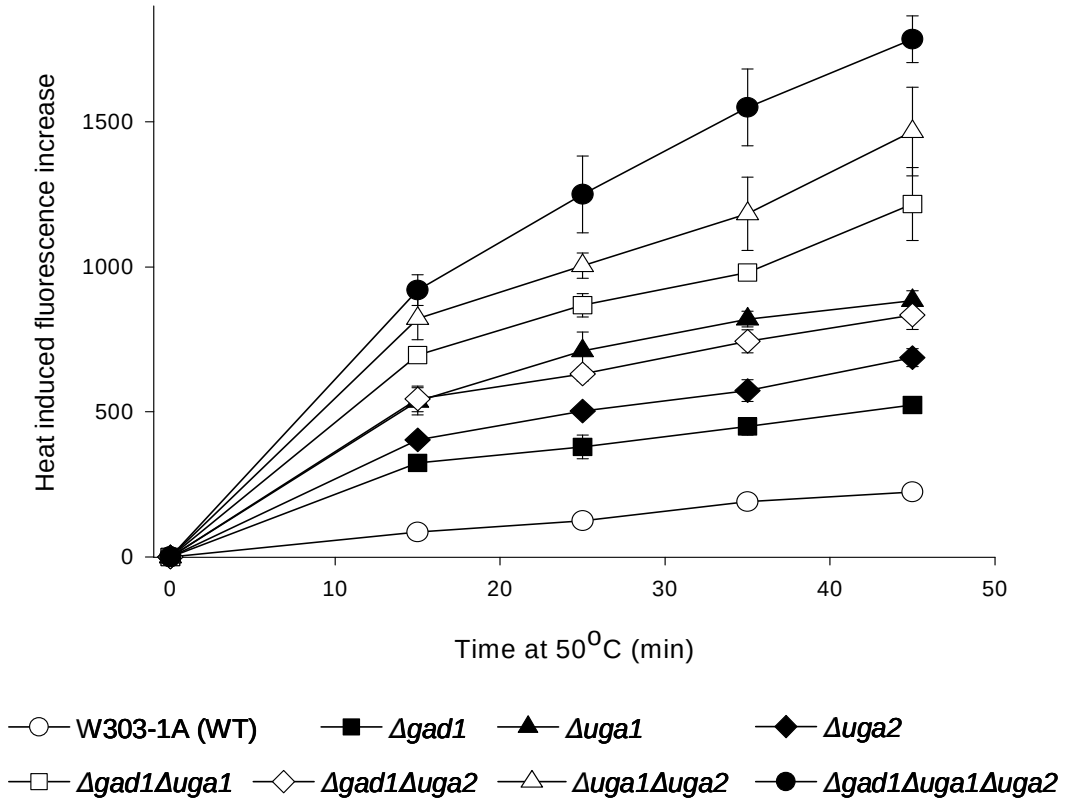

Fig. 6. Intracellular ROS levels induced by lethal heat stress at 50ºC after a sub-lethal heat stress at 40 ºC for 30min in wild-type and mutant strains. Fluorescence was recorded the same as described in Fig. 4. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

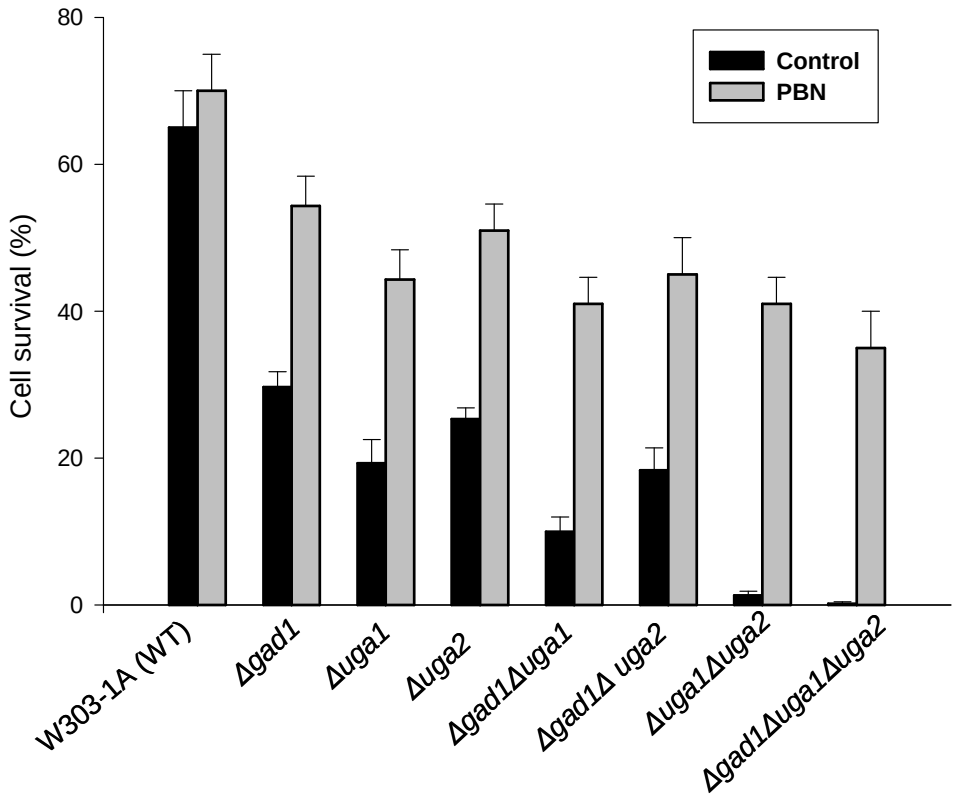

Fig. 7. Effect of PBN on lethal heat induced cell death under 45ºC for wild-type and all mutant strains. Cells of wild-type and mutants were cultured at YPD medium at 30°C. The free spin trap reagents PBN (5 mM) was added directly to the cell cultures for 5 h before heat stress. Cell survival was determined as described for a 60 min point. Cells without PBN treatment server as control. Data shown represent the mean ± SD of the results from at least three experiments.

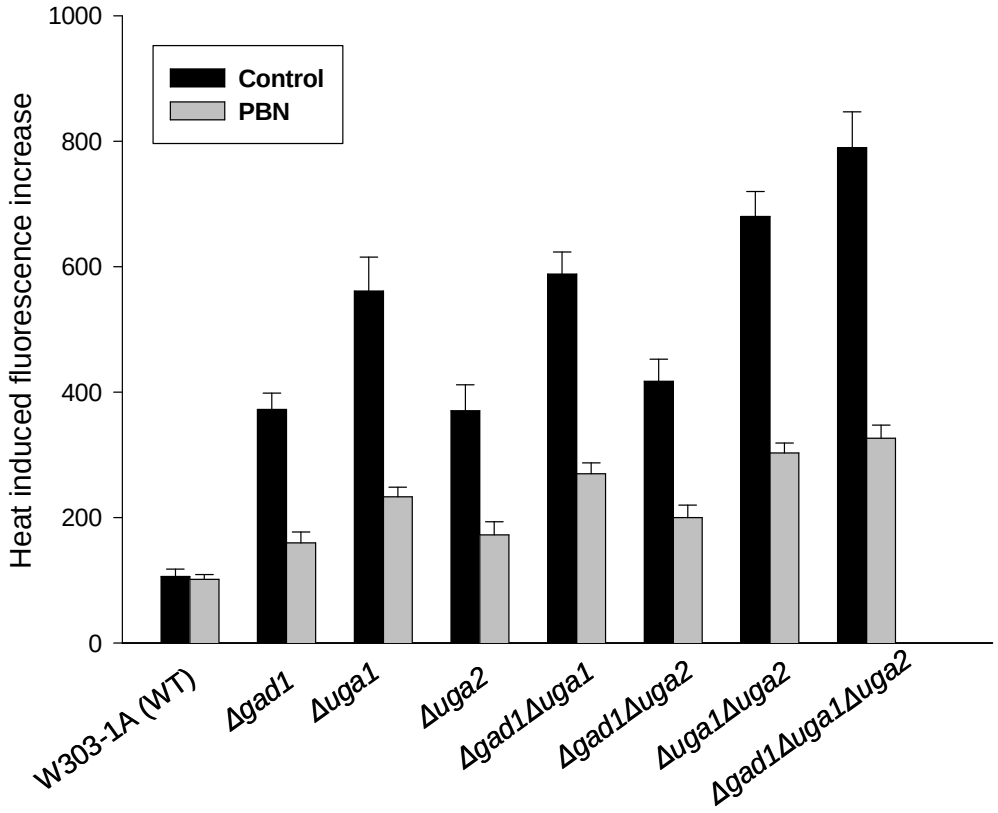

Fig. 8. Effect of PBN on production of ROS under 45ºC for wild-type and all mutant strains. Cells of wild-type and mutants were cultured at YPD medium at 30°C. The free spin trap reagents PBN (5 mM) was added directly to the cell cultures for 5 h before heat stress. Fluorescence was recorded the same as described in Fig. 4 for a 60 min point. Cells without PBN treatment server as control. Data shown represent the mean ± SD of the results from at least three experiments.

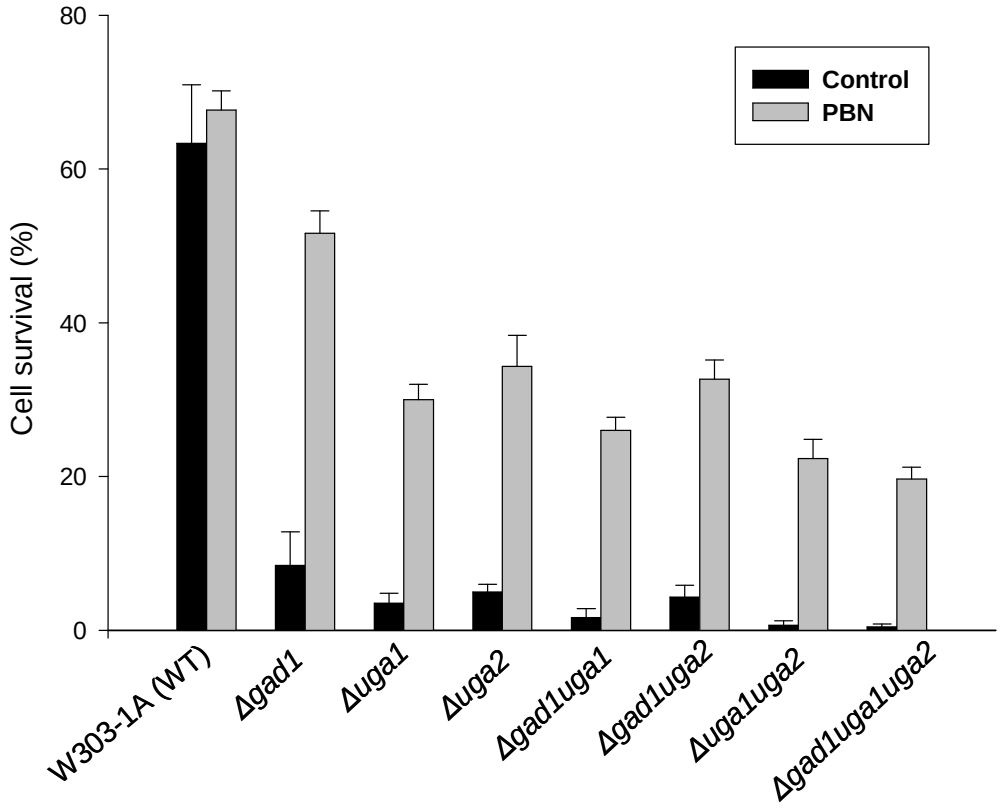

Fig. 9. Effect of PBN on heat induced cell death under 50ºC with preheat treatment at 40 ºC for 30min for wild-type and all mutant strains. Cells of wild-type and mutants were cultured at YPD medium at 30°C. The free spin trap reagents PBN (5 mM) was added directly to the cell cultures for 5 h before heat stress. Cell survival was determined as described for a 45 min point. Cells without PBN treatment server as control. Data shown represent the mean ± SD of the results from at least three experiments.

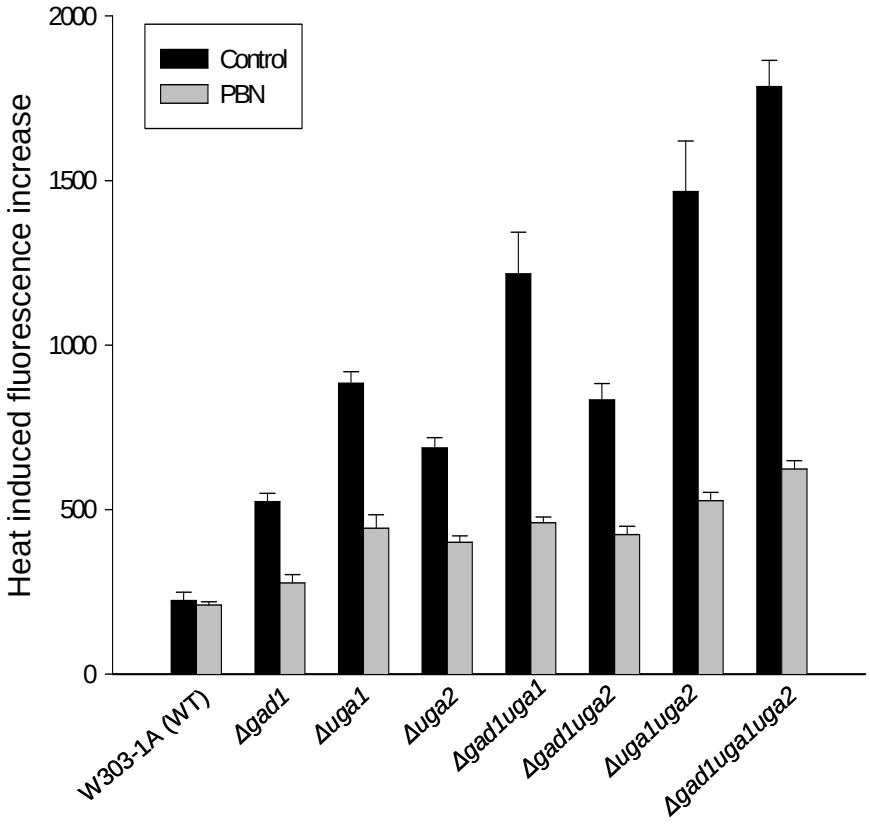

Fig. 10. Effect of PBN on production of ROS under 50ºC with preheat treatment at 40 ºC for 30min for wild-type and all mutant strains. Cells of wild-type and mutants were cultured at YPD medium at 30°C. The free spin trap reagents PBN (5 mM) was added directly to the cell cultures for 5 h before heat stress. Fluorescence was recorded the same as described in Fig. 4 for a 45 min point. Cells without PBN treatment server as control. Data shown represent the mean ± SD of the results from at least three experiments.

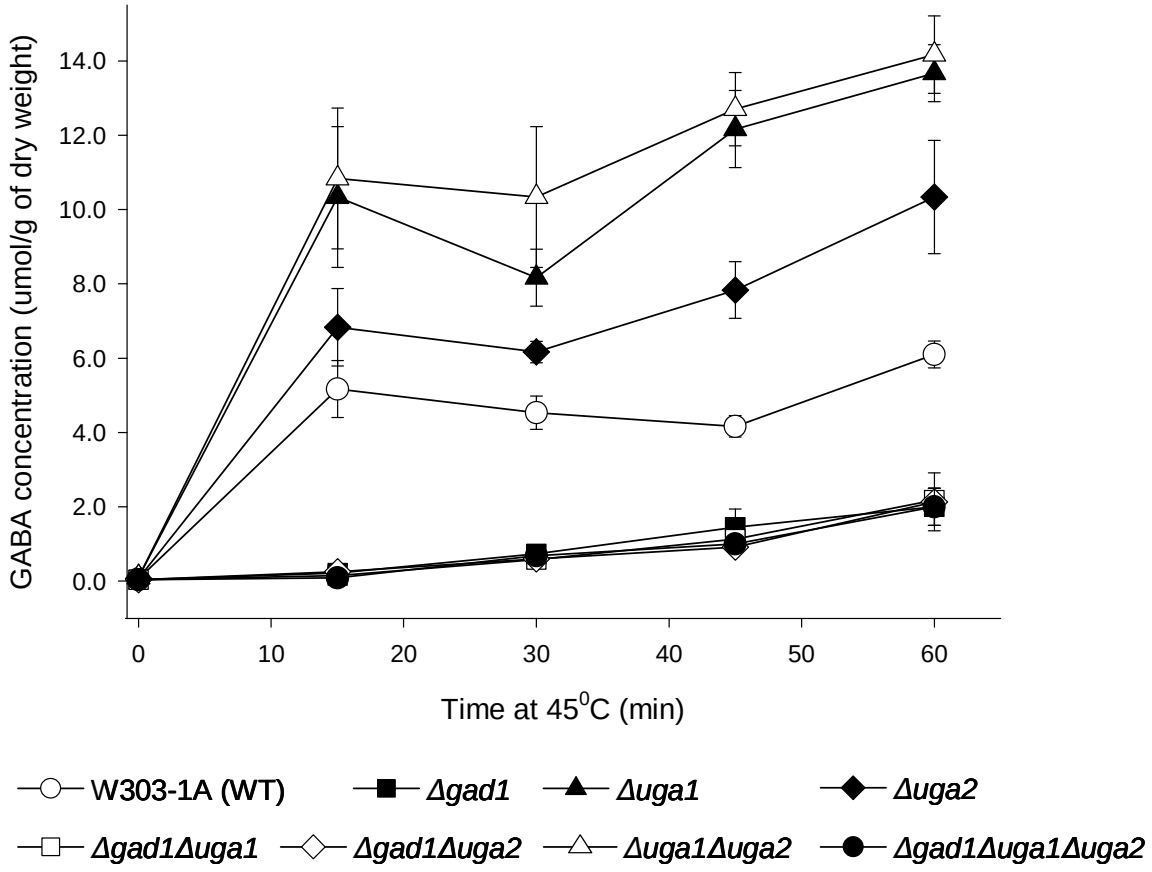

Fig. 11. Changes in the levels of GABA under lethal heat stress at 45^{o}C in wild-type and mutant strains. Intracellular GABA was extracted from heat stressed cells by boiling cells for 10 min. After centrifugation for 5 min (15,000g), supernatant was taken for determination of GABA by GABase as described. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

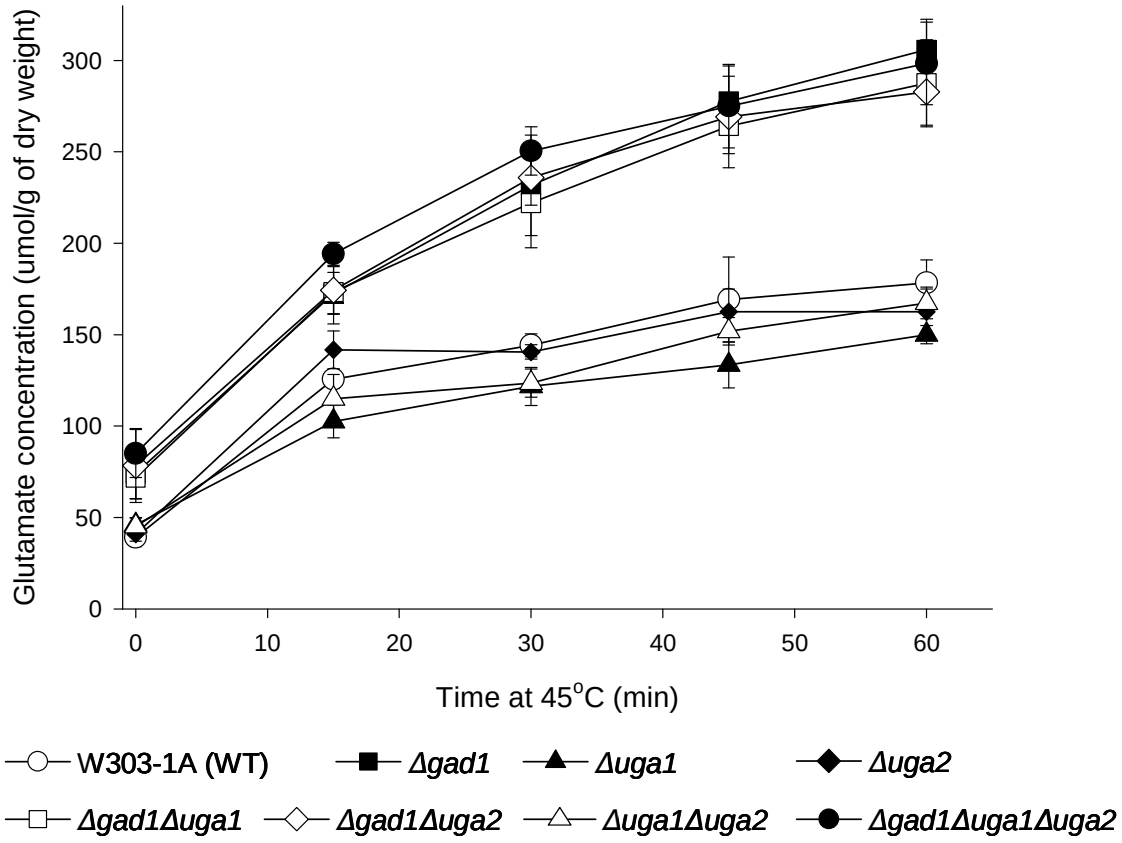

Fig. 12. Changes in the levels of Glutamate under lethal heat stress of 45°C in wild-type and mutant strains. Intracellular Glutamate was extracted from heat stressed cells by boiling cells for 10 min. After centrifugation for 5 min (15,000g), supernatant was taken for determination of Glu by glutamate dehydrogenase as described. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

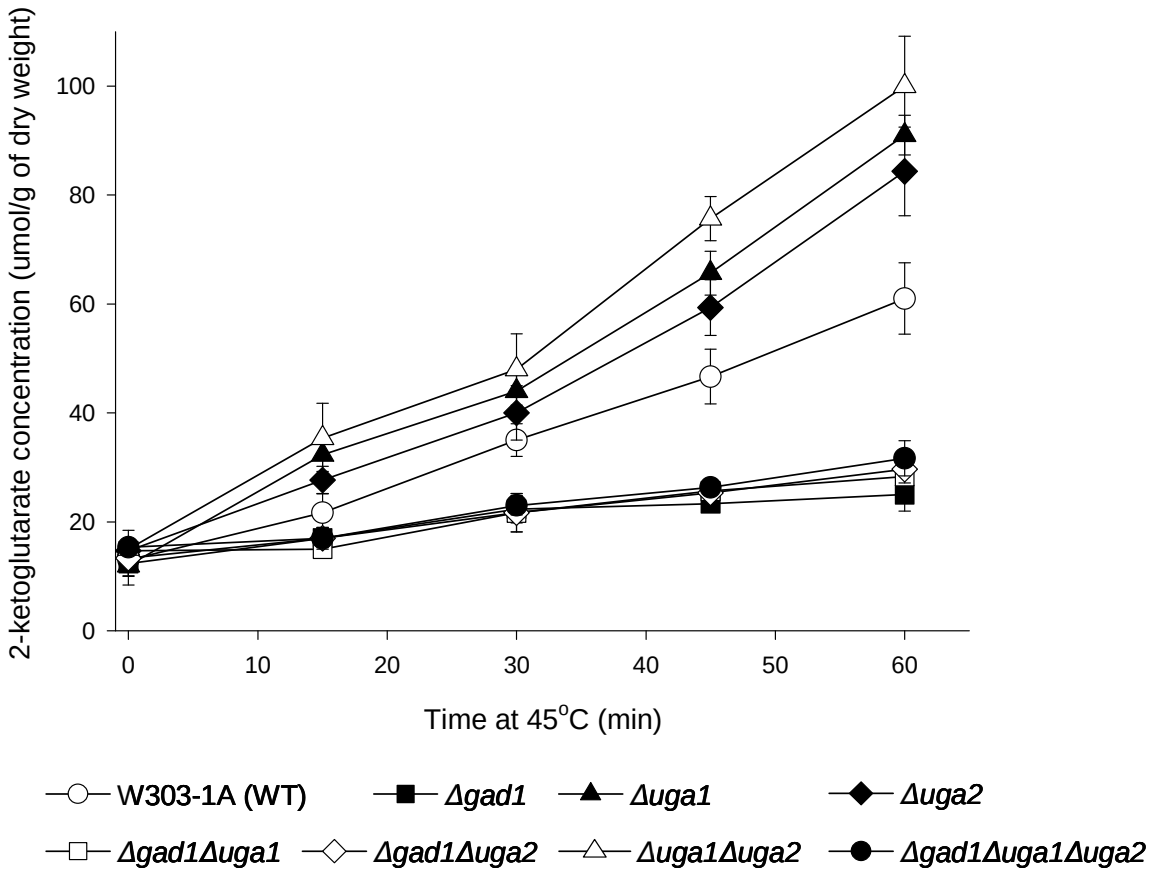

Fig. 13. Changes in the levels of a-ketoglutarate under lethal heat stress at 45°C in wild-type and mutant strains. Cell extracts for intracellular a-ketoglutarate from heat stressed cells were prepared by boiling ethanol method as described (12). α-ketoglutarate was determined by glutamate dehydrogenase assay as described. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

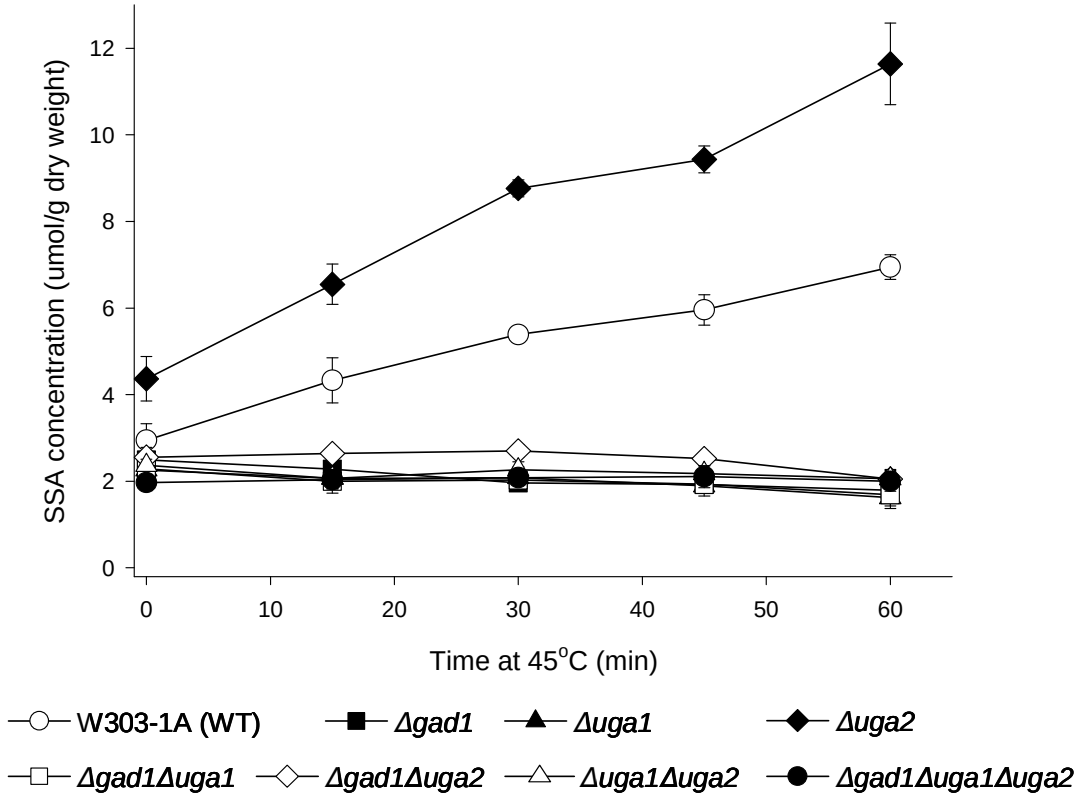

Fig. 14. Changes in the levels of succinate semialdehyde under lethal heat stress at 45°C in wild-type and mutant strains. Intracellular succinate semialdehyde was extracted by breaking the cells with glass beads**.** After centrifugation for 5 min (15,000g), supernatant was taken for determination of SSA by succinate semialdehyde dehydrogenase as described. Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes.

III. GABA SHUNT GENE EXPRESSION ANALYSIS AND HEAT STRESS RESPONSE OF THE GABA SPECIFIC TRANSCRIPTION FACTOR AND TRANSPORT GENES IN *SACCHAROMYCES CEREVISIAE*

Abstract

The GABA shunt pathway is composed of three enzymes: glutamate decarboxylase (GAD, coded by *GAD1*), GABA aminotransferase (GABA-TA, coded for *UGA1*), and succinate semialdehyde dehydrogenase (SSADH, coded by *UGA2*). In *Saccharomyces cerevisiae*, the expression of *UGA1*, *UGA2*, and a GABA permease gene, *UGA4* is regulated by the GABA specific transcription factor, *UGA3*. GABA permease *UGA4* mediates the transportation of GABA into vacuoles. The deletion mutation of *UGA3* grown in minimal medium with GABA as sole nitrogen source resulted in heat sensitivity, while *Δuga3* conferred heat tolerance if grown on YPD or minimal medium with non GABA nitrogen sources. While a deletion mutant of *Δuga4* was more heat tolerant than wild type on YPD medium is equivalent to wild type on all other media. The increased heat tolerance of the deletion mutant strains was not correlated with the expression of the enzymes of the GABA shunt, but appears to be at least partially mediated by the expression of ROS scavenging superoxide dismutases. The transcripts of GABA shunt genes, *UGA3* and *UGA4* were induced by GABA, and the expression of

UGA1, *UGA2* and *UGA4* were also induced by acidic pH. Under lethal heat stress at 45ºC, each of the GABA shunt genes together with the regulatory genes of *UGA3* and *UGA4* were up-regulated in wild-type strain. Deletion mutation of the transcription factor *UGA3* repressed the transcription activation of GABA transaminase (*UGA1*) and GABA permease (*UGA4*), but did not result in the change of the induction pattern for glutamate decarboxylase (*GAD1*) compared to the wild-type. Deletion mutation of *UGA4* did not have significant effect on the expression pattern of *GAD1*, *UGA1*, *UGA2*, or *UGA3* under lethal heat stress at 45ºC. These results suggest that GABA shunt pathway and the regulatory genes of *UGA3* and *UGA4* play an important role in utilizing GABA as a nitrogen source and the GABA shunt protects yeast cells from heat damage through transcriptional up-regulation, which is partly regulated by the transcription factor *UGA3*.

Introduction

GABA is a four carbon non protein amino acid which is widely found in all prokaryotic and eukaryotic organisms (Kumar and Puneker, 1997). The GABA shunt is a metabolic pathway consisting of three enzymes: glutamate decarboxylase (GAD, encoded by the *GAD1* gene) catalyzing the conversion of glutamate (Glu) to GABA, GABA aminotransferase (GABA-AT, encoded by the *UGA1* gene) catalyzing the conversion of GABA and α-ketoglutarate (α-KG) into succinate semialdehyde (SSA) and Glu, and succinate semialdehyde dehydrogenase (SSADH, encoded by the *UGA2* gene) catalyzing the NAD(P)-dependent conversion of SSA into succinate. These three enzymes act in concert to move carbons from α-ketoglutamate to succinate bypassing two reactions of the tricarboxylic acid cycle.

In yeast *Saccharomyces cerevisiae*, the *UGA1* and *UGA2* genes have been shown to play an essential role in the metabolism of GABA (Ramos *et al*., 1985) since these two enzyme activities are GABA-inducible. Such GABA-induced regulation is at least partially regulated through the GABA-specific transcription factor encoded by the *UGA3* gene (Vissers *et al*., 1989). The import of GABA into cells is regulated by three GABA up-take systems: the general amino acid permease (GAP1p), the proline specific permease (PUT4p), and the GABA permease (UGA4p) (Grenson *et al*., 1987). The transcriptional activation of *UGA1*, *UGA2*, and *UGA4* is positively regulated by the GABA-specific transcription factor, UGA3p, and a general nitrogen utilization related protein, UGA35p or DAL81p (Coornaert *et al*., 1991). The enzyme activity of *UGA4* has been shown to be induced by the presence of GABA (Ramos *et al*., 1985) and it was also demonstrated that the induction of *UGA4* transporter activity by GABA correlates with strong accumulation of *UGA4* RNA transcript (Andre *et al*., 1993). In addition, in the absence of GABA, UGA4p activity was also shown to be induced by acidic pH (Moretti *et al*., 2001).

To cope with the damaging effects from the environmental and physiological stresses such as heat stress, osmotic stress, or oxidative stress, yeast cells have developed rapid molecular responses by changing their gene expression patterns (Estruch, F.. 2000). Microarray analysis has shown that the expression of the three GABA shunt genes are up-regulated under mild heat stress at 37ºC (Gasch *et al*., 2000; Sakaki *et al*., 2003), while the expression of *UGA3* is unaffected (Gasch *et al*., 2000). However, these results have not been directly confirmed, and there is a lack of information on how the GABA shunt

genes are regulated under lethal heat stress temperature and the role of the GABA regulatory and transport genes (*UGA3* and *UGA4*) in the heat stress response.

Previously, we have shown that the three genes of GABA shunt are involved in thermotolerance under lethal heat stress at 45ºC by restricting the accumulation of reactive oxygen species and altering metabolite levels during episodes of lethal heat stress (Chapter II). Here, we examine the role the GABA specific transcription factor and transport genes of *UGA3* and *UGA4* in lethal heat stress and report their mediation on the expression of the GABA shunt genes under lethal heat stress temperature. Additionally, we examined the role of the *UGA3* regulated *UGA4* (GABA transporter) in lethal heat stress. Cell survival and RT-PCR analysis were conducted in the wild-type and deletion mutants of *uga3* and *uga4.*

Materials and Methods

Yeast strains and growth media

Yeast strains used in this study were all derived from stain W303-1A (*Mat a leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*) and the precise genotypes are listed in Table 1. In various experiments as detailed in text, table and figure legends, the strains were grown on either YPD medium or YNB medium at 30ºC and shaken at 250-300 rpm. YPD medium contains: 2% glucose, 1% yeast extract, 2% yeast bactopeptone, and was used for expression and cell survival analyses under heat stress. YNB medium contains: 0.67% [wt/vol] yeast nitrogen base, 2% glucose, supplemented with essential amino acids (Sherman *et al*., 1979).

Gene disruptions

The GABA specific regulatory gene *UGA3* and GABA permease gene *UGA4* were disrupted similarly as *GAD1* as described in Chapter II by replacing the open reading frame with *TRP1* gene. The *TRP1* gene was amplified from plasmid pRS414 template DNA using a forward primer (named UGA3UP45TRP1A for *UGA3* and UGA4UP45TRP1A for *UGA4*, Table 2) that consisted of the 45 nucleotides immediately upstream of the *UGA3* or *UGA4* gene fastened upstream of the 5'most portion of the *TRP1* gene contained in pRS414. The reverse primer (named UGA3DN45TRP1D for *UGA3* and UGA4DN45TRP1D for *UGA4*, Table 2) consisted of the 45 nucleotides immediately downstream of the *UGA3* or *UGA4* gene ligated to the 22 nucleotides at the 3' end of the *TRP1* gene contained in pRS414.

The PCR product was then transformed into yeast strain W303-1A by the lithium acetate method as previously described (Geitz and Woods, 2002), and TRP^+ colonies were selected for genomic DNA isolation. The correct integration was verified by isolating genomic DNA (Hofman and Winston, 1987) from each selected strain and conducting a series of PCR reactions using primers that land 300-500 nucleotides upstream and downstream of *UGA3* or *UGA4* open reading frame and within the *TRP1* gene (Table 2).

Cell growth with different nitrogen sources and pH

The standard minimal medium (without nitrogen source) as described by Ramos *et al*. (1985) was used for wild-type cell growth with ammonium sulfate, GABA, or glutamate as sole nitrogen source at pH 6.0. Minimal medium with ammonium sulfate as

sole nitrogen source at pH 4.0 was used to evaluate the acidic pH effect on gene expression. Nitrogen sources were added to a final concentration of 0.1%. Mid-log phase cells grown at 30°C on a rotary shaker (200 rpm) were collected by centrifugation at 3000 x g for 5 min at room temperature, frozen rapidly in liquid nitrogen and stored at -80ºC for total RNA isolation.

Lethal heat stress at 45ºC

For cell survival analysis, cells of wild-type and mutants of *uga3* and *uga4* were grown in YPD, YNB, minimal media with sole nitrogen sources of GABA, Glu and NH_4^+ (Ramos *et al*., 1985) respectively until mid-log phase. Cell survival assay was performed and evaluated as described in Chapter II except sample cells were stressed for 30 min. For gene expression analysis, mid-log phase cells of wild-type and mutants of *Δuga3* and *Δuga4* grown in YPD at 30°C were shifted to 45°C for 0, 5, 15, 30, 45 and 60 min. After heat stress, cells were put on ice for 1 min. Samples were then collected by centrifugation at 3000 ×g for 5 min at room temperature, frozen rapidly in liquid nitrogen and stored at -80ºC for total RNA isolation.

Total RNA extraction and reverse transcriptase PCR (RT-PCR)

Total yeast RNA was isolated as described by Peper (1997) with minor modification. Harvested yeast cells were washed with DEPC (diethylpyrocarbonate) treated water and then resuspended in 0.6 ml RNA extraction buffer (10 mM EDTA (ethylenediaminetetracetic acid), 50 mM Tris-HCl pH 7.5, 0.1M NaCl, 5% SDS), 0.6 ml of phenol:chloroform:isoamyl alcohol (50:50:1) mixture and 2 g of glass beads (0.45 mm diameter). Cells were broken by vigorous agitation for 5 min on a vortex mix set at

maximum speed with 1 min on ice between each 1 min intervals. The organic phase was separated from upper aqueous phase by centrifugation at 3000 x g for 5 min at room temperature. The supernatant was collected, extracted twice with equal volume of phenol: chloroform: isoamyl alcohol (50:50:1) and once with equal volume of chloroform: isoamyl alcohol (24:1). The RNA was precipitated by the addition of 0.1 volume of 3M NaOAc, pH 5.2, plus 2.5 volumes of ice-cold absolute ethanol and resuspended in DEPC treated water. RNA concentration was determined by measuring OD260 spectrophotometrically.

First strand cDNA was synthesized from 4 μg of total RNA using the SuperScript first-strand synthesis kit (Invitrogen, CA, USA) following manufactory's instructions. cDNA was amplified in a volume of 25 μl containing 1U Taq DNA polymerase, 1× reaction buffer, 20 pmol each gene specific primer set for *GAD1*, *UGA1*, *UGA2*, *UGA3*, *UGA4*, *SOD1*, *SOD2* and *ACT1* (Table 2), 200 μM dNTP, 1.5 mM $MgCl_2$, and 2 μl cDNA RT product. PCR products were separated on 0.8% agarose gels, and photographed under a UV light. *ACT1* gene was used for internal control.

Results

Gene expression responses to different nitrogen sources and pH

To examine the expression of the GABA shunt genes *GAD1*, *UGA1* and *UGA2*, *UGA3,* and *UGA4* by different nitrogen sources and different pH's, RT-PCR experiments were carried out in strain W303-1A growing on minimal medium with GABA, minimal medium with glutamate, and minimal medium with ammonia as the sole nitrogen source respectively. Fig. 1 shows that the expression of all of these genes was the lowest with

NH_4^+ (pH 6.0) as sole nitrogen source. The expression of all of these genes was strongly induced by the presence of GABA or glutamate when compared to ammonia, but the expression of all of the genes was greatest with GABA as nitrogen source as compared with glutamate. These results demonstrate that the GABA shunt and the transcription factor *UGA3* and the GABA permease *UGA4* play an important role in utilizing GABA as a nitrogen source in yeast *Saccharomyces cerevisiae.*

When cells were grown in minimal medium with NH_4^+ at pH 4.0, the expression of *UGA1* and *UGA4* which encode GABA transaminase and GABA permease respectively was highly induced compared to growth on the same medium at pH 6.0. The expression of *UGA2* which encodes succinate semialdehyde dehydrogenase (SSADH) was also induced, but at a lower level than *UGA1* and *UGA4*. However, *GAD1*, the first enzyme of GABA shunt and *UGA3*, which encodes a GABA specific transcription factor, were not regulated by the acidic pH. These data suggest that *UGA1*, *UGA2* and *UGA4* play more important roles than *GAD1* and *UGA3* in acid growth condition. Even though the expression of *GAD1* and *UGA3* are GABA inducible, they are regulated by a different mechanism from the *UGA1*, *UGA2*, and *UGA4* genes.

Cell survival under lethal heat stress of *Δuga3* and *Δuga4*.

UGA3 is a transcription factor that is known to positively regulate the transcription of *UGA1*, *UGA2*, and *UGA4* (Coornaert *et al*., 1991). *UGA4* is a GABA transport protein (Grenson *et al*., 1987). In Chapter II, we have shown that the GABA shunt mutant strains of *Δgad1*, *Δuga1* and *Δuga2* were sensitive to lethal heat stress at 45ºC compared to the wild-type. Here, we investigated the role of the genes of *UGA3* and *UGA4* in response to lethal heat stress.

A *UGA3* and *UGA4* wild type strain (W303-1A), and the isogenic deletion strains Δ*uga3* and Δ*uga4* grown in YPD, YNB, and minimal medium with different nitrogen sources (GABA, Glu and NH_4^+) were subjected to lethal heat stress at 45^oC for 30 min. As shown in Fig. 2., for the cells grown in rich YPD medium, after 30 min, the wild type cells showed 78% survival compared to the unstressed wild-type cells. The *Δuga3,* and *Δuga4* deletions showed 90%, and 85% survival respectively, indicating that deletions of *UGA3* and *UGA4* result in cells that are more heat tolerant than wild-type. Cells grown in other GABA-free media such as YNB, minimal medium with Glutamate as the sole nitrogen source and minimal medium with NH_4^+ as the sole nitrogen source showed similar heat-tolerance results for *Δuga3,* and *Δuga4* (data not shown). However, when cells were grown in minimal medium with GABA as the sole nitrogen sources, *Δuga3* was found to have a defective, slow growth compared to the wild-type (data not shown), while the *Δuga4* strain was found to grow normally. When cells grown on GABA as the sole nitrogen source were submitted to lethal heat stress as described above, after 30 min, wild-type and *Δuga4* cells did not have significant survival change compared to the cells grown in rich YPD medium, showing 75% and 82% survival respectively. However, *Δuga3* had a dramatic decrease in survival rate, showing 60% survival, indicating a heat sensitive phenotype when grown with GABA as the sole nitrogen source.

Analysis of gene expression pattern for GABA shunt enzymes and the GABA specific regulatory and transport genes (*UGA3* and *UGA4*) under lethal heat stress

To examine the possible effect of lethal heat stress on the expression pattern of *GAD1*, *UGA1*, and *UGA2* and the GABA specific regulatory (*UGA3)* and transport

(*UGA4*) genes, RT-PCR was performed to evaluate and compare the expression levels of transcripts during lethal heat stress at 45ºC in wild type and the *Δuga3* and Δ*uga4* mutants. Changes were observed at 5, 15, 30, 45, and 60 min following the initiation of heat stress. In the wild type strain, *GAD1* was rapidly induced upon heat stress and the expression was gradually increased at each time point (Fig. 3A). The expression level was increased above 6- fold after 60 min of heat stress. *UGA1* showed a similar gradual induction during 60 min heat treatment, but exhibited a more rapid induction upon 5 min of heating (3- fold increase) and peaked at 60 min (above 6- fold increase). *UGA2* and *UGA3* showed a similar induction pattern to *GAD1*, but the induction rates were slower for the first 15 min (1- to 2- fold increase), and then rapidly induced after 15 min. A 4-fold increase and above a 5- fold increase were observed for *UGA2* and *UGA3* respectively at 60 min. The basal expression level for *UGA4* was low, almost undetectable without heat stress. Upon heat stress, *UGA4* was gradually induced at each time point, but exhibited a slower rate of induction than any of the other genes investigated. Only a 2-fold induction was detected after 60 min heat stress. Thus, these results indicate that in the wild-type yeast strain, the expression of the three genes in the GABA shunt pathway and the GABA specific regulatory and transport genes of *UGA3* and *UGA4* respectively are all induced during heat-stress even though at slightly different levels.

To further examine how the *UGA3* and *UGA4* genes regulate the expression of GABA shunt enzymes under heat stress, the expression patterns of the same genes as above were determined in the Δ*uga3* and Δ*uga4* deletion mutant strains under lethal heat stress at 45ºC. In *Δuga3* (Fig. 3B), the loss of a functional *UGA3* coding sequence

resulted in the elimination of a detectable *UGA3* transcript. *GAD1* was rapidly and transiently induced upon heat stress at 45ºC for 5 min compared to the unstressed control, and then the expression rapidly decreased to the control level at the15 min time point. After 15 min, the expression again increased through 60 min after heat stress. *UGA2* gene expression showed a gradual induction pattern, but the induction was slow, the expression was non-detectable at the 0 and 5 min time points. However, no expression of *UGA1* and *UGA4* was observed either before or after heat stress in *Δuga3*. These results support the hypothesis that the transcription factor *UGA3* gene is required for the transcriptional induction of the *UGA1* and *UGA4* genes which is in agreement with Vissers *et al*.(1989). Additionally, the transcriptional induction of *UGA1* and *UGA4* but not *GAD1* and *UGA2*, is also mediated by *UGA3* during episodes of heat stress. The transcriptional regulation of *GAD1* and *UGA2* appears to be involved in the mechanism of heat stress defense although this regulation appears to involve other mechanisms of activation.

In the deletion mutant of Δ*uga4* (Fig. 3C), loss of the functional coding sequence resulted in non detectable expression of *UGA4* before or after heat stress. The expression levels of *GAD1* and *UGA2* were relatively low before heat stress compared to those in the wild-type but gradually increased between 15 and 60 min. and between 30 and 60 min. respectively. *UGA1* was also rapidly and gradually induced upon heat stress but to a relative higher level than in the wild-type. The expression of *UGA3* was rapidly increased after 5 min heat stress, but then decreased to a level slightly higher than the control and maintained that level until 60 min following the initiation of heat stress. These data indicate that the induction of *GAD1*, *UGA1*, *UGA2* and *UGA3* at the transcriptional level

is result of heat stress, but the induction is independent of *UGA4* although the failure to properly transport GABA may result in quantitative effects on the expression of these genes.

Disruption of the regulatory and transport genes (*UGA3* and *UGA4*) and antioxidant gene expression analysis under lethal heat stress.

Heat stress has been documented to produce oxidative stress (Davidson *et al*., 1996; Davidson and R. H. Schiestl, 2001a; 2001b), and to some extent, heat stress is equivalent to oxidative stress (Sugiya *et al*., 2000). In chapter II, it was shown that cell survival of wild-type and all GABA shunt mutant strains were correlated to the levels of ROS accumulated under lethal heat stress. Here, we analyzed the gene expression of the antioxidant genes *SOD1* and *SOD2* (coding for cytosolic and mitochondrial localized superoxide dismutases respectively) in the wild-type and mutant cells under lethal heat stress.

As shown in Fig. 4, before heat stress, the expression level of mitochondrial *SOD* (*SOD2*) was higher than the cytosolic *SOD* (*SOD1*) in all wild-type and mutant strains of *Δuga3* and *Δuga4*. However, both *SOD1* and *SOD2* had much higher levels of expression in the mutant strains of *Δuga3* and *Δuga4* (Fig. 4B and Fig. 4C) compared to the wild-type (Fig. 4A). Upon lethal heat stress at 45ºC, both *SOD1* and *SOD2* were gradually induced for the time points analyzed (Fig. 4A) in the wild type. However, the expression of *SOD1* and *SOD2* in the mutant strains *Δuga3* and *Δuga4* were constitutively and highly expressed before and during heat stress (Fig. 4B and Fig. 4C). These results indicate that an intact GABA-specific transcription factor (*UGA3*) or GABA permease (*UGA4*) is

required for the regulation of expression of both superoxide dismutases, and that when *UGA3* or *UGA4* are deleted this negative regulation is removed leading to high level, constitutive expression of the superoxide dismutases subsequently affecting the thermotolerance and ROS production by these mutants.

Discussion

Previously, GABA was reported to induce the enzyme activities of *UGA1* and *UGA2* (Ramos *et al*., 1985). The expression of all three of GABA shunt genes (*GAD1*, *UGA1*, and *UGA2*) and the GABA-specific regulatory and transport genes (*UGA3* and *UGA4* respectively) was determined on media with and without GABA (glutamate, or NH_4^+) (Fig. 1). The expression of all 5 of the above genes was greatest with GABA as sole nitrogen source. The *UGA1, UGA2, UGA3,* and *UGA4* genes were also up-regulated on media containing GABA plus glutamate or GABA plus NH_4^+ but to a slightly lower level than on media with GABA alone. The lowest expression levels of these 4 genes were observed with glutamate or NH_4^+ alone. *GAD1* showed essentially the same pattern of regulation except that the expression levels of *GAD1* were greater on glutamate-containing media than on NH_4^+-containing media.

These results confirm the essential roles of all 5 of these genes in GABA utilization as a nitrogen source and demonstrate that there may be at least 2 different mechanisms for regulating the expression of the GABA utilization genes. The induced expression of *UGA1* and *UGA2* is coordinated with the increased enzyme activities of Uga1p and Uga2p in the presence of GABA (Ramos *et al*., 1985). The induction of *UGA3* and *UGA4*

was coupled with the induction of *UGA1*and *UGA2* further confirming the positive regulatory role of *UGA3* on the transcription of GABA-related genes.

GABA is incorporated into yeast cells through three permeases (Grenson *et al*., 1987). Among these, *UGA4* is the only GABA–specific transporter. The synthesis of *UGA4* has also been shown to be GABA-inducible (Grenson *et al*., 1987) and was regulated by at least two positive factors, UGA3p and the pleiotropic UGA35p factor (also referred to as Dal81p/DurLp) (André *et al*., 1995). Without the presence of GABA in the medium, the expression of *UGA4* has also been shown to be induced by acidic pH (Moretti *et al*., 2001). However, the expression of the other GABA-related genes by acidic media has not been investigated. Here we have shown that the expression of *UGA1, UGA2, and UGA4* were all increased at acidic pH on media containing NH_4^+ as the nitrogen source. Acidic pH does not appear to have significant effect on the induction of *GAD1* and *UGA3* genes. These results indicate that the induction of *UGA1*, *UGA2*, and *UGA4* by low pH is mediated by a mechanism different from the expression of the other two GABA-related genes (*GAD1* and *UGA3*). Whether this mechanism involves Uga3p cannot be determined from the data presented here. However, since acidic pH does not effect *UGA3* expression it is a reasonable hypothesis that acid regulation does not involve Uga3p.

Mutation of GABA specific transcription factor (*UGA3*) and GABA-specific permease (*UGA4*) resulted in an increase in tolerance of yeast cells to a lethal heat stress at 45ºC when cells were grown in rich YPD medium (Fig. 2). Deletion of *UGA3* resulted in more increase in stress tolerance than the deletion of *UGA4*. Essentially similar results were obtained when cells were grown on synthetic complete medium or minimal medium

with glutamate or ammonium sulfate as sole nitrogen source (data not shown) indicating that the rich source of amino acids present in YPD medium does not contribute to the heat stress tolerance phenotype of wild type or the mutants. However, the *UGA3* deletion generated a decrease in heat stress tolerance when cells were grown on GABA as the sole nitrogen source. This is likely because the expression of *UGA1* and *UGA4* are eliminated in *Δuga3* (Fig 3B), and it has previously been shown that the loss of function of GABA transaminase (*UGA1)* in a deletion mutant (Chapter II) decreases thermotolerance. Notably in *Δuga4*, GABA transaminase is not down-regulated as it is in *Δuga3.*

In chapter II and IV, we demonstrated that the three enzymes of the GABA shunt were involved in different levels of lethal heat stress tolerance. Under mild heat stress at 37ºC, microarray data show that the three enzymes in the GABA shunt pathway are highly up-regulated (Gasch *et al*., 2000; Sakaki *et al*., 2003). These results are further substantiated and expanded here by the observation that the transcripts of the three GABA shunt genes were gradually increased under lethal heat stress at 45ºC compared to the unstressed control in the wild-type strain (Fig. 3A). The GABA specific transcription factor gene and the GABA permease gene showed expression patterns under lethal heat stress (Fig. 3A) similar to the genes for the GABA shunt enzymes.

Deletion of GABA permease (encoded by *UGA4*) did not change the induction pattern of the three GABA shunt enzymes and the GABA specific transcription factor (encoded by *UGA3*) under lethal heat stress (Fig. 3C). However, compared to the wild-type, the transcript level of GABA transaminase *UGA1* was higher and the induction of *GAD1, UGA2* and *UGA3* were slower. This effect may be explained by the existence of

three GABA permeases in yeast, simultaneous deletion of the three GABA permeases is needed for further investigation.

In unstressed control cells, deletion of *UGA3* did not result in a change in *GAD1* expression. However, *UGA1* and *UGA4* were virtually unexpressed during heat stress (Fig. 3B). The expression of *UGA2* gene was induced more slowly than in the wild-type strain even though the expression was undetectable before heat stress (Fig. 3B). Thus, it can be concluded that under lethal heat stress, the transcription factor *UGA3* gene is required to activate the transcription of *UGA1, UGA2,* and *UGA4,* but *UGA3* not directly involved in the induction of *GAD1*. Apparently, factors in addition to *UGA3* are involved in regulating the expression of *UGA2* which are probably activated by prolonged heat stress.

Given the gene expression pattern of the *Δuga3* mutant, the heat stress tolerance phenotype is unexpected. Thus, an alternative overriding pleiotrophic factor must be involved in the heat stress phenotype of *Δuga3.* The heat stress tolerant phenotypes of the mutant strains were further supported by the expression levels of the superoxide dismutase *SOD1* and *SOD2* antioxidant genes in the *Δuga3* and *Δuga4* mutant strains (Fig. 4). Cells grown in YPD medium showed higher levels of transcripts for both *SOD1* and *SOD2* in the mutant strains than in the wild-type before heat stress. The expression of both genes was induced upon heat stress in the wild-type strain but after the initiation of stress. These data indicate a negative regulatory role for the GABA specific transcription factor and/or permease proteins on the expression of superoxide dismutases in yeast. The induced expression of *SOD1* and *SOD2* may be necessary for detoxifying ROS induced by heat assisting in the reduction of damage caused by the initial production

of ROS. The higher transcript level of *SOD2* than *SOD1* in all the wild-type and mutant strains is consistent with the hypothesis that superoxide dismutase is required for the detoxification of ROS in mitochondria more than in cytosol.

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Table 1. Yeast strains used in this study

Strain	Genotype	Source and description
W303-1A	Mat a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*	ATCC[a], WT
Δ*uga3*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga3::TRP1*	This study, *uga3* disruptant
Δ*uga4*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga4::TRP1*	This study, *uga4* disruptant

Table 2. Primers used for *UGA3* and *UGA4* gene deletions and verifications

Primer Name	Sequence (5’ to 3’)
	CACGTCGCTCTTAACAATCCAGGCT
UGA3UP45TRP1A	GAACAAAACAAGGAATAATGGGAAGCATTTAATAGACAGCATCGT
	TACATACATATAGGGGGCGGTA
UGA3DN45TRP1D	TATTGGATGACCTTTTCAACTCAAGGCAAGTGCACAAACAATACT
UGA3/TRP1KO_A	GGTAAGAAATGAAACAATAGGACGA
UGA3/TRP1KO_B	GAATATTTCAATTTCAGCTTCTCCA
UGA3/TRP1KO_C	GCATTCAGGTAGAAGATATGGAGAA
UGA3/TRP1KO_D	TCATGATATAGATATGATTGGCGG
UGA4UP45TRP1A	TTGTGAAGTGTAACAAGGTCTTATA
	ATTTATTATTACTAACAATGGGAAGCATTTAATAGACAGCATCGT
UGA4DN45TRP1D	TTATAAACTCTGAATATAAAAT
	CTTTATAAAGGTTTGAACATTTAAGGCAAGTGCACAAACAATACT
UGA4/TRP1KO_A	TTTATCGAATAAGGGGAGAACCTAC
UGA4/TRP1KO_B	AAATTTCCATCTTTGGTTAATGTGA
UGA4/TRP1KO_C	ATTATTTTGGTTATGTTCCCCTCTC
UGA4/TRP1KO_D	TAATGGAATGGAGAGTGATGATTTT
TRP1B	TCTGCAAGCCGCAAACTTT
TRP1C	AGTTCCTCGGTTTGCCAGTTATT
ACT1F	GGTATGTGTAAAGCCGGTTTT
ACT1R	AGGATGGAACAAAGCTTCTGG

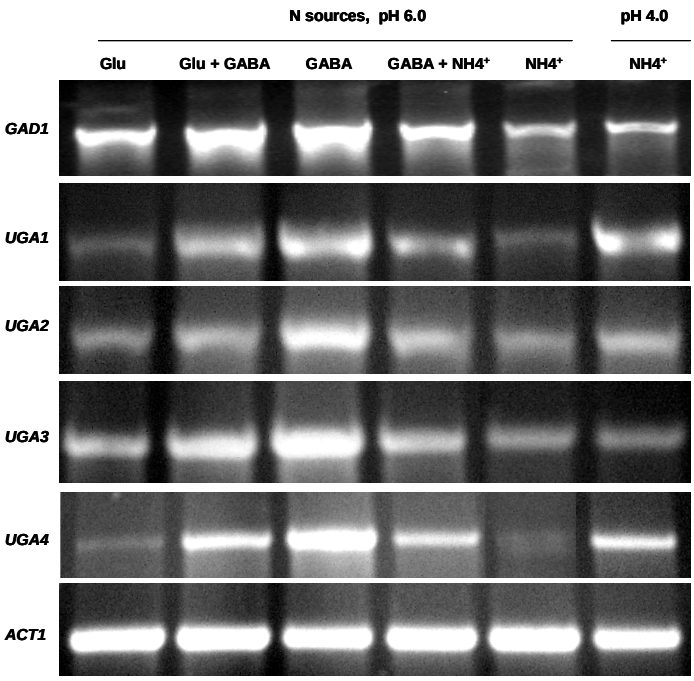

Fig. 1. Transcript levels of GABA shunt genes (*GAD1*, *UGA1*, *UGA2*), the GABA specific transcription factor (*UGA3*) and GABA permease (*UGA4*) in response to minimal media with different nitrogen sources and acidic pH. RT-PCR analysis was used to determine transcript abundance in mid-log phase cells growing at 30ºC as described in Materials and Methods. The *ACT1* gene was served as internal control.

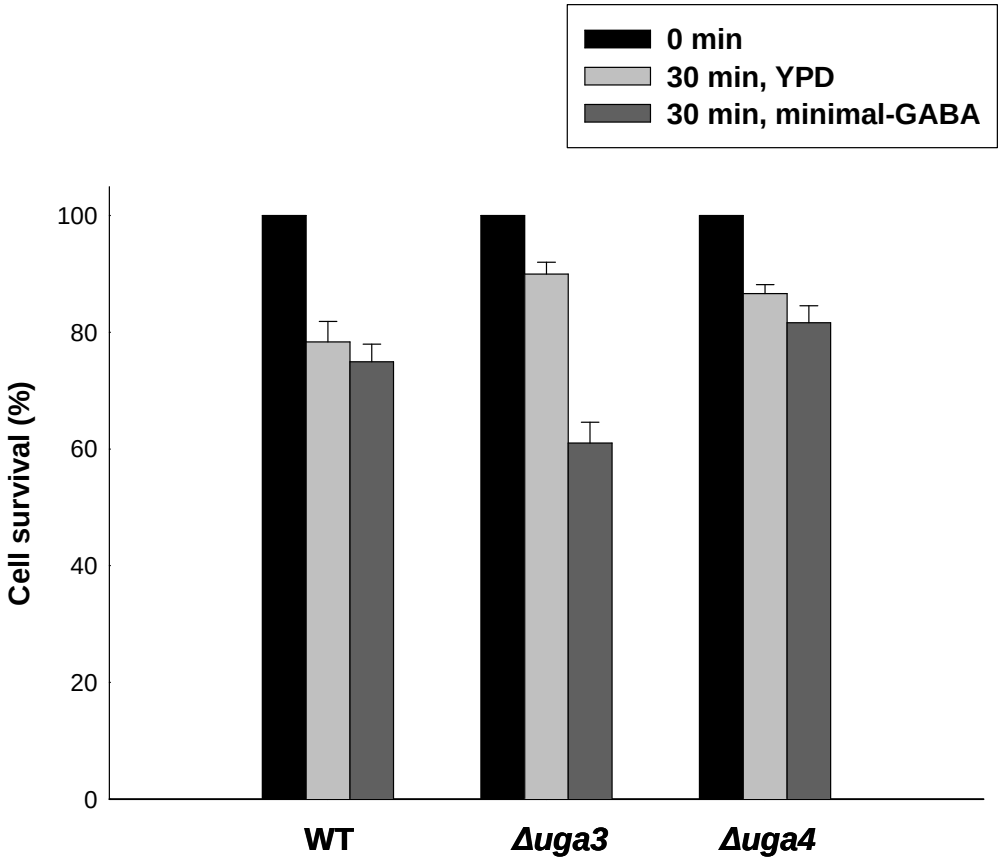

Fig. 2. Viability of wild-type, *Δuga3*, and *Δuga4* mutant strains after lethal heat treatment at 45°C. Strains were grown to mid log phase (5×10^6 cells/ml) in either YPD medium or minimal medium with GABA as sole nitrogen source, washed in 0.87% NaCl and concentrated to 2×10^8 cells/ml. Aliquots of 100 µl were heated at 45°C in a thermocycler for 30 min. Viability was determined as described in Materials and Methods. Cell survival percentage was determined prior to heat stress (black bar), 30 min after heat stress in YPD medium (light grey bar), or 30 min after heat stress in minimal medium with GABA as the nitrogen source (dark grey bar). Each point represents the mean ± SD of the results from at least three experiments.

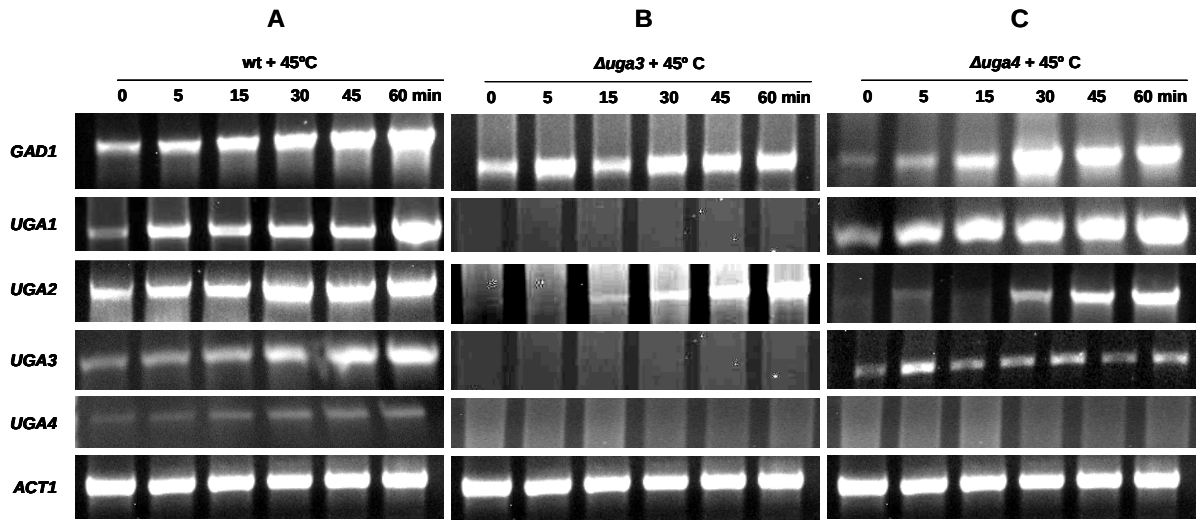

Fig. 3. Transcript levels of GABA shunt genes (*GAD1*, *UGA1* and *UGA2*) and the GABA specific regulatory and transport genes (*UGA3* and *UGA4* respectively) in response to lethal heat stress at 45ºC. RT-PCR analysis (see Materials and Methods) was used to determine transcript abundance in mid-log phase yeast cells grown in YPD from (A) wild-type; (B) *Δuga3* mutant; (C) *Δuga4* mutant following exposure to heat stress (45°C) for the time points indicated.

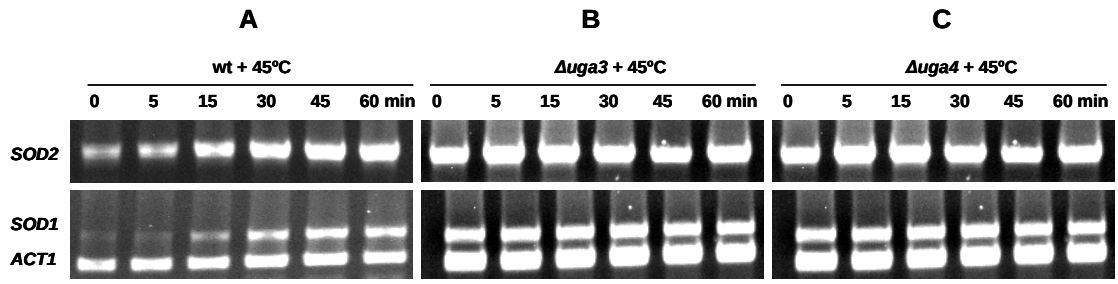

Fig. 4. Transcript levels of the cytosolic and mitochondrial superoxide dismutases (*SOD1* and *SOD2* respectively) in response to lethal heat stress at 45ºC. RT-PCR analysis (see Materials and Methods) was used to determine transcript abundance in mid-log phase yeast cells grown in YPD from (A) wild-type; (B) *Δuga3* mutant; (C) *Δuga4* mutant following exposure to heat stress (45°C) for the time points indicated.

IV. GABA TRANSAMINASE: COMPLEMENTATION ANALYSIS AND INTRACELLULAR LOCALIZATION IN *SACCHAROMYCES CEREVISIAE*

Abstract

GABA transaminase is the enzyme which catabolizes GABA conversion to succinate semialdehyde (SSA) in the GABA shunt pathway. GABA transaminase is α-ketoglutarate dependent (GABA-TKG), localized in the cytosol, and encoded by *UGA1* gene in yeast while in plants it is pyruvate dependent (GABA-TP) and localized in mitochondria. The web-based utilities TargetP 1.1 and PSORT predicts that GABA transaminase from *Arabidopsis* (AtGABA-TP) is localized in the mitochondria with a mitochondrial targeting peptide sequence localized in 54 nucleotides at the 5'end of the coding sequence. Yeast GABA transaminase (Uga1p or ScGABA-TKG) is predicted to be localized in cytosol. To examine the impact of localization differences between ScGABA-TKG and AtGABA-TP on physiological function, expression vectors were constructed that altered organellar targeting information to obtain expression of. Physiological function was evaluated by complementation of yeast GABA transaminase mutant *Δuga1* and *Δuga2* phenotype of: GABA growth defect, thermosensitivity and heat induced production of reactive oxygen species (ROS). Our studies revealed that AtGABA-TP is functionally interchangeable with ScGABA-TKG for GABA growth,

thermotolerance, and limiting production of ROS production whether they are located in mitochondria or cytosol in yeast.

Introduction

GABA is a ubiquitous non protein amino acid which is widely distributed from prokaryotic to eukaryotic organims (Kumar, 1997). It was first identified in 1950 in plants (Hulme & Arthington, 1950). GABA is produced from glutamate by glutamate decarboxylase (GAD), and further transaminated by GABA tranaminase to succinate semialdehyde (SSA) which is subsequently converted to succinate (SUCC) feeding into the TCA (Krebs) cycle. The role of GABA in animals is well established as a neuronal transmission inhibitor, the deficiency in catabolizing GABA through GABA transaminase (GABA-T) and SSADH is characterized by non-specific neurological disorder including psychomotor retardation, language delay, occasional seizures (Hogema *et al*., 2001; Gropman, 2003). In plants, GABA is catabolized to SSA by a pyruvate-dependent GABA transaminase (AtGABA-TP) (Shelp *et al*., 1999). *Arabidopsis* mutants of SSADH are hypersensitive to environmental stress such as heat and accumulate reactive oxygen species (ROS) during episodes of heat stress (Bouche *et al*., 2003). In yeast, GABA is catabolized to SSA by α-ketoglutarate-dependent GABA transaminase (GABA-TKG), the product of the yeast *UGA1* gene. Subsequently, SSA is converted to SUCC by SSADH, the gene product of the yeast *UGA2* gene. Yeast mutants deficient in *UGA1* and *UGA2* were shown to be sensitive to the oxidative stress (Coleman *et al*., 2001). Mutant strains lacking *UGA1* or *UGA2* were found to be incapable of growth on minimal medium with

GABA as the sole nitrogen source. This growth defect was not observed during growth on ammonium sulfate as the sole nitrogen source (Ramos *et al*., 1985).

Previously, we have shown yeast *uga1* and *uga2* deletion mutants were more sensitive to heat stress than is wild type and the *uga1* mutant is more sensitive to heat stress than is the *uga2* mutant. This thermosensitivity phenotype was shown to correlate with high levels of intracellular ROS; In that the *uga1* deletion mutant produces more ROS than does *uga2* during heat stress although both produce more ROS than wild type (Chapter II).

Bioinformatic analysis utilizing the BLAST search utility at NCBI suggests that plants do not contain orthologs of yeast ScGABA-TKG and that yeast contains no orthologs of plant GABA-TP. TargetP 1.1. and PSORT predicts *Arabidopsis* AtGABA-TP is localized in the mitochondria and that it contains an 18 amino acid amino-terminal mitochondrial targeting peptide. These same utilities also predict that ScGABA-TKG and ScSSADH enzymes are localized in cytosol. This leaves 2 prominent hypotheses to explain this comparative difference between the behavior of plant and yeast mutants in GABA shunt enzymes during heat stress. First, mitochondrial localization of the plant GABA transaminase and SSADH may result in this difference, or alternatively the fact that the plant GABA transaminase utilizes pyruvate while the yeast transaminase utilizes α-ketoglutarate or both may result in the stress sensitivity and ROS production differences between plants and yeast.

To test these hypotheses, expression plasmids were constructed to alter the cellular locations of plant GABA-TP and yeast GABA-TKG in yeast. Thus, we overexpressed a set of 4 constructs in yeast *uga1*, *uga2*, and *uga1uga2* deletion mutants. The 4 constructs

consisted of the yeast *UGA1* coding region; the yeast *UGA1* coding region also containing a 54 nucleotide sequence that would code for the mitochondrial transit peptide from AtGABA-TP; the AtGABA-TP coding region; and the AtGABA-TP coding region lacking the 54 nucleotides coding for the mitochondrial targeting peptide. All of these constructs were placed under the control of the strong constitutive GPD promoter. The mutant strains with GABA growth defect, thermosensitivity phenotype and ROS production during heat stress were assayed to determine the phenotype of each of theses constructs. Physiological analyses were conducted to determine the extent that differently localized enzymes compensate for these phenotypes.

Materials and methods

Bacterial and yeast strains and growth media.

The *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA, USA) was used for molecular cloning. Bacteria were grown on standard Luria Broth medium (LB medium) supplemented with antibiotics as required.

All yeast strains used in this study and their genotypes are listed in Table 1. The yeast strain W303-1A was purchased from American Type Culture Collection, and Δ*uga1, Δuga2, and Δuga1Δuga2* were derived from W303-1A as previously described (Chapter II). The strains were grown on either YPD medium or YNB minimal medium. YPD medium contains: 2% glucose, 1% yeast extract, 2% yeast bactopeptone, and was used for growth, cell survival, and ROS detection. YNB medium contains: 0.67% [wt/vol] yeast nitrogen base and 2% glucose supplemented with essential amino acids

(Sherman *et al*., 1979). YNB medium was used for cells grown on GABA as the sole nitrogen source.

Gene cloning and plasmid construction.

The endogenous *S. cerevisiae UGA1* open reading frame (*UGA1* ORF) was PCR amplified from the genomic DNA isolated from the wild type strain W303-1A using UGA1FOR and UGA1REV primers (Table 2). Yeast *UGA1* with the AtGABA-TP (NC_003074.4) 5'-54 nucleotide sequence coding for the 18 amino acid mitochondrial transit peptide added 5' to the UGA1 ORF sequence was constructed by a two-round PCR procedure. In the first round of PCR W303-1A genomic DNA was used as template and mTP15 UGA1FOR and UGA1REV (Table 2) were used as forward and reverse primers respectively. mTP15 UGA1FOR consisted of 15 nucleotides from 3'-end of the mitochondrial targeting peptide 54 nucleotide sequence in front of the 5'-most 27 nucleotides of *UGA1*. The PCR product was purified using a GENECLEAN kit (Biogene) and used as template for the second round of PCR. The second round of PCR was conducted using mTP54 UGA1FOR which has 54 nucleotides from the mitochondrial targeting peptide nucleotide sequence as the forward primer, and the same reverse primer UGA1REV (Table 2) as above. To facilitate plasmid construction, two restriction sites for *Bam*HI and *Hind*III (underlined, Table 2) were introduced at each end of the specific primers utilized. Following PCR amplification, the 1.778-kb (UGA1 orf) or 1.832-kb (mTP+UGA1 orf) *Bam*HI / *Hind*III fragment containing the entire gene was ligated into the pGEM Teasy vector using DNA ligase (Promega) according to manufacturer's procedure, and the ligated vector was transformed into bacterial strain

DH5α competent cells by $CaCl_2$ as described (Sambrook and Rusell, 2001). Plasmid DNA was isolated and purified by QIAGEN Plasmid Mini Kit(QIAGEN, USA), and then digested by *Bam*HI / *Hind*III (Biolabs, New England) according to manufacturer's procedures and ligated into the unique *Bam*HI / *Hind*III sites of yeast shuttle vector p425 GPD (American Type Culture Collection) using DNA ligase (Promega) according to manufacturer's procedure to create plasmids p425 GPD-UGA1 and p425 GPD-mTP54 UGA1.

The full length AtGABA-TP orf sequence (NC_003074.4) was PCR amplified using p416 GPD-pGAT (Barbosa, 2001) as template DNA which contains the entire cDNA sequence of AtGABA-TP using FL-pGATFOR and pGATREV (Table 2) as forward and reverse primers respectively. The truncated pGAT without mitochondrial targeting peptide sequence was also PCR amplified from plasmid p416 GPD-pGAT by using FL-51-pGATFOR (Table 2) as forward primer and the same reverse primer as above. To facilitate plasmid construction, two restriction sites for *BamHI* and *HindIII* (underlined, Table 2) were introduced at each end of the specific primers. Following PCR amplification, the 1.515-kb or 1.464-kb *BamHI* / *Hind*III fragment containing the entire gene was replicated in pGEM Teasy vector, digested, and ligated into the unique *Bam*HI / *Hind*III site of yeast shuttle vector p425 GPD (American Type Culture Collection) as described above to create plasmid p425 GPD-pGAT and p425 GPD-pGAT-mTP.

Gene disruptions

UGA1 was disrupted by replacing the coding sequences with *HIS3* gene as described (Chapter II). *UGA2* was disrupted by replacing the coding sequences with *URA3* gene as described (Chapter II).

Yeast transformation

The plasmid constructs p425 GPD-UGA1, p425 GPD-mTP54UGA1, p425 GPD-pGAT and p425 GPD-pGAT-mTP were used to transform the Δuga1, Δuga2, and Δuga1Δuga2 strains using lithium acetate method as described (Geitz *et al*., 1995) to generate the complementation strains of *Δuga1*-p425 GPD-UGA1, *Δuga1*-p425 GPD-mTP54UGA1, *Δuga1*- p425 GPD-pGAT, *Δuga1*- p425 GPD-pGAT-mTP; *Δuga2*-p425 GPD-UGA1, *Δuga2*-p425 GPD-mTP54UGA1, *Δuga2*- p425 GPD-pGAT, *Δuga2*- p425 GPD-pGAT-mTP; and *Δuga1Δuga2*-p425 GPD-UGA1, *Δuga1Δuga2*-p425 GPD-mTP54UGA1, *Δuga1Δuga2*- p425 GPD-pGAT, *Δuga1Δuga2*- p425 GPD-pGAT-mTP.

To minimize the nutritional effect of amino acids supplied from the medium as nitrogen source which are not needed for the growth of *uga1* and *uga2* mutants, the empty shuttle vectors which carry HIS(p423 GPD), URA(p426 GPD), LEU(p425 GPD) and TRP(p424 GPD) were used to transform into wild-type strain, URA(p426 GPD) and TRP(p424 GPD) were used to transform into *Δuga1* complementation strains, and HIS(p423 GPD) and TRP(p424 GPD) were used to transform into *Δuga2* complementation strains. The transformed strains were then grown on minimal media plus essential amino acids (Ade, Lys, Met) with 10mM GABA as sole nitrogen source to test the complementation of GABA growth phenotype for strains lacking *UGA1* or *UGA2* (Ramos *et al*., 1985).

Lethal heat stress survival assays

Lethal heat stress survival assays were performed as described in Chapter II using the strains in Table 1 and the complementation strains outlined above.

Measurement of ROS production.

Intracellular ROS generated during heat stress were measured as described in Chapter II using the strains in Table 1 and the complementation strains outlined above.

Subcellular fractionation

An overnight culture grown at 30ºC with vigorous shaking was diluted into 500 ml YPD media for wild type and mutant strains and grown until the OD_{600} reached 2.0. Cellular fractionation to produce mitochondrial and cytosolic fractions was performed as described (Daum *et al*., 1982). Briefly, mid-log phase cells were harvested by centrifugation at room temperature for 5 min at 3,000 g**,** washed once with distilled water, resuspended in DTT buffer (0.1 M Tris.SO4, pH 9.4, 10 mM dithiothreitol) at 2 ml per gram of wet weight cells, and incubated at 30ºC for 20 min. Cells were centrifuged for 5 min at 3,000 g, and the pellets were then washed once with Lyticase buffer (1.2 M sorbitol, 20 mM KPO4, pH 7.4) to give 7ml/g wet weight. Cells were resuspended in Lyticase buffer containing 0.5 mg Lyticase/ml (Sigma Aldrich), the suspension was incubated at 30ºC with gentle shaking for 60 min until all the cells had been converted to spheroplasts. Spheroplasts were harvested by centrifugation at room temperature for 5 min at 3,000 g, and the pellets were washed twice with Lyticase buffer. Cells were centrifuged at room temperature for 5 min at 3,000 g, and pellets were resuspended in ice cold homogenization buffer (0.6 M sorbitol, 10 mM Tris-C1, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% bovine serum albumin (BSA), 1 mM phenylmethylsulfonyl fluoride (PMSF)) to a concentration of 6.5 ml/ g wet weight. Spheroplasts were homogenized by 10 -15 strokes in a tight-fitting Dounce homogenizer.

From this point on, all operation were carried at 0 – 4ºC. The homogenate was diluted with 1 volume of ice cold homogenization buffer and centrifuged for 5 min at 1500 g to pellet the cell debris and nuclei. Supernatants were centrifuged for 5 min at 4000 g, and the pellets were discarded. The resulting supernatant was referred to as the *total yeast protein fraction*. The mitochondrial fraction was isolated by centrifugation of the supernatant at 12,000 g for 15 min. The resulted supernatant was referred to *cytosolic protein fraction*. The crude mitochondrial pellet was resuspended in cold SEM buffer (250mM sucrose, 1mM EDTA, 10mM MOPS-KOH, pH 7.2) to a final concentration of 5-10 mg/ml protein. For further purification, the mitochondrial suspension was loaded on top of the sucrose step gradients: 1.5 ml 60%, 4 ml 32%, 1.5 ml 23%, and 1.5 ml 15% (w/v) sucrose in EM buffer (1mM EDTA, 10mM MOPS-KOH, pH 7.2), and centrifuged for 60 min at 2ºC at 134,000 g. The purified mitochondria was recovered from 60% / 32% interface, concentrated by centrifugation to a protein concentration of 5-10 mg/ml, quickly frozen in liquid nitrogen, and stored at -80ºC for further use. For enzyme assays, mitochondrial pellets were broken by thawing and freezing, subcellular fractionations were assayed for cross contamination of fractions using NAD^+ dependent isocitrate dehydrogenase (NAD-IDH) as a mitochondrial marker and hexokinase 1 as a cytosolic marker.

Enzyme activity assays

Published procedures were used to assay GABA-transaminase (GABA-T), succinate semialdehyde dehydrogenase (SSADH), mitochondrial NAD^+ dependent isocitrate dehydrogenase (NAD-IDH) and cytosolic hexokinase 1. Protein concentrations were

determined by Bradford assay (Bradford, 1976) with crystalline bovine serum albumin (BSA) as the standard.

The activity of GABA-T was assayed as described (Ramos *et al*., 1985). The standard reaction mixture contains 100 mM potassium phosphate buffer, pH 8.2; 0.2 mM EDTA; 7.5 mM potassium 2-oxoglutarate or pyruvate; 0.2 mM β-NAD^+; 1 unit of yeast SSADH (Chapter V); and an appropriate amount of each cell fraction. The reaction was started by the addition of 7.5 mM GABA after pre-incubation of components at 30^{o}C for 5 min.

The activity of SSADH was assayed as described (Ramos *et al*., 1985). The standard reaction mixture contains 100 mM potassium phosphate buffer, pH 8.2; 0.2 mM EDTA; 0.2 mM NAD^+; and an appropriate amount of each cell fraction. The reaction was started by the addition of 0.1 mM SSA after pre-incubation of components at 30^{o}C for 5 min.

NAD-IDH, a mitochondrial marker, was assayed as described (Illingworth *et al*., 1972). The standard reaction mixture contains 40 mM Tris-HCl buffer, pH7.6; 4 mM-$MgCL_2$; 0.25 mM NAD^+ and an appropriate amount of each cell fraction. The reaction was started by addition of 2.5 mM trisodium DL-isocitrate after pre-incubation of components at 30^{o}C for 5 min.

Hexokinase 1, a cytosolic marker, was assayed as described (Sigma Technical Bulletin SPGLYC100, Bergmeyer, 1983). The standard reaction mixture contains 39 mM triethanolamine, 0.74 mM adenosine 5'-triphosphate (ATP), 7.8 mM magnesium chloride, 1.1 mM β-nicotinamide adenine dinucleotide phosphate (β-NADP), 2.5 units glucose-6-phosphate dehydrogenase (Sigma Aldrich), and an appropriate amount of each cell

fraction. The reaction was started by the addition of 216 mM D-glucose after pre-incubation of components at 30^{o}C for 5 min.

The enzymatic activity assays were based on the change in absorbance at 340 nm caused by the reduction of NAD(P)$^{+}$ to NAD(P)H. All enzymatic reactions were performed at 30^{o}C for 60 min and the change of absorbance at 340 nm was monitored on spectrophotometer (Beckman DU 640, USA). One unit of enzyme for GABA transaminase and succinate semialdehyde dehydrogenase was defined as the amount of enzyme required to produce 1 μmol of NADH per hour at 30°C. One unit of enzyme for NAD-IDH and hexokinase 1 was defined as the amount of enzyme required to reduce 1 μmol of NAD^{+} per min at 30°C. All assays were repeated at least three times and data represented the average of the results from at least three experiments.

Results

Subcellular localization of GABA shunt enzyme activities in *Saccharomyces*

In order to investigate the interchangeable function of *Arabidopsis thaliana* AtGABA-TP and yeast ScGABA-TKG, plasmid constructs were made as indicated in Materials and Methods that contained AtGABA-TP with (+P) and without the mitochondrial targeting peptide (+P-mtp) or that contained ScGABA-TKG (+Y) or ScGABA-TKG with the AtGABA-TP mitochondrial targeting peptide (+Y+mtp). The intent was to be able to examine functional complementation of a set of yeast mutants overexpressing these two proteins localized in either the mitochondria or the cytosol. Prior to this investigation the proper localization of the proteins made by these constructs was investigated.

The TargetP 1.1 (Nielsen *et al*., 1997; Emanuelsson *et al*., 2000) and PSORT (Nakai and Horton, 1999) web based utilities predict AtGABA-TP to be localized in mitochondria with an 18 amino acid (54 nucleotide) mitochondrial trageting peptide in *Arabidopsis thaliana*. These utilities also indicate that these sequences will localize to mitochondria in yeast. The yeast GABA degrading enzymes ScGABA-TKG and ScSSADH were predicted by Target P 1.1 and PSORT to localize to the cytosol.

To further verify the subcellular localization predictions of UGA1p, UGA2p and whether AtGABA-TP is localized in yeast mitochondria when expressed in yeast, we tested isolated subcellular fractions derived from appropriate stains for GABA-TKG, SSADH, and GABA-TP activities as described in Methods. Mitochondria could be easily separated from cytosol by differentiation centrifugation after cell lysis (Daum *et al*., 1982). Cytosolic hexokinase 1 was employed as a marker enzyme for the cytosol fraction, and mitochondrial NAD^+-dependent isocitrate dehydrogenase was employed as a marker enzyme for mitochondria. Transformants and wild type strains were cultivated on YPD medium, and whole yeast cells were fractionated into mitochondria and cytosolic fractions. The specific enzyme activities in each fraction for each strain are listed in Table 3. In all strains which contain intact UGA2 gene, over 99% activity of the SSADH activity was found in cytosol fraction with specific activities of 2.22 to 2.52 U/mg protein. SSADH activity was almost non-detectable in the mitochondrial fraction suggesting that yeast UGA2p is localized in cytosol and not in mitochondria.

In wild type yeast cells, significant GABA-TKG activity (>99%, 1.63 U/mg protein) was found in the cytosol. GABA-TKG enzyme activity was almost non detectable in the mitochondrial fraction. In the *Δuga1* deletion mutant, no GABA-TKG activity was found

in either fraction supporting the complete loss of GABA-TKG activity with the absence of a functional UGA1 gene. In the *Δuga1* mutant overexpressing full length ScGABA-TKG (*Δuga1*+Y) and overexpressing the truncated AtGABA-TP (*Δuga1*+P-mTP), GABA-TKG and GABA-TP specific activity was increased 6-fold and 0.5-fold relative to wild type respectively suggesting both constructs play a functional role in yeast and the truncated AtGABA-TP is not as highly expressed as the ScGABA-TKG in wild type. Similar to the wild type, no GABA-T activity was found in the mitochondria for both *Δuga1*+Y and *Δuga1*+P-mTP. Over 90% of GABA-TP activity was found in cytosol for *Δuga1*+P-mTP indicating that functional GABA transaminases without mitochondrial transit peptides are located in the cytosol.

We then tested whether the plant mitochondrial transit peptide will cause yeast and plant GABA transaminases to localize into yeast mitochondria. Over 90% of the GABA-TKG and GABA-TP activity was found in mitochondria for both *Δuga1*+Y+mTP and *Δuga1*+P transformants respectively. For example, *Δuga1*+Y+mTP had a specific enzyme activity of 9.4 and 0.15 U/mg protein in mitochondria and cytosol respectively and *Δuga1*+P had a specific enzyme activity of 0.92 and 0.08 U/mg protein in mitochondria and cytosol respectively. These data suggest that both yeast endogenous GABA-TKG and AtGABA-TP were efficiently imported into yeast mitochondria directed by the presence of the AtGABA-TP mitochondrial targeting peptide sequence.

Complementation of the GABA growth phenotype by cytosolic and mitochondrial GABA transaminases

Previous studies have demonstrated that *Saccharomyces* mutants lacking *UGA1*,

UGA2, and *UGA3* are incapable of growth on minimal medium with GABA as the sole nitrogen source but grow normally on minimal medium with ammonium sulfate as the sole nitrogen source (Ramos *et al*., 1985; Coleman *et al*., 2001). To test the extent of interchangeable function of *Arabidopsis thaliana* AtGABA-TP and yeast ScGABA-TKG in both the cytosol and mitochondria, mutant strains bearing *Δuga1*, *Δuga2*, or the *Δuga1Δuga2* double mutant were transformed by the set of plasmid constructs containing AtGABA-TP with and without the mitochondrial targeting peptide or containing ScGABA-TKG or ScGABA-TKG with the AtGABA-TP mitochondrial targeting peptide.

Cells transformed with ScGABA-TKG (*Δuga1*+Y, *Δuga2*+Y, *Δuga1Δuga2*+Y) were grown on YNB medium containing 10 mM GABA or $NH4^+$ as the sole nitrogen source (Fig 1B). Growth was fully rescued to at least wild type level for *Δuga1*+Y while growth was restored to the same extent as *Δuga2* for the *Δuga1Δuga2*+Y transformants, and as expected, overexpressing ScGABA-TKG did not recover the growth defect for the *Δuga2* mutant. Similar growth recovery effects were also observed for cells transformed with plasmids overexpressing ScGABA-TKG with the mitochondrial targeting peptide from AtGABA-TP (*Δuga1*+Y+mTP, *Δuga2*+Y+mTP, *Δuga1Δuga2*+Y+mTP), indicating that overexpressing ScGABA-TKG with an *Arabidopsis* mitochondrial targeting peptide is functionally similar to overexpressing ScGABA-TKG alone in recovering the GABA growth defect in the mutants lacking *UGA1*.

Similarly, cells transformed with full length *Arabidopsis* AtGABA-TP (*Δuga1*+P, *Δuga2*+P, *Δuga1Δuga2*+P) and AtGABA-TP with a truncated mitochondrial transit peptide (*Δuga1*+P-mTP, *Δuga2*+P-mTP, *Δuga1Δuga2*+P-mTP) were examined for GABA growth complementation. Transformation with AtGABA-TP (*Δuga1*+P and

Δuga1Δuga2+P) which putatively localized in mitochondria partially rescued the growth defect for mutants lacking *UGA1* (Fig. 1B). A 3- to 4- fold increase in growth was observed in liquid media (data not shown). However, no significant growth recovery was found for the *Δuga2* mutant when transformed with the AtGABA-TP constructs (Fig. 1B). Equivalent growth recovery was observed whether the transformants were overexpressing the full length AtGABA-TP or the AtGABA-TP with the truncated mitochondrial targeting peptide. These results indicate that AtGABA-TP targeted to either the mitochondria or cytosol in yeast has a similar function to yeast endogenous ScGABA-TKG in supporting growth on media containing GABA as the sole nitrogen source although the use of endogenous, α-ketoglutarate-utilizing ScGABA-TKG has a stronger growth supporting effect than does the pyruvate-utilizing AtGABA-TP. Mitochondrial versus cytosolic localization for both enzymes appears to be less critical than either the use of α-ketoglutarate versus pyruvate as substrate or the expression level of the specific enzyme in yeast cells.

Complementation of the thermosensitivity and ROS production phenotype by cytosolic and mitochondrial forms of GABA transaminases

Our previous studies showed that under lethal heat at 45ºC, yeast mutants of *Δuga1* lacking GABA transaminase and *Δuga2* lacking succinate semialdehyde dehydrogenase are more heat sensitive than wild type. and *Δuga1* is dramatically more heat sensitive than *Δuga2*. The *Δuga1Δuga2* double mutant is more heat sensitive than either of the single mutants. The thermo sensitivities were correlated to the level of ROS production (Chapter II). To test the function and localization of AtGABA-TP and ScGABA-TKG in yeast in thermotolerance and limiting ROS production, the three mutants above which are

defective in GABA degradation were transformed with the four plasmid constructs as described and characterized above.

Overexpression of ScGABA-TKG in *Δuga1* (*Δuga1*+Y) led to heat stress survival comparable to the wild type after 60 min (Fig 2A). Comparably, overexpression of ScGABA-TKG in the double mutant *Δuga1Δuga2* (*Δuga1Δuga2*+Y) led to heat stress survival comparable to the *Δuga2* mutant alone after 60 min. However, overexpression of ScGABA-TKG in the *Δuga2* mutant (*Δuga2*+Y) did not enhance heat stress cell survival. In all cases, ROS production was comparable to that expected from the survival data (Fig 2A). Overexpression of ScGABA-TKG with the AtGABA-TP mitochondrial transit peptide attached also produced comparable results to the expression of ScGABA-TKG in the cytosol (Fig. 2B compared to Fig 2A and Fig 3B compared to Fig. 3A).

To assay the complementation by AtGABA-TP yeast mutants were transformed with plasmid constructs containing either full length AtGABA-TP (*Δuga1*+P, *Δuga2*+P, and *Δuga1Δuga2*+P) or the truncated GABA-TP lacking a mitochondrial targeting peptide (*Δuga1*+P-mtp, *Δuga2*+P-mtp, and *Δuga1Δuga2*+P-mtp), these strains were tested for their ability to compensate for the thermosensitivity (Fig. 4) and ROS production (Fig. 5) phenotypes. When *Δuga1*+P was heat stressed for up to 60 min at 45°C, cell survival for *Δuga1*+P significantly improved (19% to 50%, survival) but did not achieve wild type levels (71%) of survival (Fig. 4A). Clearly AtGABA-TP did not complement the yeast *Δuga1* deletion to the same extent as did ScGABA-TKG or ScGABA-TKG plus the mitochondrial targeting peptide. Similar partial levels of complementation were observed for *Δuga1Δuga2*+P (Fig 4A) and *Δuga1Δuga2*+P-mtp (Fig 4B) since the levels of complementation did not reach the survival level of *Δuga2*. No significant cell survival

recovery was observed for the *Δuga2* mutant transformants (*Δuga2*+P or *Δuga2*+P-mtp). Additionally, the production of ROS (Fig 5) in these mutants and transformants was appropriately proportional to the heat stress survival data as was the case for the ScGABA-TKG transformations above.

Taken together these results show that AtGABA-TP with or without a mitochondrial targeting peptide compensated for ScGABA-TKG under lethal heat stress for both survival and ROS accumulation. However, AtGABA-TP, regardless of its intracellular location was less effective than the native ScGABA-TKG form of GABA transaminase (regardless of its location).

Discussion

In this study, the cellular localization of yeast ScGABA-TKG and ScSSADH in wild type yeast strain were investigated based on the location of enzyme activity in the mitochondrial or cytosol subfractions. For both proteins, over 90% of specific enzyme activities were detected in the cytosol fraction (Table 3), the mitochondrial fractions possessed almost non measurable enzyme activities (<0.01 U/mg protein), suggesting both GABA-TKG and SSADH are localized and functioning in cytosol, not mitochondria. This finding is consistent with the localization predicted by TargetP and PSORT, and the cytosolic location of SSADH previously reported (Huh *et al*., 2003). Deletion of GABA-TKG in yeast resulted in completely loss of enzyme activities in cytosol. TargetP and PSORT predict that AtGABA-TP is localized in the mitochondria in *Arabidopsis* and would also be in yeast based on the presence of an 18 amino acid mitochondrial targeting peptide at the amino-terminus of the protein. Using both native

and recombinant DNA constructs and transformation of appropriate yeast gene deletions we have shown that the mitochondrial localization of both ScGABA-TGK and AtGABA-TP is mediated by this 18 amino acid mitochondrial targeting peptide. Since all of our plasmid constructs placed the cloned sequences under the control of the strong constitutive GPD promoter it is unclear why AtGABA-TP activity levels were reduced when compared to even wild type expression of ScGABA-TKG. The addition of the mitochondrial targeting peptide did not appear to reduce the stability of ScGABA-TGK, nor did the deletion of the mitochondrial targeting peptide from AtGABA-TP appreciably alter specific activity relative to the full length construct. Thus it seems most likely that the lower expression of AtGABA-TP is the result of either posttranscriptional processing or the posttranslational stability of the *Arabidopsis* protein in yeast.

In complementation assays, transformants targeting ScGABA-TKG to mitochondria were found to be as efficient as the endogenous or ectopically expressed cytosolic form of ScGABA-TKG in supporting growth on GABA medium (Fig. 1A), thermotolerance (Fig 2), or ROS accumulation (Fig 3). Thus, the production of succinate semialdehye from GABA which is normally a cytosolic process in *Saccharomyces* appears to be equally functional when it occurs in mitochondria. Thus, it can be concluded that the production of GABA and/or succinate semialdehyde in the mitochondria is no more responsible for heat stress tolerance or ROS accumulation than the production of these compounds in the cytosol.

Complementation of the *Δuga1* mutant by AtGABA-TP with or without its mitochondrial targeting peptide was found to be partially effective in supporting growth on GABA as the sole nitrogen source (Fig 1), heat stress tolerance (Fig 4), and ROS

accumulation (Fig. 5) supporting again the idea that localization in the mitochondria was not critical nor detrimental to the function of this pathway in *Saccharomyces*. This finding was not consistent with our hypothesis that the conversion of pyruvate to alanine by AtGABA-TP ectopically expressed in mitochondria should limit carbon flux through the TCA cycle, and thus reduce mitochondrial ROS production. Rather this finding is consistent with the hypothesis that carbon flux through the TCA cycle at least from pyruvate to α-ketoglutarate is not related to the production of ROS during heat stress, and that the accumulation of succinate semialdehyde or some metabolite downstream is likely the major factor playing a role in abating ROS production during heat stress.

The fact that AtGABA-TP only partially complements the *Δuga1* mutation while ScGABA-TKG fully complements the mutant further supports the above hypothesis. However, it is unclear whether this is from the difference in substrate specificity of the two enzymes or from the difference in enzyme activity content in cells transformed with the two different constructs (Table 3).

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Table 1. Yeast strains used in this study

Strain	Genotype
W303-1A	Mat a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*
Δ*uga1*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga1::HIS3*
Δ*uga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga2::URA3*
Δ*uga1Δuga2*	Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga1::HIS3 uga2::URA3*

Table 2. Primers used for yeast cytolic and mitochondrial forms of GABA-TKG and plant GABA-TP expression

Primer Name	Sequence (5' to 3')
UGA1FOR	GAGAGGATCCATGTCTATTTGTGAACAATACTACCCA
mTP15 UGA1FOR	ACTCAGGTTCATTTGATGTCTATTTGTGAACAATACTACCCA
mTP54 UGA1FOR	GGATCCATGGTCGTTATCAACAGTCTCCGACGCTTGGCGCGTACCACTCAGGTTCATTTG
UGA1REV	GTGTAAGCTTTCATAATTCATTAACTGATTTGGCTAA
FL-pGATFOR	GAGAGGATCCATGGTCGTTATCAACAGTCTCCG
FL-51-pGATFOR	GAGAGGATCCATGCACAGTAGGTATGCCACTT
pGATREV	GTGTAAGCTTTCACTTCTTGTGCTGAGCCTT

Table 3. Enzyme activities in yeast wild type, *Δuga1* deletion mutant strain and *Δuga1* mutant transformants[a]

Preparation	Protein	Specific activity[b] (U/mg protein)					
		wild type	*Δuga1*	*Δuga1*+Y	*Δuga1*+Y+MTP	*Δuga1*+P	*Δuga1*+P-MTP
Total cell extract	NAD-IDH	0.03	0.028	0.032	0.029	0.030	0.033
	Hexokinase	3.1	2.5	3.0	3.08	3.1	3.2
	GABA-T(P)	0.16	0	0.80	0.88	0.10	0.09
	SSADH	0.29	0.27	0.238	0.24	0.26	0.26
Mitochondria	NAD-IDH	0.19	0.185	0.20	0.0188	0.0195	0.21
	Hexokinase	0	0	0	0	0	0
	GABA-T(P)	<0.01	0	0	9.4	1.52	0.06
	SSADH	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Cytosol	NAD-IDH	0	0	0	0	0	0
	Hexokinase	25	23	28	27.5	28	26
	GABA-T(P)	1.63	0	9	0.15	0.08	1.48
	SSADH	2.52	2.48	2.22	2.35	2.42	2.45

[a] Yeast strains were grown on rich YPD medium. Cell fractionation was performed as described by Daum *et al* (Daum, 1982).

[b] The enzymes assayed as described in Materials and Methods, were NAD^+ dependent isocitrate dehydrogenase (NAD-IDH) as mitochondrial marker protein, hexokinase as cytosolic marker protein, yeast endogenous GABA-TKG or plant GABA-TP, succinate semialdehyde dehydrogenase (SSADH). The strains used were W303-1A (wild type), yeast deletion mutant *Δuga1*, and the mutant overexpressing yeast endogenous GABA-TKG (*Δuga1*+Y), GABA-TKG with plant mitochondrial targeting peptide sequences (*Δuga1*+Y+MTP), plant GABA-TP (*Δuga1*+P), or GABA-TP without mitochondrial targeting peptide sequences (*Δuga1*+P-MTP). The values shown represent averages of three independent determinations.

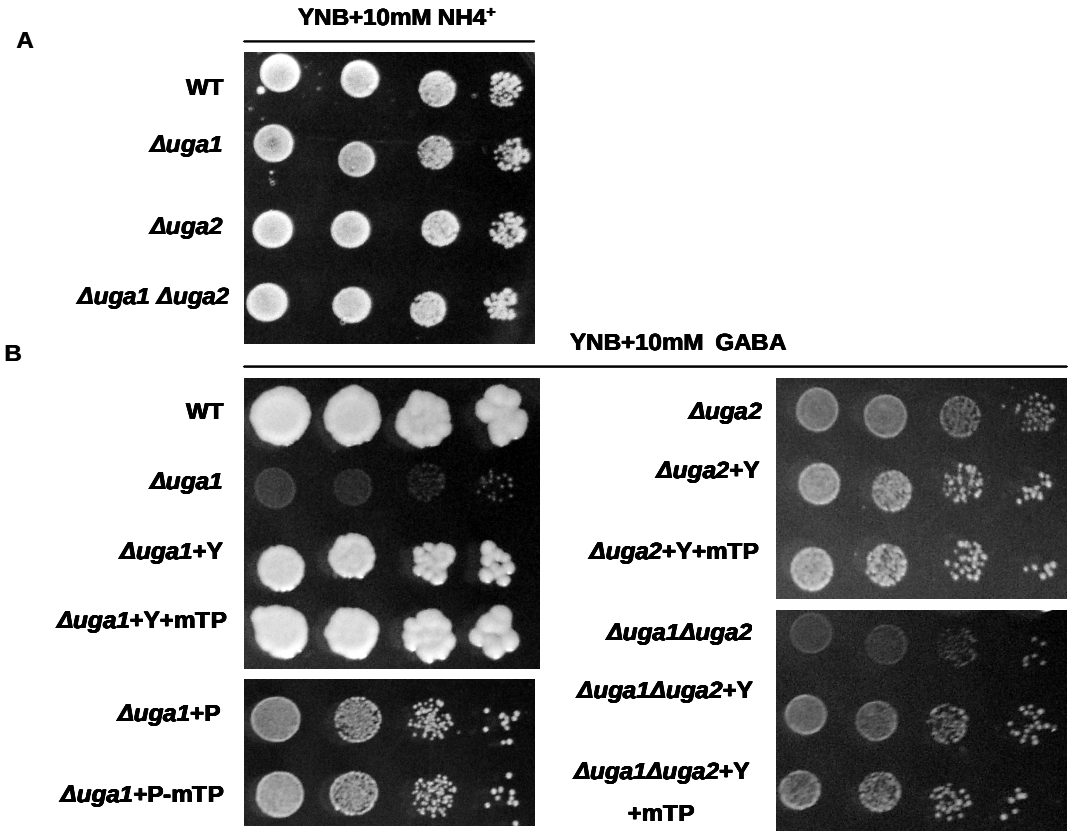

Fig. 1. (A) Growth of wild type and mutant strains on YNB medium with $NH4^+$ as the sole nitrogen source; (B) YNB medium with GBAB as the sole nitrogen source. Each strain contains vectors of HIS(p423 GPD), URA(p426 GPD), LEU(p425 GPD), TRP(p424 GPD) and LEU(p425 GPD), LEU(p425 GPD) vector for mutant strains carries the cytolic and mitochondrial forms of yeast endogenous GABA-TKG or cytosolic and mitochondrial forms of plant GABA-TP. All strains are able to grow on YNB media with $NH4^+$ as the sole nitrogen sources, however, *Δuga1*, *Δuga2* and *Δuga1uga2* can't grow on GABA as the sole nitrogen source as well as the wild type. Overexpression of plasmids bearing cytosolic and mitochondrial forms of yeast endogenous GABA-TKG fully recovered the GABA growth phenotype (B, left panel); Overexpression of plasmids bearing cytosolic and mitochondrial forms of plant GABA-TP partially recovered the GABA growth phenotype (B, right panel). "+Y" indicates expression of P425 GPD-UGA1, "+Y+mTP" indicates expression of P425 GPD-mTP54UGA1, "+P" indicates expression of P425 GPD-pGAT; "+P-mTP" indicates expression of P425 GPD-pGAT-mTP.

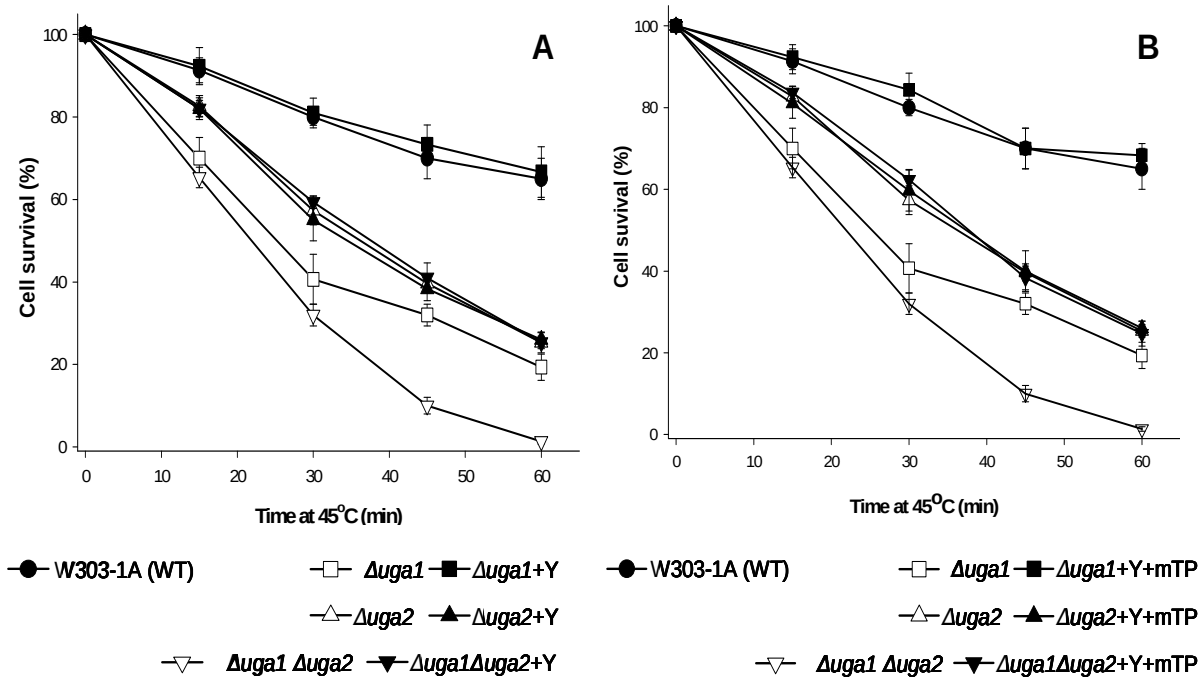

Fig. 2. Viability of wild-type, mutant strains and mutant strains transformed with (A) cytosolic and (B) mitochondrial forms of yeast endogenous GABA-TKG after lethal heat treatment at 45°C. Strains were grown to mid log phase (5×10^6 cells/ml) in YPD, washed in 0.87% NaCl, and concentrated to 2×10^8 cells/ml in fresh YPD. Aliquots of 100 µl were heated at 45°C in a thermocycler for 0, 15, 30, 45 and 60 min. Viability was determined as described. Each point represents the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes. "+Y" indicates expression of P425 GPD-UGA1, "+Y+mTP" indicates expression of P425 GPD-mTP54UGA1.

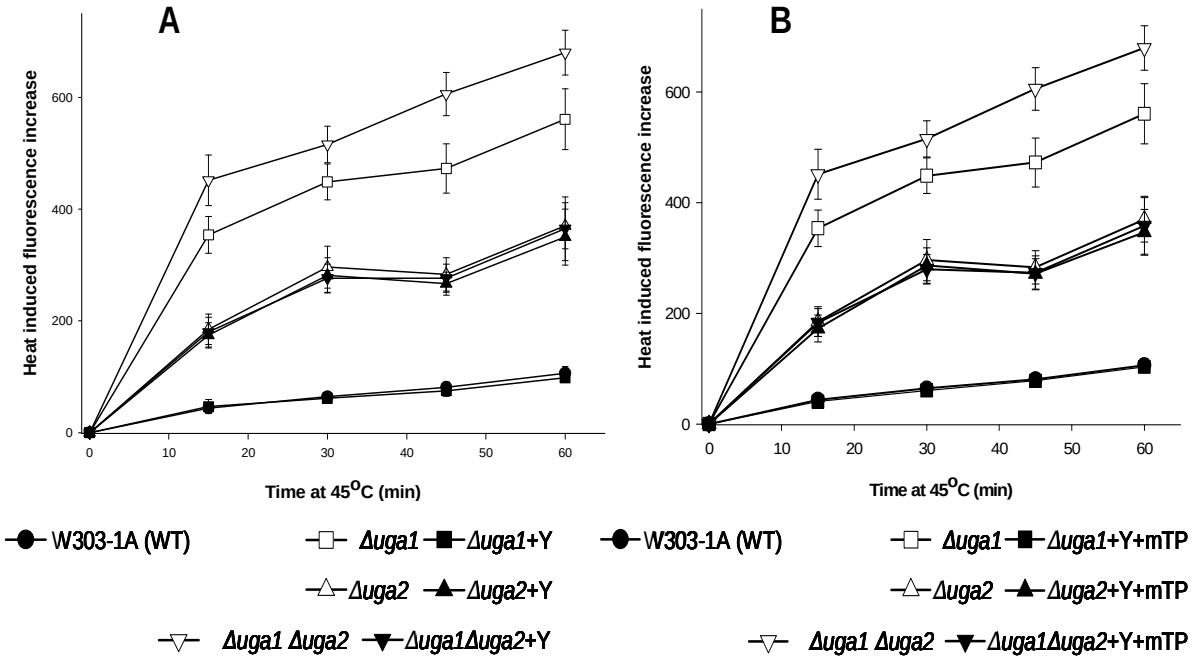

Fig. 3. Intracellular ROS levels induced by lethal heat stress at 45ºC in wild type, mutant strains and mutant strains transformed with cytosolic (A) and mitochondrial (B) forms of yeast endogenous GABA-TKG. Fluorescence was measured at an excitation wavelength of 504nm and an emission wavelength of 524nm in crude cell extracts as described. As a control, fluorescence was recorded during heat stress without cells and cells without DCFH-DA (date not shown). Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes. "+Y" indicates expression of P425 GPD-UGA1, "+Y+mTP" indicates expression of P425 GPD-mTP54UGA1.

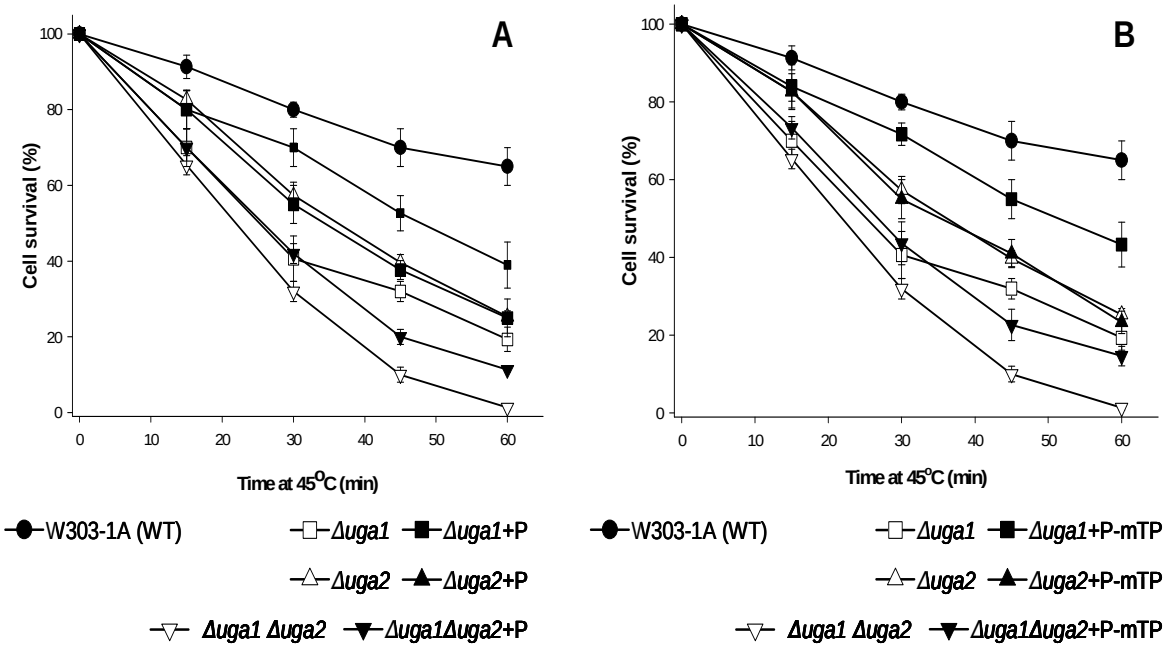

Fig. 4. Viability of wild-type, mutant strains and mutant strains transformed with (A) cytosolic and (B) mitochondrial forms of plant GABA-TP after lethal heat treatment at 45°C. Strains were grown to mid log phase (5×10^6 cells/ml) in YPD, washed in 0.87% NaCl, and concentrated to 2×10^8 cells/ml in fresh YPD. Aliquots of 100 µl were heated at 45°C in a thermocycler for 0, 15, 30, 45 and 60 min. Viability was determined as described. Each point represents the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes. "+P" indicates expression of P425 GPD-pGAT; "+Y-mTP" indicates expression of P425 GPD-pGAT-mTP.

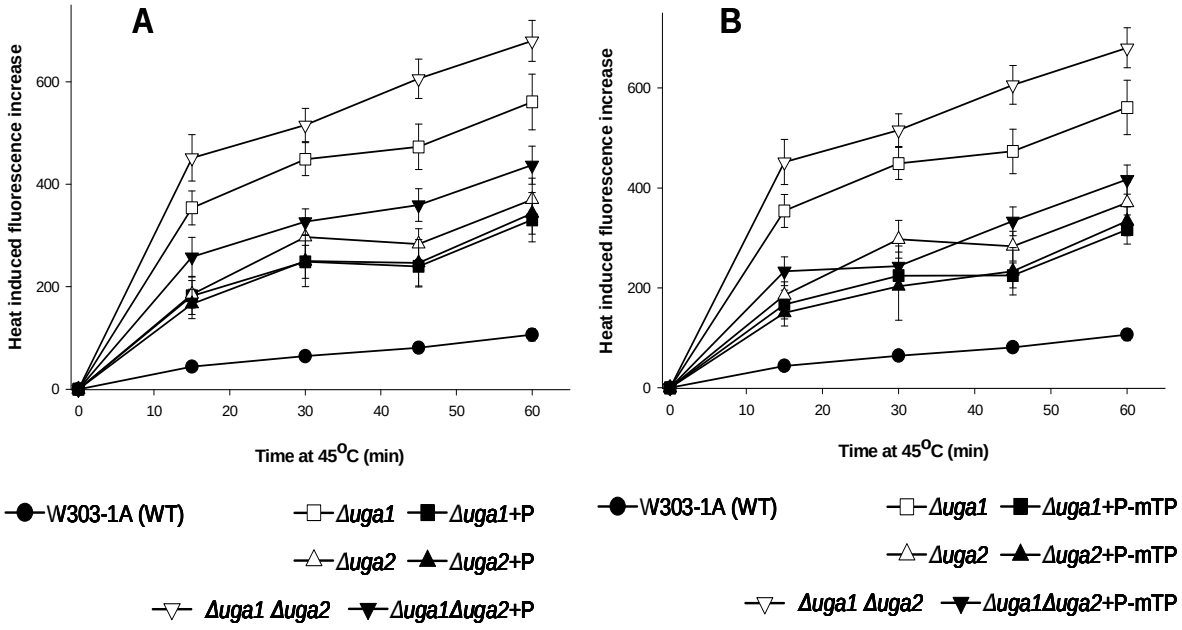

Fig. 5. Intracellular ROS levels induced by lethal heat stress at 45ºC in wild type, mutant strains and mutant strains transformed with (A) cytosolic and (B) mitochondrial forms of plant GABA-TP. Fluorescence was measured at an excitation wavelength of 504nm and an emission wavelength of 524nm in crude cell extracts as described. As a control, fluorescence was recorded during heat stress without cells and cells without DCFH-DA (date not shown). Data shown represent the mean ± SD of the results from at least three experiments, some error bars are smaller than the symbol sizes. "+P" indicates expression of P425 GPD-pGAT; "+P-mTP" indicates expression of P425 GPD-pGAT-mTP.

V. MOLECULAR CLONING, EXPRESSION AND CHARACTERIZATION OF RECOMBINANT YEAST SUCCINIC SEMIALDEHYDE DEHYDROGENASE

Abstract

The yeast succinic semialdehyde dehydrogenase gene (SSADH; EC 1.2.1.16) was cloned and overexpressed in *E. coli*. Based on SDS-PAGE, the molecular mass of the subunit was around 54 kDa, and the purified recombinant enzyme has a tetrameric molecular mass of approximately 200 kDa. The specific activity of the recombinant enzyme was 1.90 μmol NADH formed min^{-1} mg^{-1}, and showed maximal activity at pH 8.4. The recombinant protein was highly specific for succinate semialdehyde (*K*m = 15.48 ± 0.14 μM, can use both NAD^+ and $NADP^+$ as a cofactor with *K*m values of 579.06 ± 30.1 μM and (1.017 ± 0.46) mM respectively. Initial velocity studies show NADH was a competitive inhibitor with respect to NAD^+ (*K*i = 129.5 μM), but non-competitive inhibitor with respect to succinate semialdehyde. Adenine nucleotides of AMP, ADP and ATP inhibited the yeast SSADH activity with *K*i of 1.13 ~ 10.2 mM and showed competitive inhibition with respect to NAD^+ and mixed-competitive and non-competitive, and non-competitive inhibition respectively with respect to succinate semialdehyde. The kinetic data suggest a ping-pong mechanism.

Introduction

γ-Aminobutyrate (GABA) is a ubiquitous non protein amino acid widely found in prokaryotic and eukaryotic organisms. It is well known as a major inhibitory neurotransmitter in mammals. GABA plays a role in the nitrogen and carbon metabolism in bacteria. In plants, GABA accumulates rapidly in response to various abiotic and biotic stresses such as hypoxia, cold, and mechanical stimulation. However, in most organisms, its physiological role is uncertain.

In yeast, GABA is produced from glutamate in the cytosol, catalyzed by glutamate decarboxylase (GAD; EC 4.1.1.15) in the GABA shunt pathway. GABA is then further transaminated to succinate semialdehyde (SSA) by GABA transaminase (GABA-T; EC 2.6.1.19), and finally, SSA is oxidized by succinate semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) to the final product of succinate which then enters the tricarboxylic acid cycle. Clearly, SSADH is involved in the utilization of GABA as a nitrogen source. In human, SSADH deficiency results in 4-hydroxybutyric aciduria, an autosomal recessive disorder due to an accumulation of GABA and 4-hydroxybutyric acid in the central nervous systems (Jakobs *et al.,* 1981). Recently, mutation studies show SSADH in plants (Jakobs *et al.,* 2003) and yeast (Coleman *et al*., 2001) is critical for normal oxidative stress tolerance.

SSADH has been shown to be present and has been purified from mammalian tissues (Blaner and Churchich, 1979; Ryzlak and Pietruszko, 1988; Chambliss and Gibson, 1992; Lee *et al.,* 1995), plants (Busch and Fromm, 1999; Satya and Nair, 1989; Yamaura *et al.,* 1988) and microorganisms (Steinbuchel and Lutke-Eversloh, 1999; Hidalgo *et al.,* 1991; Ramos *et al.,* 1985). The reaction catalyzed by SSADH is

essentially irreversible, and the enzyme activity is inhibited by the substrate succinate semialdehyde (SSA) and the product NADH in mammalian brain tissues (Ryzlak and Pietruszko, 1988; Chambliss and Gibson, 1992; Lee *et al.,* 1995). In plants, SSADH activity is also inhibited by adenine nucleotides (Busch and Fromm, 1999). In fungi, the first enzyme of the GABA shunt (GAD) was purified and best studied in *N. crassa* (Christensen and Schmit, 1980), GABA-T was purified and characterized from *Candida* species (Der *et al*., 1986). However, the last enzyme of pathway (SSADH) has received relatively little attention. Its properties could not be determined in crude cell extracts (Baldy, 1977; Vissers *et al.,* 1989), and it has only been partially purified in yeast and not well characterized (Ramos *et al.,* 1985).

We reasoned that cloning of the yeast gene encoding SSADH enzyme and detailed characterization of the purified recombinant protein would provide significant information on the physiological and functional properties of SSADH, and its specific regulation in the GABA shunt pathway. Here, we describe the cloning and purification of a yeast SSADH and detailed kinetic studies of the purified recombinant enzyme.

Materials and methods

Cloning of yeast *SSADH* gene

The *Saccharomyces* Genome Database (SGD) showed a reference sequence (GenBank Accession No. NC_001134) encoding a full-length ORF sequence for yeast SSADH. This sequence was used to design genomic PCR primers for the amplification of a DNA fragment of the SSADH gene containing this ORF. To facilitate expression vector construction, two restriction sites of *Xho*I and *Bam*HI were introduced at each end

of primers. The sense primer for PCR amplification contained an XhoI site: 5'-GAGAC TCGAGATGACTTTGAGTAAGTATTCTAAACCAACTC-3', and the antisense primer contained a *Bam*HI site: 5'-GAGAGGATCCTTAAATGCTGTTTGGCAAATTCC-3'. Yeast genomic DNA was prepared as described by Sherman *et al.*(1989). PCR was carried out using a PCR Thermal Cycler PTC-1000^{TM} for 30 cycles in a 25 µl reaction mixture containing 10 pmol of primers, 1 unit of High Fidelity Taq DNA polymerase (Roche Applied Sciences), and 100 ng of yeast genomic DNA under the following conditions: denaturation at 95^{o}C for 10 s, annealing at 50^{o}C for 30 s and extension at 72^{o}C for 2 min, each for 30 cycles. The PCR product was purified on 0.8% agarose gel, cloned into the p-GEM®-T Easy vector (Promega, Madison, WI) and sequenced.

Expression and purification of His6-tagged yeast SSADH

A yeast SSADH expression clone was constructed by inserting the corresponding open reading frame after amplification by PCR into the bacterial expression vector pET-16b (Novagen, Madison, WI). The amplified PCR product and the pET-16b expression vector were digested with *Xho*I and *Bam*HI, and the SSADH ORF was ligated into the vector using T4 DNA ligase (Promega) according to manufacturer's procedure.

Expression of the recombinant protein was initiated by adding 0.5 mM IPTG to the cultures of transformed BL21 cells in LB medium containing 50 mg/L ampicillin. After the addition of IPTG, the culture was further grown for 6 h to induce the expression of His6-tagged SSADH protein. Cells were harvested and resuspended in Binding Buffer (pH8.0, 50 mM sodium phosphate containing 300 mM NaCl, 10 mM 2-mercaptoethanol, and 10 mM imidazole and 200 µg/ml lysozyme). Final lysis was achieved by incubation

the cell suspension with lysozyme for 15 min at 30^{o}C. The lysate was cleared by centrifugation at 12,000 rpm in a SS34 rotor for 20 min. The supernatant was loaded onto a nickel-nitrilo triacetic acid (Ni-NTA) agarose column (Qiagen). The column was washed three times with Washing Buffer (pH 8.0, 50 mM sodium phosphate containing 10% (v/v) glycerol, 300 mM NaCl, 10 mM 2-mercaptoethanol, and 50 mM imidazole). The enzyme was then eluted with 250 mM imidazole. The purity of the eluted protein was evaluated by 12% SDS-PAGE using Coomassie blue staining to visualize the protein.

Enzyme Activity Assay

Succinate semialdehyde dehydrogenase activity was assayed in the direction of aldehyde oxidation by measuring the reduction of NAD^+ or $NADP^+$ spectrophotometrically at 340 nm (Beckman, model DU 640) in a 600 μl reaction volume at 25°C. The standard enzyme assay contained 100 μM potassium pyrophosphate buffer (PH 8.4), 50 μM SSA, and 714 μM NAD^+. The reaction was started with the addition of succinate semialdehyde dehydrogenase (0.13 μg). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 μmol of $NAD(P)^+$ per minute at 25°C and specific activity is expressed as units / mg protein. The kinetic parameters, *K*m and *K*cat values were calculated from Lineweaver-Burk plots. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. The dependence of succinate semialdehyde dehydrogenase on pH was determined in 0.1 M potassium phosphate buffer (pH 6 to 9), 0.1 M potassium pyrophosphate buffer (pH 8.5 to 9.5) and 0.1 M glycine-NaOH buffer (pH 9 to 12). Kinetic studies were repeated at least three times with different preparations of the purified recombinant SSADH.

Zymogram Staining Analysis

Purified recombinant protein was separated on 8% native PAGE at 4°C. The gel was then stained for SSADH activity by incubation at room temperature in a solution containing 100 mM potassium pyrophosphate, pH 8.4, 300 μM SSA, 0.2 mg/ml nitroblue tetrazolium (NBT), 0.06 mM phenazine methosulfate (PMS), and 1.5 mM $NAD(P)^+$ at room temperature for 15min. The excess stain was removed by washing the gels in ddH_2O. SSADH activity appeared as a dark band.

FPLC gel filtration analysis

FPLC gel filtration analysis was performed on a Bio-Rad HPLC equipped with an OD280 monitor at a flow rate of 0.4 ml / min. A 0.5 x 30 cm column packed with Sephacryl S-300-HR (Sigma Aldrich) was used in the FPLC analysis. The mobile phase was 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride (Jeong *et al.*, 2005). A molecular mass standard curve was constructed by calibrating the column with protein standards from Sigma Aldrich (Blue dextran: 2,000 kDa, Thyroglobulin: 670 kDa, Apoferritin: 443 kDa, β-Amylase: 200 kDa, Alcohol dehydrogenase: 150 kDa, Albumin: 66 kDa). The molecular mass of the native recombinant SSADH was estimated from the equation for the line of best fit ($y=-0.39x+7.79$, where y is $\log_{10}$ molecular mass and x is the retention time [V_e / V_o]).

Results

Expression and purification of SSADH in *E. coli*

The yeast SSADH gene was cloned from genomic DNA isolated from the W303-1A wild- type strain. The full sequence of the SSADH DNA encodes a 497-amino acid

protein with a molecular mass of 54,189 Da, and a computer calculation predicts the isoelectric point is 6.61.

Expression of SSADH in bacterial strain BL21/pET-16b-SSADH was induced by 0.5 mM IPTG at 37°C. Most of the expressed protein was found in the soluble extract after cell lysis. A nickle metal-affinity resin column was used for the purification of a His-tagged yeast SSADH. The purity of the protein was estimated by SDS-PAGE (Fig. 1.). The His6-tagged recombinant SSADH yielded one single band with an apparent molecular mass of 54-kDa, and the overall yield of the enzyme in the purification procedure was 76% (Table 1). The purification resulted in approximately 10 mg of pure SSADH from 100 ml of cultured cells. The addition of 5% glycerol was essential, but 5 mM β-mercaptoethanol was not required for maintaining the stability of the protein for a long time.

Properties of yeast SSADH

Gel filtration on a Sephacryl S-300-HR column (FPLC) was used to determine the native molecular weight of recombinant yeast SSADH. In three independent runs, the purified enzyme preparation eluted as a single peak with an elution volume of 20 μl. A line of best fit equation (y=-0.39x+7.79) from the protein standards was used to calculate the native molecular weight. It was found to be 200 kDa (Fig. 2). These data suggest that the recombinant yeast SSADH is a homotetramer with four 54-kDa subunits. The effect of pH on the SSADH activity was determined with the purified enzyme, the optimum pH was 8.4 in 0.1 M potassium pyrophosphate buffer.

Lactate, butonal and a number of aldehydes including formaldehyde, glyceraldehyde, propanal, hexanal, valeraldehyde and acetalaldehyde were tested as substrates for the purified recombinant yeast succinate semialdehyde dehydrogenase. The purified enzyme was highly specific for SSA and had no activity for the other tested substrates.

The recombinant yeast SSADH could utilize both NAD^+ or $NADP^+$ as a coenzyme. Howeverr, the enzyme had a 2.5-fold lower activity observed with $NADP^+$ than with NAD^+ at a concentration of 0.2 mM, which was in good agreement with the crude preparations and partially purified yeast SSADH (Ramos *et al.*, 1985).

Kinetic experiments

Kinetic studies for characterizing the recombinant yeast SSADH were carried out in the direction of $NAD(P)^+$ reduction. Initial velocity experiments were carried out by varying one substrate at several fixed substrate concentrations of the other substrate. Data from experiments with varied moderate concentrations of SSA at different fixed concentrations of $NAD(P)^+$ resulted in intersecting lines as analyzed by double-reciprocal plots (Fig. 3). Further experiments with increasing the varied substrate SSA concentration (above 100 μM) resulted in reciprocal plots which curve up as they approach the 1/v axis indicating substrate inhibition. In contrast, varied concentrations of $NAD(P)^+$ at different fixed concentrations of SSA revealed a set of parallel lines (Fig. 4 and Fig. 5). No substrate inhibition was observed when NAD^+ or $NADP^+$ was used as substrate. Kinetic constants obtained from double-reciprocal plots are presented in table 2.

The GABA shunt bypasses 2 reactions in the tricarboxylic acid cycle (α-ketoglutarate dehydrogenase and succinate thiokinase) by removing α-ketoglutarate from

the cycle and feeding succinate and NADH produced by SSADH back into the cycle. An inhibitory effect of NADH on SSADH activity is widely known for several mammalian and plant SSADH enzymes (Blaner and Churchich, 1979; Duncan and Tipton, 1971; Rivett and Tipton, 1981; Busch and Fromm, 1999; Jeong *et al.*, 2005). Similar inhibition of recombinant yeast SSADH with increasing NADH was observed here. SSADH activity was decreased significantly (Fig. 6 and Fig. 7). Further investigation revealed that NADH was a strong competitive inhibitor (Ki=129.5μM) with respect to NAD^+, while it was found to be a non-competitive inhibitor (Ki=110.7 μM) with respect to SSA at fixed concentrations of NAD^+ (Table 3). Interestingly, succinate did not cause detectable inhibition at concentrations of up to 10 mM at pH 8.6 with respect to either NAD^+ or SSA which was similar to the rat brain SSADH (Rivett and Tipton, 1981). This finding suggests that the concentrations of SSA, NAD^+, and NADH in yeast cells might partly regulate the GABA shunt through SSADH activity, but not the product, succinate.

The inhibitory effect of adenine nucleotides on the regulation of yeast SSADH was investigated. It has been reported that SSADH activity was inhibited by AMP (Rivett and Tipton, 1981; Busch and Fromm, 1999; Satya and Nair, 1989), ADP and ATP (Busch and Fromm, 1999), but no report for yeast SSADH. Similar to *Arabidopsis* SSADH, the activity of yeast SSADH was inhibited by all three adenine nucleotides. 5'-AMP was found to be a competitive inhibitor with respect to NAD^+ with a Ki value of 4.18×10^3 μM (Fig. 8) and a mixed inhibitor with respect to SSA (Fig. 9). Secondary intercept and/ or slope re-plots were linear indicating mixed inhibition. The inhibition by ADP was competitive with respect to NAD^+ (Fig. 10) and non-competitive with respective to SSA (Fig. 11) and Ki values of 5.92×10^3 μM and 7.57×10^3 μM respectively were

determined. Since most adenine-dependent enzymes require Mg^{2+} bound to the nucleotides, we then tested the inhibition by ADP with the addition of Mg^{2+}. Similar to *Arabidopsis* SSADH, in the presence or absence of 5 mM Mg^{2+}, no difference was detected for the inhibition by ADP on the yeast SSADH activity suggesting that the binding of NAD^+/ SSA was not hindered by ADP and vice versa. Inhibition by ATP was competitive with respect to NAD^+ (Fig. 12) and non-competitive with respective to SSA (Fig. 13). The *K*i value was 8.89×10^3 μM for NAD^+ and 10.21×10^3 μM for SSA. In the presence or absence of 5 mM Mg^{2+}, no difference was detected for the inhibition by ATP on the yeast SSADH activity again suggesting that the binding of NAD^+ / SSA was not hindered by ATP and vice versa.

Discussion

Yeast SSADH plays a pivotal role in the metabolism of GABA, providing the mitochondria with succinate. Physiological and biochemical studies on the role of the GABA shunt have been hampered by the lack of detailed characterization of GABA shunt enzymes. In fungi, the first two enzymes, GAD1 and UGA1, have been purified and well characterized (Christensen and Schimt, 1980; Der Garabedian, 1986). However, limited information was reported on the last enzyme SSADH (Ramos *et al.*, 1985).

In this study, we report the biochemical characterization of the recombinant yeast SSADH. The enzyme is constituted of four subunits of similar size with an apparent relative molecular mass of 200 kDa (Fig. 2). This is comparable with several other SSADH enzymes from barley, *Arabidopsis*, and potato (Blaner and Churchich, 1979; Busch and Fromm, 1999; Satya and Nair, 1989) and mammalian species (Ryzlak and

Pietruszko, 1988; Chambliss and Gibson, 1992; Jeong *et al.,* 2005). However, different results have been reported for other organisms, in *E. coli* SSADH was a dimeric protein (Doonelly and Cooper, 1981) while human brain SSADH was a heterotetramer composed of non-identical sized subunits (Ryzlak and Pietruszko, 1988). The results presented here suggest that yeast SSADH is a homotetramer. The optimum pH is similar to that reported from partially purified yeast SSADH (Ramos *et al.,* 1985), human SSADH (Jeong *et al*., 2005), and *Arabidopsis* SSADH (Busch and Fromm, 1999).

Like the partially purified yeast SSADH preparation (Ramos *et al.,* 1985), the purified recombinant yeast SSADH is highly specific for SSA. None of the other aldehyde analogs (C3 to C6 straight chain aldehyde) gave any detectable activity as a substrate. The *K*m value obtained for SSA is 15.3 µM. This is similar to, but slightly higher than the previously reported recombinant enzymes from human, *K*m = 6.3 µM (Jeong *et al*., 2005) and rat, *K*m = 3.5 µM (Murphy *et al.,* 2003), and slightly lower than the recombinant *Arabidopsis* SSADH, $K_{0.5} = 15 \pm 5$ (Busch and Fromm, 1999). Generally, it seems that eukaryotic SSADHs uses NAD^+ as cofactor (Rivett and Tipton, 1981; Busch and Fromm, 1999), rather than $NADP^+$ which is typically required by bacterial SSADHs. The recombinant yeast SSADH can utilize both NAD^+ and $NADP^+$ as a cofactor but has a much higher affinity for NAD^+, which is in good agreement with the partially purified yeast SSADH (Ramos *et al.,* 1985), and SSADH from rat (Cash *et al.,* 1977) and human brains (Cash *et al.,*1978). However, the mechanism leading to the variance with these results between these organisms is unknown.

In the direction of aldehyde oxidation, substrate inhibition was observed with SSA at concentrations above 100 µM (Fig. 3). No substrate inhibition with NAD^+ or $NADP^+$

was observed. Similarly, substrate inhibition with higher concentrations of SSA was also reported for the crude and partially purified yeast SSADH, the SSADHs from mammalian species, the SSADHs from plants, and bacterial SSADHs.

SSADH product inhibition by NADH was reported from mammalian species (Blaner and Churchich, 1979; 1980; Rivett and Tipton, 1981; Jeong *et al.*, 2005) and plants (Busch and Fromm, 1999). Here, similar result for a yeast SSADH is reported that it has a comparatively high affinity for NADH. NADH is a strong competitive inhibitor with respect to NAD^+ (Ki = 129.5 μM), but is a non-competitive inhibitor with respect to SSA (Ki = 110.7 μM). However, similar to rat brain SSADH (Rivett and Tipton, 1981), no product inhibition by succinate could be detected for yeast SSADH, which makes it difficult to accurately determine the inhibition by NADH at the SSA substrate concentrations at Km levels for NAD^+. The high substrate inhibition is more likely caused by a compulsory-order mechanism in which NADH is the last product to be released from the enzyme and the inhibition is resulted from the formation of an abortive ternary complex between enzyme, NADH and SSA (Rivett and Tipton**,** 1981).

Adenine nucleotide inhibition by AMP was reported for rat-brain SSADH (Rivett and Tipton, 1981) and potato SSADH (Satya and Nair, 1989). *Arabidopsis* SSADH was inhibited by all three adenine nucleotides (Busch and Fromm, 1999), and it was also reported that succinate dehydrogenase in mitochondria is regulated by ATP (Singer *et al.*, 1973). Our kinetic studies show that yeast SSADH was inhibited by AMP, ADP and ATP similar to the plant enzyme. The inhibition pattern given by AMP, ADP and ATP is competitive with respect to NAD^+, mixed-competitive and non-competitive, and non-competitive respectively with respect to SSA. We may reason that AMP inhibition is

regulated by an ordered or random mechanism through which AMP could bind to the free enzyme or bind to the enzyme-SSA complex or both in the latter case. For the type of inhibition by ADP and ATP, we assume that the inhibitor could only bind to the enzyme-NAD^+ binary complex in a compulsory-order mechanism rather than in a random-order mechanism. The inhibition pattern given by AMP is comparable with rat-brain SSADH (Rivett and Tipton, 1981). However, the inhibition by ADP and ATP differs from that previously reported for *Arabidopsis* SSADH (Busch and Fromm, 1999) with respect to NAD^+ which was mixed-competitive and non-competitive respectively. However, like *Arabidopsis* SSADH (Busch and Fromm, 1999), it was found that yeast SSADH was not dependent on the complex of the nucleotide with Mg^{2+}.

The inhibition constants of yeast SSADH for all the three adenine nucleotides were in the milimolar range. It was found in plants that ATP regulates some enzymes in the TCA cycle in the millimolar concentration range (Raymond *et al.,* 1987). In addition, AMP and ATP inhibited *Arabidopsis* SSADH in the milimolar range (Busch and Fromm, 1999). In animals, the concentrations of total nucleotides in mitochondria are in the milimolar range (Pradet and Raymond, 1983; Hutson *et al.,* 1989). In yeast, the levels of ATP determined by Theobald *et al* (1996**)** during steady state growth in both cytosol and mitochondrial were in milimolar range (2.1 ± 0.1 mM and 7.8 ± 0.3 mM respectively), and the levels of AMP and ADP were 0.11 ± 0.03 mM and 0.47 ± 0.05 mM respectively in cytosol and for ADP the level in mitochondria was 0.8 ± 0.1 mM. The levels of NAD^+ in both cytosol and mitochondria were 1.07 mM and 1.73 mM, while the concentration of NADH was 0.04 mM in the cytosol and 6.28 mM in mitochondria. These values indicate

the possibility that adenine nucleotides and pyridine nucleotides inside yeast cells and other organisms are in the range to affect the SSADH activity.

Kinetic experiments with recombinant yeast SSADH showed an interesting substrate saturation pattern in double reciprocal plots versus initial velocity. These experiments revealed intersecting lines with SSA as the varied substrate, while parallel lines were obtained with various concentrations of $NAD(P)^+$. Two ping pong mechanisms previously reported (Brigitte and Gerhard, 1993; Cleland, 1979; Fromm, 1967**)** are in accord with our kinetics, in which, the binding of the substrate which gives parallel lines in double reciprocal plots is followed by the release of a product. The substrate may bind before the last product is released or bind followed by the first product released (Brigitte and Gerhard, 1993). In our kinetic data for yeast SSADH, varied $NAD(P)^+$ gave parallel lines, we predict that binding of the $NAD(P)^+$ is followed by the release of NAD(P)H at the beginning or in the end of the reaction. However, more investigation is needed to provide evidence for this proposed mechanism.

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Table 1. Single step purification of recombinant yeast SSADH

Purification step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	*units*	*Units* mg^{-1}	*fold*	*%*
Crude extract	10	5	0.5	1	100
Ni-NTA agarose	2	3.80	1.90	3.8	76

Table 2. Kinetic parameters of recombinant yeast SSADH

substrate	*K*m	*V*max	*k*cat/*K*m
	µM	µmol.min^{-1}.mg^{-1}	$M^{-1}s^{-1}$
SSA	15.48 ± 0.14	15.93 ± 0.55	7.28 x 10^5 ± 210
NAD^+	579.06 ± 30.1		2.38 x 10^4 ± 108
$NADP^+$	(1.017 ± 0.46) x 10^3		1.15 x 10^4 ± 152

Table 3. Inhibition of recombinant yeast SSADH by the products and nucleotides

Inhibitor	Varied substrates	Pattern	*K*i[a] or *K*ui[b] (μM)
NADH	SSA	Non-competitive	110.7
NADH	NAD^+	competitive	129.5
SUCC	SSA	No inhibition[c]	
SUCC	NAD^+	No inhibition[c]	
AMP	SSA	Mixed-competitive	*K*i = 1.13×10^3, *K*ui = 2.37×10^3
AMP	NAD^+	competitive	4.18×10^3
ADP	SSA	Non-competitive	7.57×10^3
ADP	NAD^+	Competitive	5.92×10^3
ATP	SSA	Non-competitive	10.21×10^3
ATP	NAD^+	Competitive	8.89×10^3

[a]*K*i, competitive inhibition constant; [b]*K*ui, non-competitive inhibition constant; [c]No inhibition, no inhibition at succinate concentration up to 10 mM.

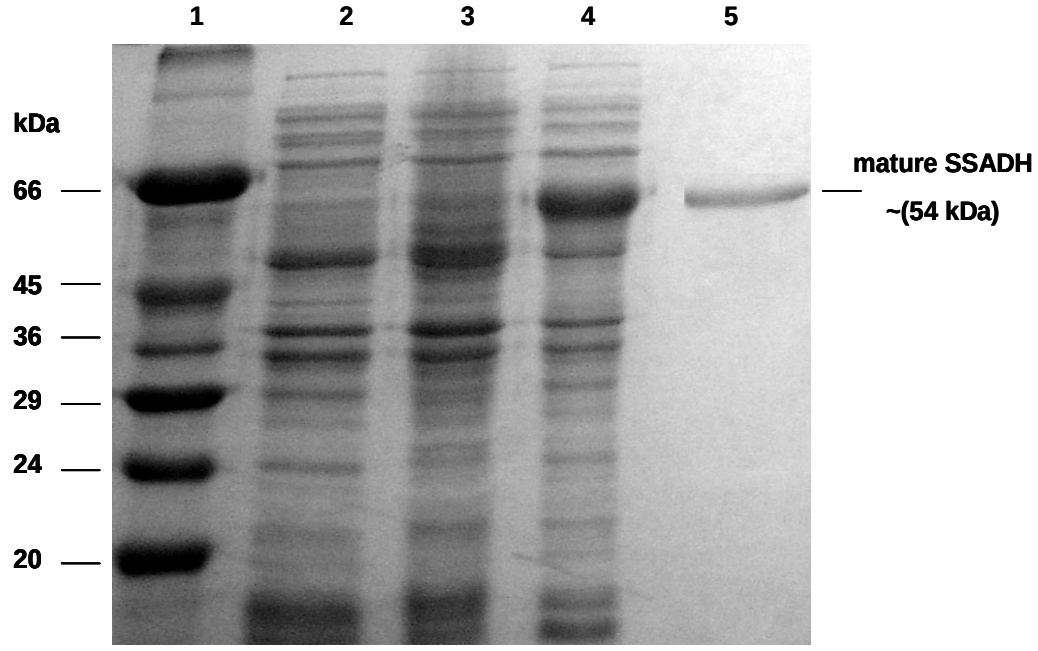

Fig. 1. Expression and purification of recombinant yeast SSADH. 12% of SDS-PAGE analysis of crude cell extracts of BL21 transformed with expression vector pET16b containing the SSADH coding sequence. Lane 1, marker protein; Lanes 2, 3 and 4, cell free extracts (50 μg) before induction, without and with the presence of 0.5 mM IPTG; Lane 5, purified His6-tagged recombinant enzyme (5 μg).

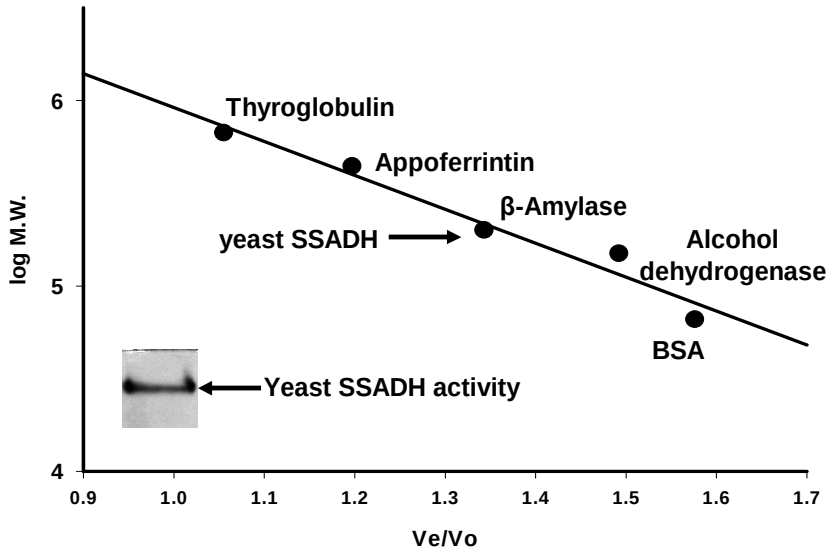

Fig. 2. Molecular Mass determination of native SSADH by FPLC gel filtration on Sephacryl S-300-HR. Sephacryl S-300-HR was used to determine the molecular mass of purified SSADH as described under "Materials and Methods". The molecular mass of the proteins used to calibrate the column are: Thyroglobulin (670 kDa), Appoferrintin (443 kDa), β-Amylase (200 kDa), Alcohol dehydrogenase (150 kDa), BSA (66 kDa). The void volume (Vo) was determined with blue dextran (2000 kDa). The arrow points to the position of SSADH on the standard curve. Inset shows the zymogram staining of yeast SSADH activity as described under "Materials and Methods".

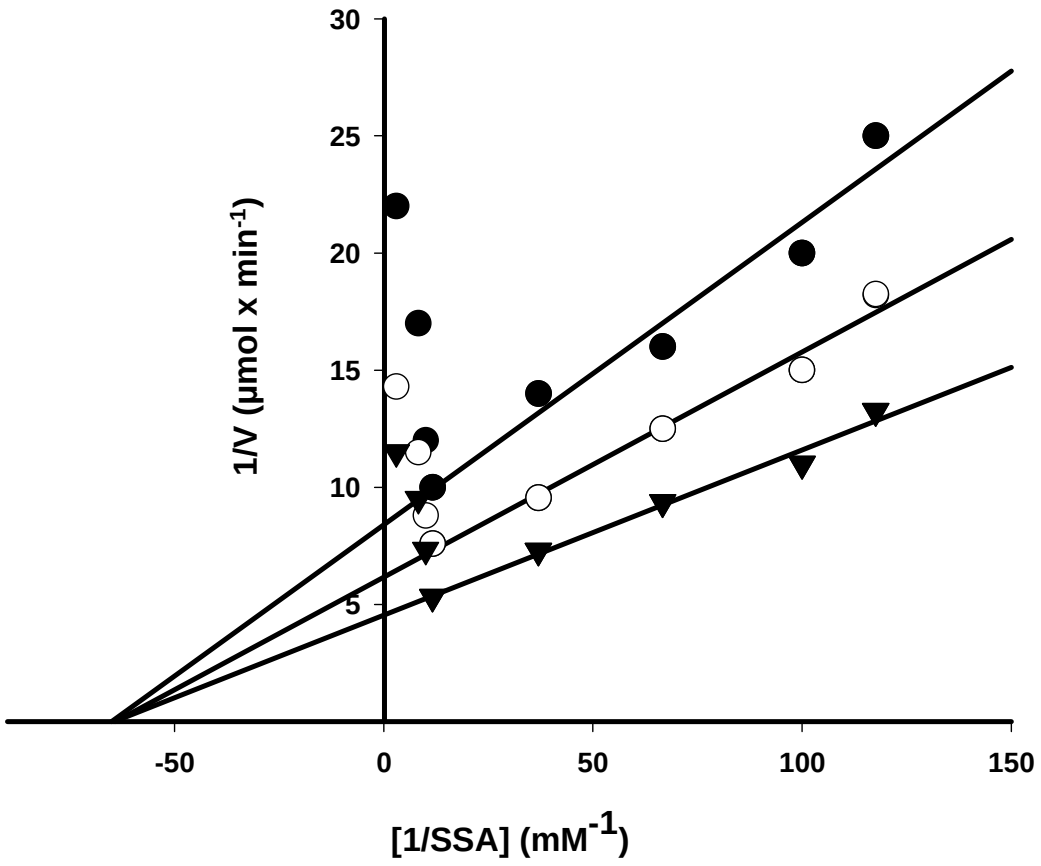

Fig. 3. Double reciprocal plot from initial velocity experiments of yeast recombinant succinate semialdehyde dehydrogenase with varied concentrations of SSA. Enzyme activities were assayed in the direction of SSA oxidation as described in Materials and Methods. Substrate concentrations for SSA were varied over 0.0067-0.317 mM as indicated in the figure. Concentrations of NAD+ were 0.225 mM (●), 0.416 mM (O) and 0.712 mM (▼). Note the decrease in velocity at the highest substrate levels indicating substrate inhibition.

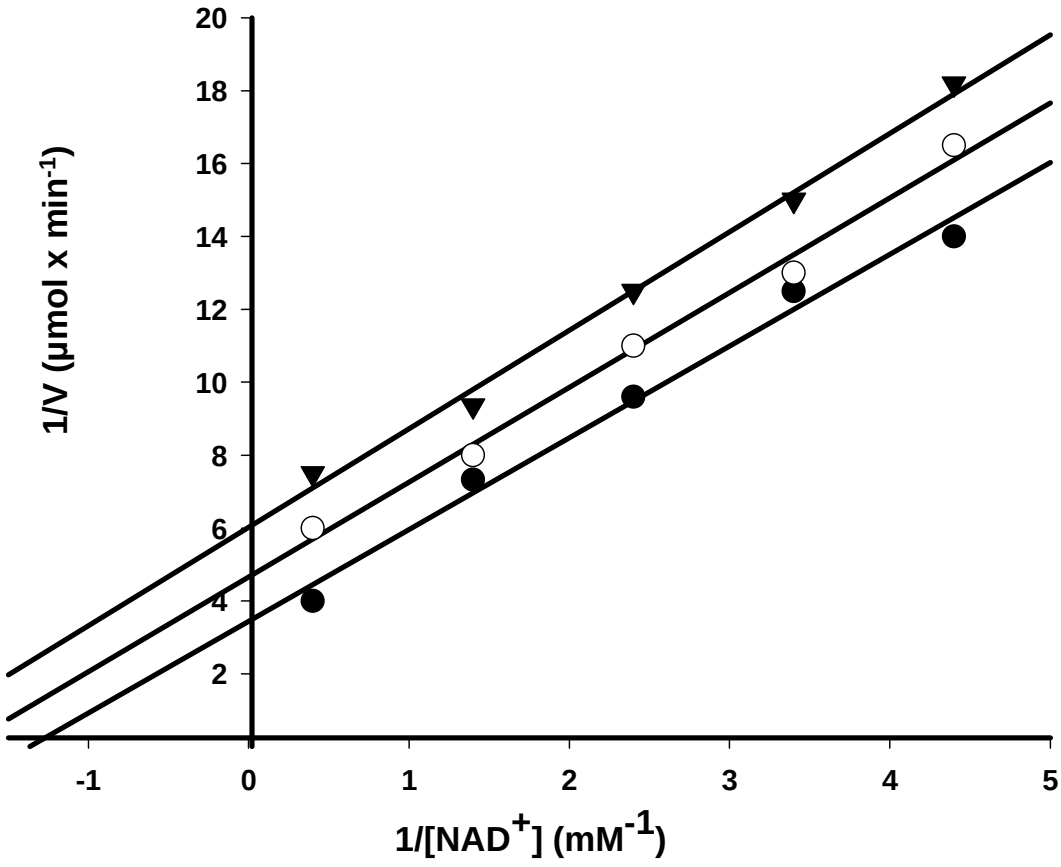

Fig. 4. Double reciprocal plot from initial velocity experiments of yeast recombinant succinate semialdehyde dehydrogenase with varied concentrations of NAD^+. Enzyme activities were assayed in the direction of SSA oxidation as described in Materials and Methods. Substrate concentrations for NAD^+ were varied over 0.227-2.5 mM as indicated in the figure. Concentrations of SSA were 0.090 mM (●), 0.045 mM (O) and 0.030 mM (▼).

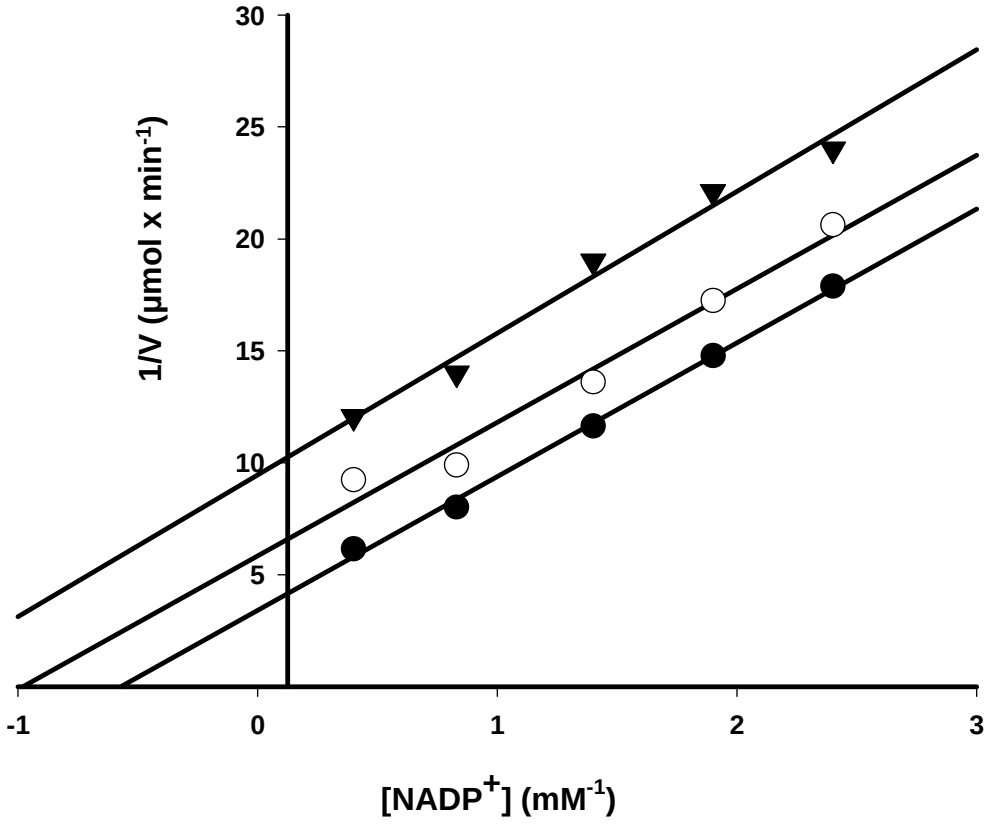

Fig. 5. Double reciprocal plot from initial velocity experiments of yeast recombinant succinate semialdehyde dehydrogenase with varied concentrations of $NADP^+$. Enzyme activities were assayed in the direction of SSA oxidation as described in Materials and Methods. Substrate concentrations for $NADP^+$ were varied over 0.417-2.5 mM as indicated in the figure. Concentrations of SSA were 0.085 mM (●), 0.027 mM (O) and 0.015 mM (▼).

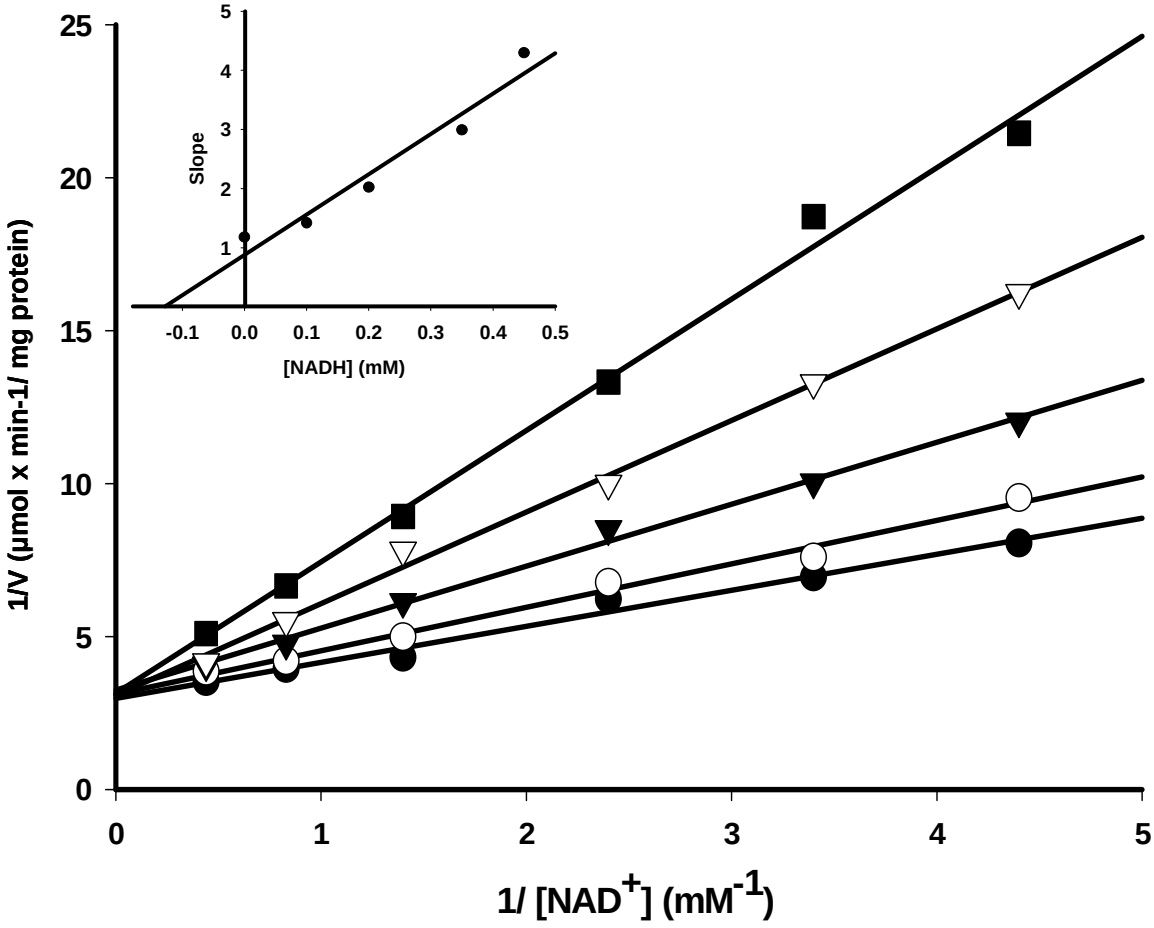

Fig. 6. Inhibition of yeast recombinant SSADH by NADH. Double-reciprocal plots of the rate of NADH formation *vs* NAD^+ concentration at different NADH concentrations: 0 µM NADH (●), 0.1 mM NADH (O), 0.2 mM NADH (▼), 0.35mM NADH (∇), 0.45 mM NADH (■). Assays were carried out as described in Fig. 2 in the presence of 85 µM of SSA. The inset shows the secondary plot of the line slope *vs* NADH concentration to determine the inhibition constant.

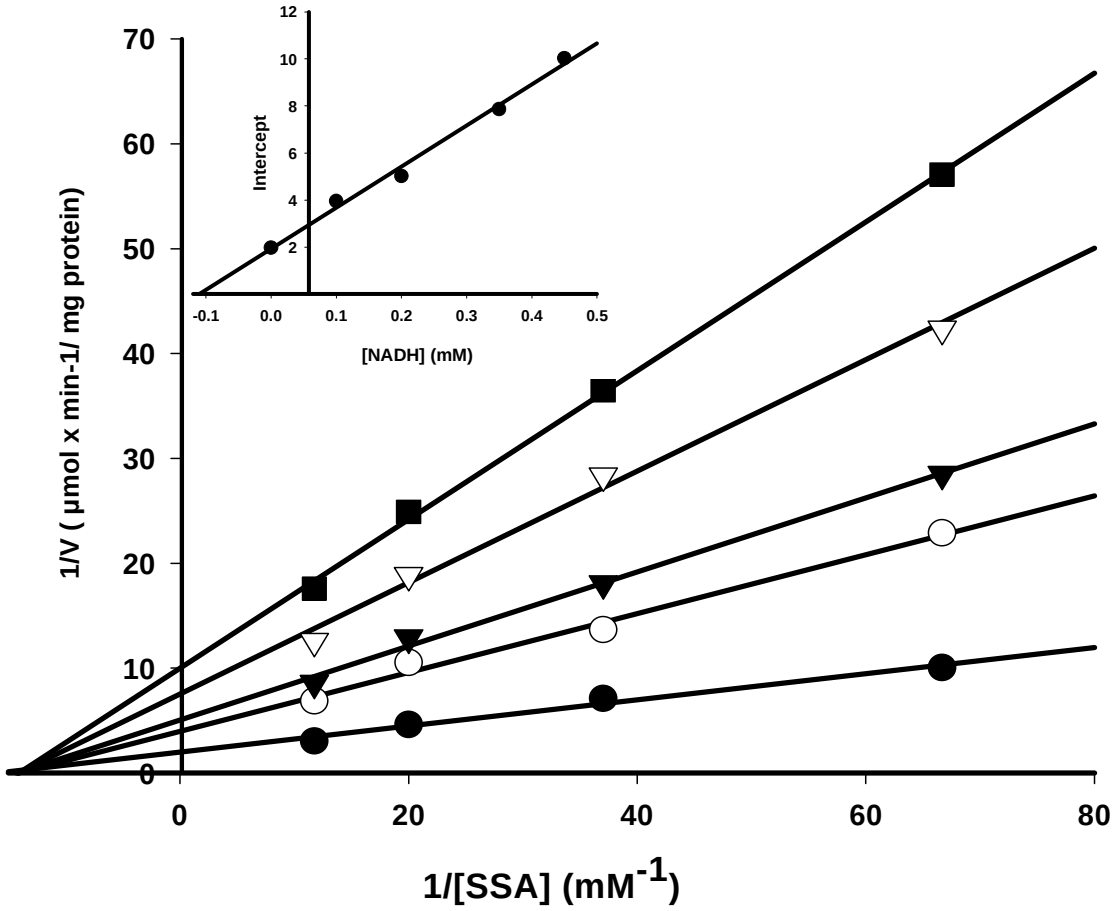

Fig. 7. Inhibition of yeast recombinant SSADH by NADH. Double-reciprocal plots of the rate of NADH formation *vs* SSA concentration at different NADH concentrations: 0 µM NADH (●), 100 µM NADH (O), 200 µM NADH (▼), 350 µM NADH (∇), 450 µM NADH (■). Assays were carried out as described in Fig. 2 in the presence of 712 µM of NAD. The inset shows the secondary plot of the line intercept *vs* NADH concentration to determine the inhibition constant.

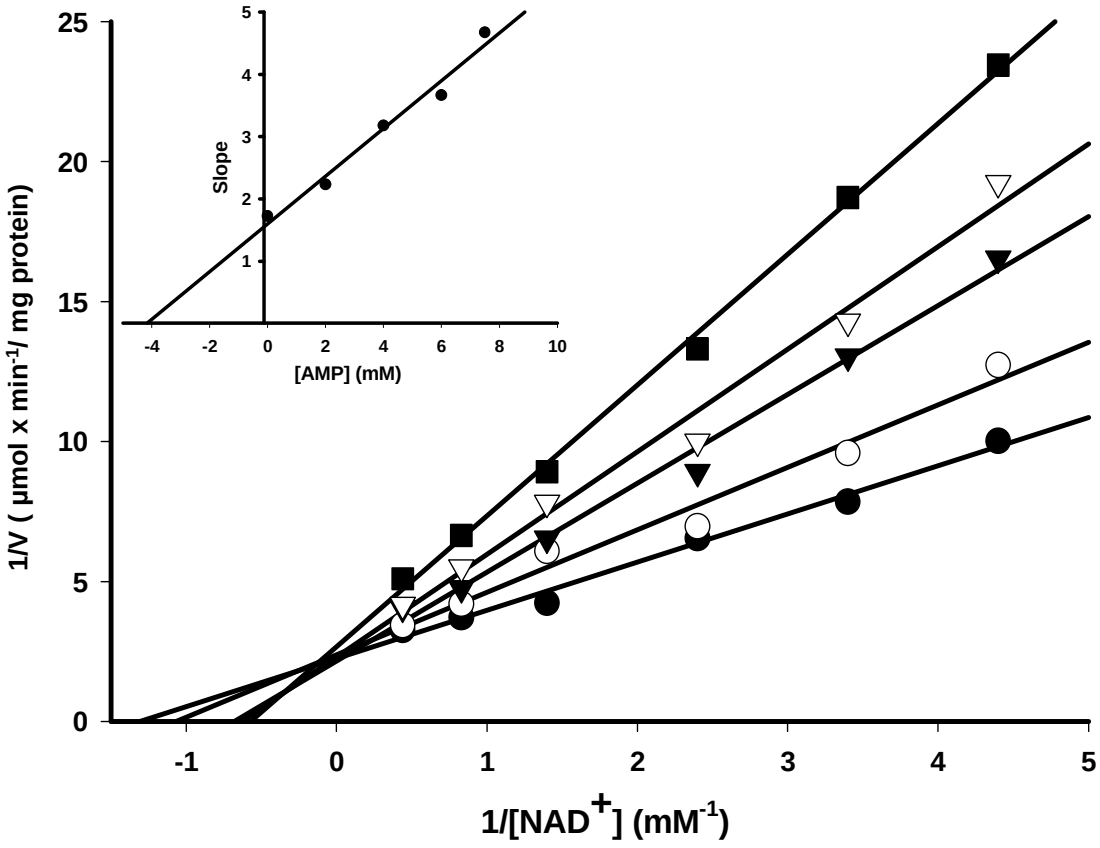

Fig. 8. Inhibition of yeast recombinant SSADH by AMP. Double-reciprocal plots of the rate of NADH formation vs NAD^+ concentration at different AMP concentrations: 0 mM AMP (●), 2mM AMP (O), 4 mM AMP (▼), 6 mM AMP (∇), 7.5 mM AMP (■). Assays were carried out as described in Fig. 2 in the presence of 85 µM of SSA. The inset shows the secondary plot of the line slopes vs AMP concentration to determine inhibition constant.

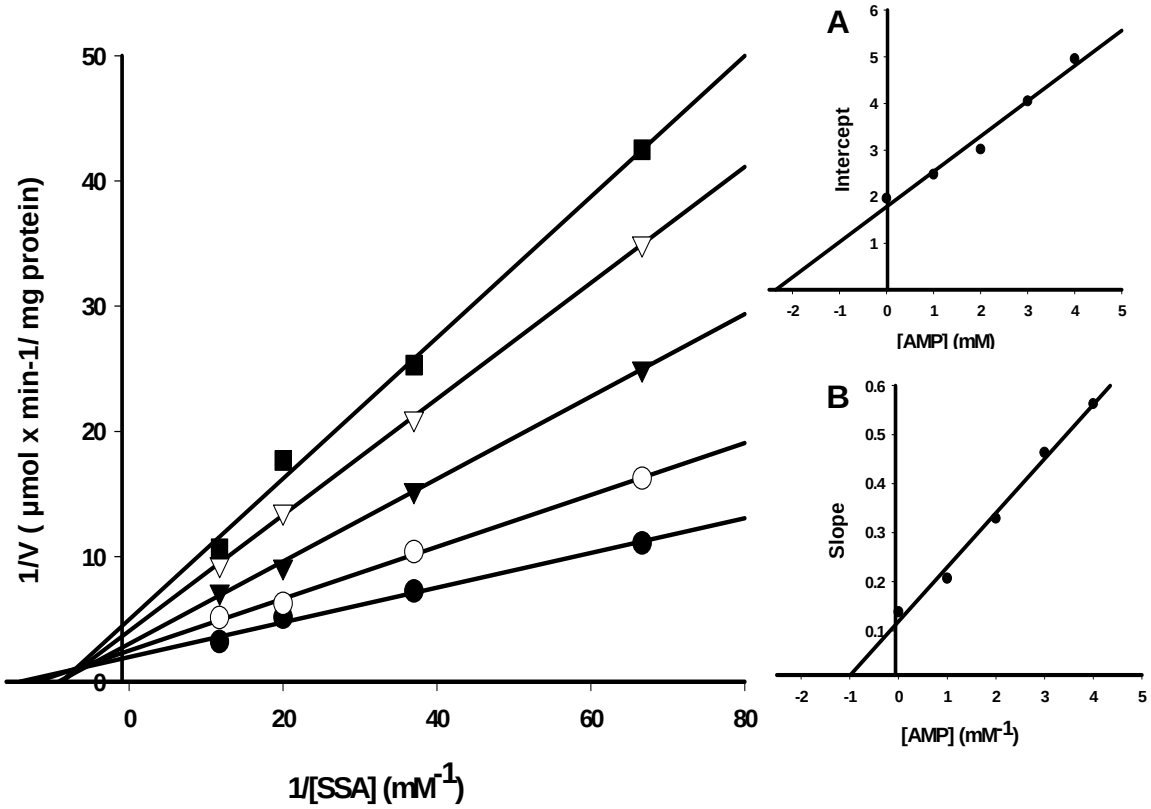

Fig. 9. Inhibition of yeast recombinant SSADH by AMP. Double-reciprocal plots of the rate of NADH formation *vs* SSA concentration at different AMP concentrations: 0 mM AMP (●), 1mM AMP (O), 2 mM AMP (▼), 3 mM AMP (∇), 4 mM AMP (■). Assays were carried out as described in Fig. 2 in the presence of 712 µM of NAD. The insets show the secondary plot of the line intercepts (1/Vmax) (A) or slope (B) *vs* AMP concentration to determine the inhibition constants.

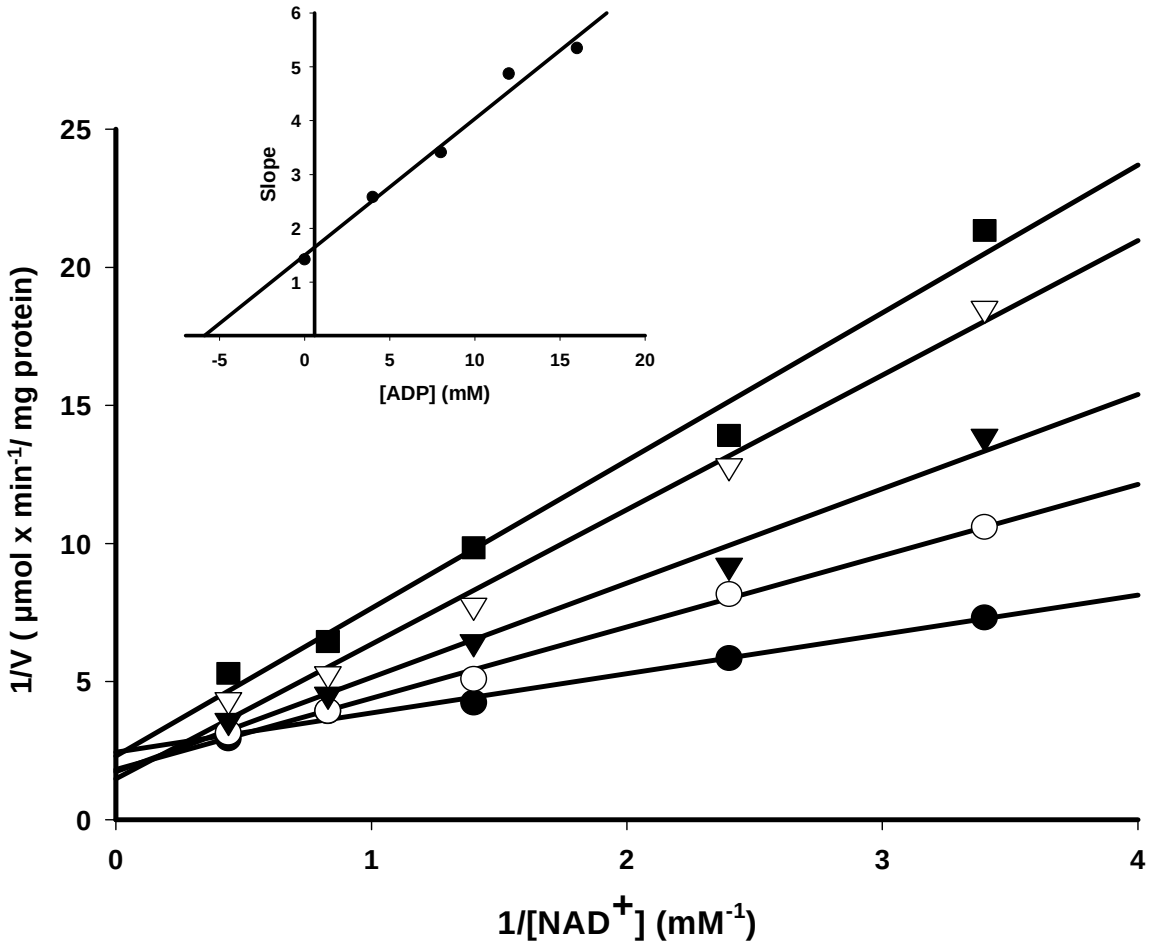

Fig. 10. Inhibition of yeast recombinant SSADH by ADP. Double-reciprocal plots of the rate of NADH formation *vs* NAD concentration at different ADP concentrations: 0 mM ADP (●), 4mM ADP (O), 8 mM ADP (▼), 12 mM ADP (∇), 16 mM ADP (■). Assays were carried out as described in Fig. 2 in the presence of 85 µM of SSA. The inset shows the secondary plot of the line slopes *vs* ADP concentration to determine the inhibition constant.

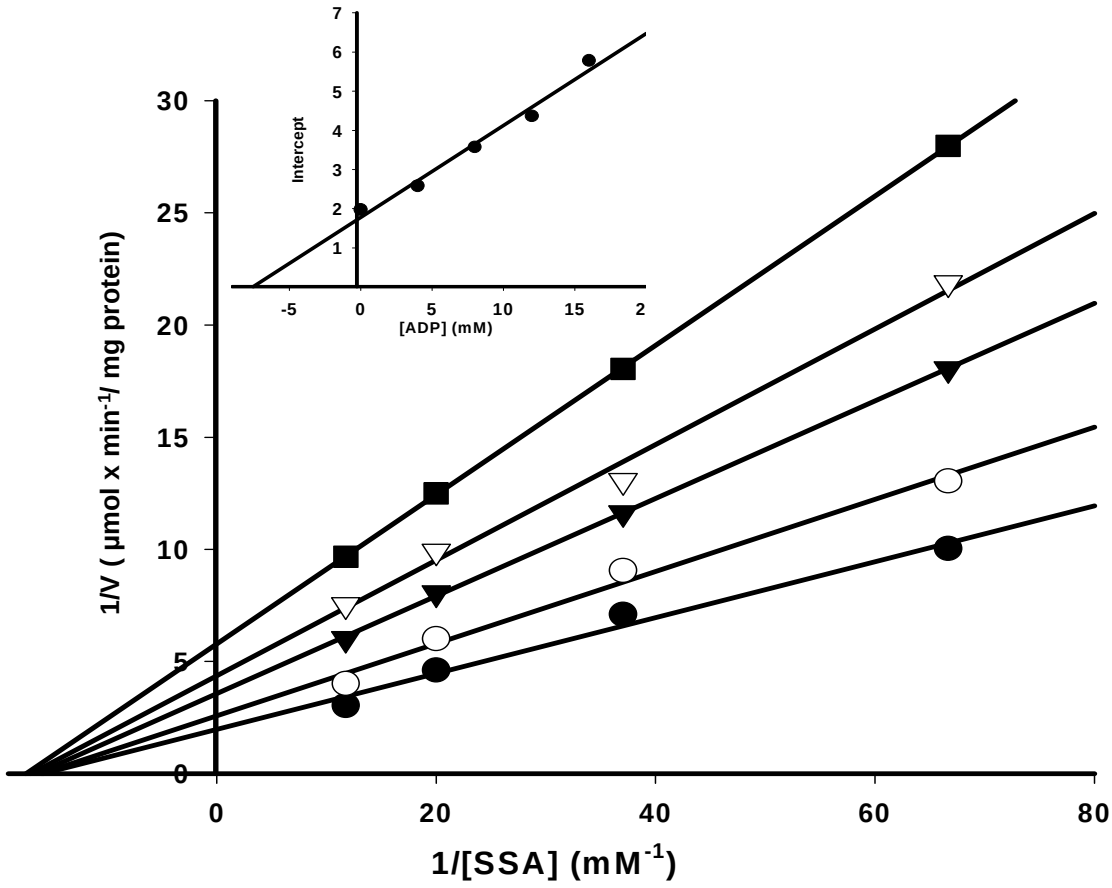

Fig. 11. Inhibition of yeast recombinant SSADH by ADP. Double-reciprocal plots of the rate of NADH formation vs SSA concentration at different ADP concentrations: 0 mM ADP (●), 4mM ADP (O), 8 mM ADP (▼), 12 mM ADP (∇), 16 mM ADP (■). Assays were carried out as described in Fig. 2 in the presence of 712 µM of NAD. The inset shows the secondary plot of the line intercept (1/Vm) *vs* ADP concentration to determine the inhibition constant.

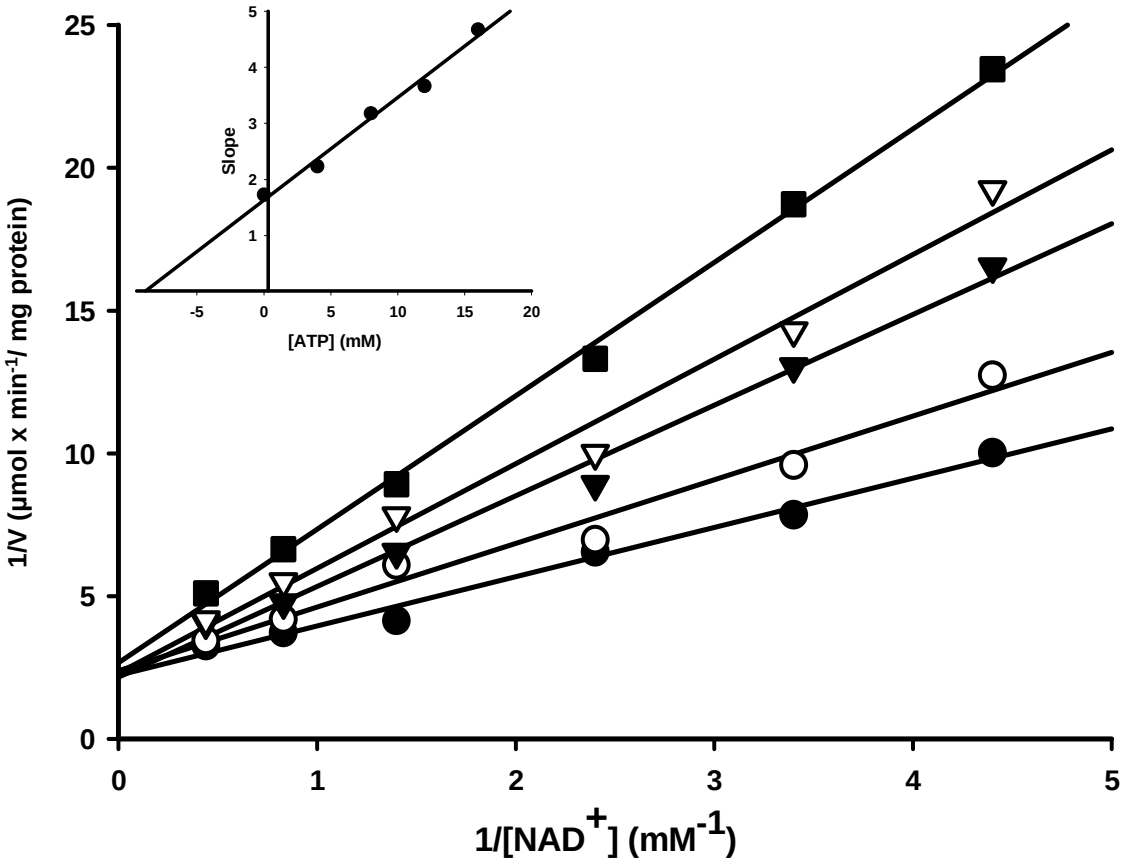

Fig. 12. Inhibition of yeast recombinant SSADH by ATP. Double-reciprocal plots of the rate of NADH formation vs NAD concentration at different ATP concentrations: 0 mM ATP (●), 4mM ATP (O), 8 mM ATP (▼), 12 mM ATP (∇), 16 mM ATP (■). Assays were carried out as described in Fig. 2 in the presence of 85 µM of SSA. The inset shows the secondary plot of the line slopes *vs* ATP concentration to determine the inhibition constant.

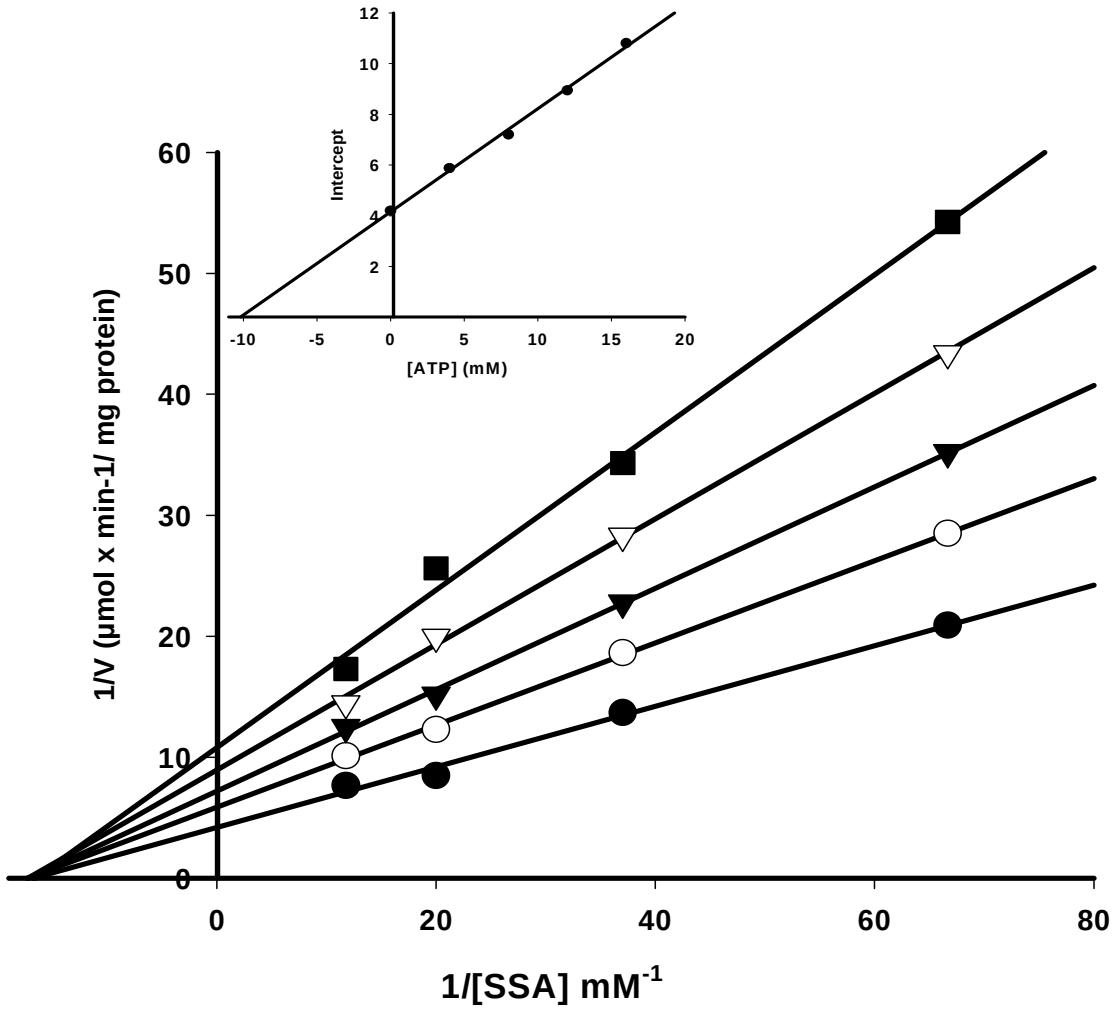

Fig. 13. Inhibition of yeast recombinant SSADH by ATP. Double-reciprocal plots of the rate of NADH formation *vs* SSA concentration at different ATP concentrations: 0 mM ATP (●), 4mM ATP (O), 8 mM ATP (▼), 12 mM ATP (∇), 16 mM ATP (■). Assays were carried out as described in Fig. 2 in the presence of 712 µM of NAD. The inset shows the secondary plot of the line intercept (1/Vmax) *vs* ATP concentration to determine the inhibition constant.

VI. IN GEL STAINNING METHOD FOR DETECTING GABA TRANSAMINASE ACTIVITY

Abstract

A method was developed to detect GABA transaminase activity in native polyacrylamide gels. This method was then tested using homogenates from *Arabidopsis thaliana* for the identification of plant GABA-TP substrates. GABA transaminases in cell-free crude extract samples were resolved by native polyacrylamide gel and then the protein gels were incubated with the succinate semialdehyde dehydrogenase coupling substrate solution containing GABA as the amino donor and α-ketoglutarate or pyruvate as the amino acceptor, the resulting product succinate semialdehyde was reduced by succinate semialdehyde dehydrogenase causing the oxidation of nitroblue tetrazolium. The resulting enzyme activity appeared as a dark band against an opaque background. The whole procedure can be completed within 15 to 20 min after protein gel electrophoresis, and is a rapid, semiquantitative method to examine the activity of GABA aminotransferase activity in crude extracts.

Introduction

GABA is a ubiquitous, four carbon non protein amino acid which is widely found from all prokaryotic and eukaryotic organisms (Satya and Nair 1990; Bown and Shelp 1997; Shelp *et al*., 1999). It is well known as a neurotransmission inhibitor in mammals (Varju *et al*., 2001), but its role in most organisms remains unknown. For decades, GABA has been found to accumulate rapidly in plants in response to various biotic and abiotic stresses, such as hypoxia, cold, heat, and mechanical stimulation (Kinnersley and Turano, 2000).

GABA is produced from glutamate by glutamate decarboxylase (GAD), and then transaminated by GABA aminotransferase (GABA-TP) to succinate semialdehyde (SSA) which is finally converted to succinate by succinate semialdehyde dehydrogenase (SSADH) (Bown and Shelp, 1997). These reactions constitute the GABA shunt pathway, which moves carbon from α-ketoglutarate through glutamate to succinate in the TCA (Krebs) cycle.

In *Saccharomyces*, GABA transaminase was found to play an important role in stress tolerance (Coleman *et al*., 2003; Chapter 1) and is highly specific for α-ketoglutarate as a substrate (Ramos *et al*., 1985). In plants, the only well characterized GABA-T is a pyruvate-dependent form (Van Cauwenberghe *et al*., 1999). However, an α-ketoglutarate-dependent GABA-T activity has been observed in tobacco crude preparations (Van Cauwenberghe *et al*., 1999). Pyruvate-dependent activity (GABA-TP) may have been partially separated from an α-ketoglutarate-dependent GABA-T activity by FPLC anion exchange chromatography (Van Cauwenberghe *et al*., 1999). However,

the α-ketoglutarate dependent GABA-T activity was so unstable that such conclusions are uncertain and have not been verified. To date no plant (in the Viridiplantae) cDNA or genomic sequences with homology to bacterial, fungal, or animal α-ketoglutarate-dependent GABA-T are found in the nonredundant database at the National Center for Biotechnology information or have been found including plants with complete genome sequences. Thus, it is unclear whether an α-ketoglutarate-dependent GABA-T exists in any higher plant.

A number of assay methods for GABA-T activity have been recorded. The transamination activity was detected by measuring product formation such as alanine, glutamate, or SSA which can be determined either by HPLC (Van Cauwenberghe *et al*., 1995) or a second enzyme coupled assay based on the formation of NAD(P)H which absorbs at 340 nm spectrophotometrically (Ansari *et al*., 2005; Ramos *et al*., 1985). An activity staining of a filter paper print of electrophoresis resolved proteins has been used to identify GABA-T from plant tissues based on the fluorescence of NADPH under short wave ultraviolet light (Van Cauwenberghe *et al*., 2002), but none of the above techniques has met wide acceptance.

In-gel activity staining or zymography is a useful technique for the detection of enzyme activities in non-denaturing polyacrylamide gels. It involves protein separation by electrophoresis followed by in gel assay of enzyme activities (Gabriel and Gersten, 1992; Gabriel and Gersten, 1992). It has proved invaluable in assessing the enzyme activity in non-fractionated cell extracts and for estimating the molecular weight and isoelectric point of the corresponding polypeptides and their isoforms (Kaberdin and McDowall, 2003). An in gel activity method for the detection of succinate semialdehyde

dehydrogenase from rat brain has been reported (Kammeraat and Veldstra, 1986). In this paper, we have extended the in-gel activity staining for succinate semialdehyde dehydrogenase activity to the detection of GABA transaminase activity by using our previously characterized yeast GABA-T knockout and overexpression of yeast strains and *Arabidopsis* plants as the enzyme sources to demonstrate the ability of the in gel staining assay system to detect GABA-T activity from crude protein mixtures.

Materials and methods

Plant material, growth condition, and Media

Seeds of *Arabidopsis thaliana* ecotype 'Columbia' were obtained from Lehli Seeds.. Standard procedures were employed for plant germination and growth. The plants were grown on basal salts medium of Murashige and Skoog (Murashige, 1962) at approximately 22ºC under continuous light provided by cool-white fluorescent lamps for two weeks. The collected plant seedlings were rapidly frozen in liquid N_2 and stored at -80ºC before extraction.

Yeast strains, growth conditions and media

GABA transaminase deletion mutant *Δuga1* (Mat a *leu2-3112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met uga1::HIS3*) derived from wild type yeast strain W303-1A (Mat a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1, lys2, met*) was generated as described (Chapter I). A plasmid P425 GPD-UGA1 bearing yeast GABA transaminase gene *UGA1* was transformed into *Δuga1* and wild type W303-1A as described (Chapter II) to generate the overexpression of UGA1p. The wild type, mutant and overexpression transformant strains were grown on YPD medium containing 2% glucose, 1% yeast extract, 2% yeast

bactopeptone, or YNB medium containing 0.67% [wt/vol] yeast nitrogen base and 2% glucose supplemented with essential amino acids (Sherman *et al*., 1979).

Enzyme and protein crude extract preparations

The enzyme succinate semialdehyde dehydrogenase (SSADH) cloned and purified as described (Chapter V) was used for the assay of GABA-transaminase activity.

Two weeks old *Arabidopsis* seedlings were homogenized as described (Wattebled *et al*., 2005) in cold extraction buffer (100 mM MOPS, pH 7.2; 1 mM EDTA; 1 mM dithiothreitol; and 10% glycerol). The homogenate was centrifuged twice for 20 min at 10,000*g* at 4°C. The resulting supernatant was immediately used to perform zymograms (after protein assay).

Mid-log phase yeast cells were harvested and washed once in ddH_2O. The washed cells were suspended in in lysis buffer containing 53.35 mM sodium phosphate, pH 8.0; 47.8 mM sodium chloride; 5 mM potassium chloride; 61 mM glucose, 0.1% Triton X-100 and 0.1 mM EDTA and broken using glass beads as described (Kharade *et al*., 2005). The homogenate was centrifuged at 4°C for 10 min at 10,000*g*. The protein content of the resulting supernatant was determined, and it was immediately used to perform zymograms as described below.

Protein concentration was determined spectrophotometrically at 595 nm by Bradford method (Bradford, 1976) using the Bio-Rad Protein Assay Dye Reagent concentrate (Bio-Rad, Hercules, CA).

Zymograms of GABA transaminase activities

Protein gels were prepared according to the standard protocol (Sambrook *et al*., 1989). Protein mixtures were separated under non-denaturing conditions using 8% polyacrylamide gels with 5% stacking gel. For detection of GABA transaminase activity, gels was soaked at room temperature with gentle shaking in a 10 ml solution containing 100 mM potassium pyrophosphate buffer, pH 8.4, 0.1mM pyridoxal 5-phosphate (PLP), 16mM GABA and 4 mM pyruvate or α-ketoglutarate for 5 min for the first step GABA transaminase reaction, the resulting product succinate semialdehyde (SSA) was determined based on the oxidation of nitroblue tetrazolium (NBT) with the addition of 0.2 mg/ml NBT, 0.06 mM phenazine methosulfate (PMS), 1.5 mM NAD^+ and 1.5 unit of SSADH. The gel was incubated with this solution at room temperature for another 10 to 15 min until dark bands appeared. The excess stain was removed by washing the gels in ddH_2O.

Results and Discussion

Published methods for assaying the enzyme activity of GABA transaminse are mostly based on the production of NADH or NADPH (Ramos *et al*., 1985; Ansari *et al*., 2005) or measuring the formation of product of alanine, glutamate or SSA. Most of these assays do not work well on crude cell extracts because the assays are not very specific requiring separation of the protein mixture to accurately assay the activity of interest, and such assays can also be so time consuming. Zymogram staining is a common technique for the detection of many enzyme activities in acrylamide gels under non-denaturing conditions based on the production of colored products immediately following protein

separation by gel electrophroesis. We have developed a novel method using zymogram in gel staining to overcome many of these drawbacks and avoid possible artifacts from spectrophotometric measurements. The detection GABA-T activity requires relatively short incubation time (15 ~ 20 minutes), and many samples can be assayed at once.

The detection of GABA-transaminase from various yeast sources indicated that in gel staining can be effectively employed. Yeast GABA-transaminase is α-ketoglutarate specific and encoded by the *UGA1* gene. The zymogram shown in Fig. 1 demonstrates only a single band of UGA1p activity in the cell free crude extracts from yeast cells. One single band was detected in the wild type cells, and no band was observed in the *Δuga1* deletion mutant due to the deletion of the coding sequence, confirming the loss of function of UGA1p in the *Δuga1* deletion mutant. When the plasmid bearing *UGA1* gene under a strong constitutive promoter (P425 GPD-UGA1) was transformed into the wild type and mutant strains, the wild type cells showed a much stronger band with UGA1p activity. Similarly, more UGA1p activity was recovered in the mutant transformant cells than detected wild type level. However, it should be noted that some similar enzyme isoforms may migrate together with the detected protein because the separation of the proteins by native polyacrylamide gel is based on the electric charge density. The specificity of the detection of UGA1p activity was confirmed by the reaction when no GABA was added, no band was detected (data not shown).

It has been widely accepted that plant GABA-transaminase is pyruvate specific and can only use pyruvate as a substrate. Interestingly, α-ketoglutarate dependent plant GABA-T activity was shown to be present in the crude extracts from tobacco leaf, but not detected in the partially purified preparation (Van Cauwenberghe *et al*., 1999). This

suggests the existence of a second α-ketoglutarate-specific GABA-T in plants, or alternatively that the α-ketoglutarate-dependent plant GABA-T activity was an artifact due to some combination of aminotransferase activities in crude preparations. To further identify the substrates of plant GABA-T and identify the number of GABA-T activities in plants crude cell-free extracts from 2 weeks old Arabidopsis seedlings were separated on 8% native polyarcylamide gels, and zymogram staining was conducted for GABA-T activity. In Fig. 2, when pyruvate was present as substrate in the staining solution, a single dark band was detected showing the pyruvate-dependent GABA-T activity (lane 1). When α-ketoglutarate instead of pyruvate was present, even darker bands were detected in a different location (lane 2) showing the α-ketoglutarate-dependent enzyme activities. To further investigate whether the detected bands were related to GABA-T activity, SSADH (lane 3, Fig. 2) and GABA (lane 4, Fig. 2) were separately removed from the staining solution. Unexpectedly, the enzyme activity-related bands were not observed at the same location as in lane 1, suggesting that α-ketoglutarate-dependent GABA-T activity was not observed. However, it was interesting to find bands were disappeared without the presence of co-factor NAD^+ (lane 6, Fig. 2), α-ketoglutarate (lane 7) or both (data not shown). Possibly an α-ketoglutarate dependent dehydrogenase activity was detected instead. These results from in gel staining indicated to us that the plant GABA-T from *Arabidopsis* was highly pyruvate-dependent, and that there was no detectable GABA-T activity for the substrate α-ketoglutarate, which was contradictory to Van Cauwenberghe *et al*.'s finding (1999).

Conclusion

We describe a simple and rapid zymogram technique for detection of GABA-transaminase activity after electrophoresis under native conditions in polyacrylamide gels. The technique involves incubation of the native gel with a SSADH staining solution containing GABA-T and SSADH substrates. The assays were based on the production of SSA from the first reaction by GABA transaminase which was subsequently detected by excess SSADH leading to the production of NADH and the subsequent reduction of NBT. The reduced product in the gel appeared as a dark blue band. The method was shown to be useful for monitoring of GABA-T activities from cell crude extracts including plants and yeast and possibly other organims as well as for characterization of GABA-T and checking the biochemical purity and substrate specificity of GABA-T preparations.

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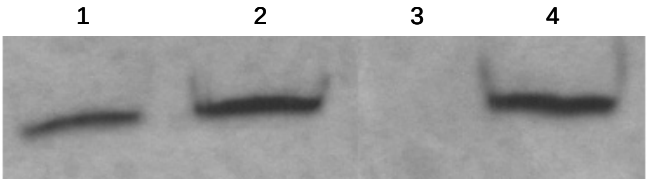


Fig. 1. Zymogram staining analysis of GABA transaminase activities from various yeast sources. Cell-free extracts prepared from WT yeast cells (lane 1, 100 µg), WT yeast overexpressing yeast GABA transaminase cells (lane 2, 50 µg), yeast GABA transaminase deletion mutant cells (lane 3, 100 µg), and yeast GABA transaminase deletion mutant cells overexpressing yeast GABA transaminase (lane 4, 50 µg). The cells were all grown on YPD medium. After electrophoresis, the gel was soaked in staining solution containing 100 mM potassium pyrophosphate buffer, pH 8.4, 0.1mM pyridoxal 5-phosphate (PLP), 16mM GABA and 4 mM a-ketoglutarate for 5 min at room temperature. Then 0.2 mg/ml nitroblue tetrazolium (NBT), 0.06 mM phenazine methosulfate (PMS), 1.5 mM NAD^+ and 1.5 unit of SSADH were added into the solution, followed by incubation at room temperature for another 15 min.

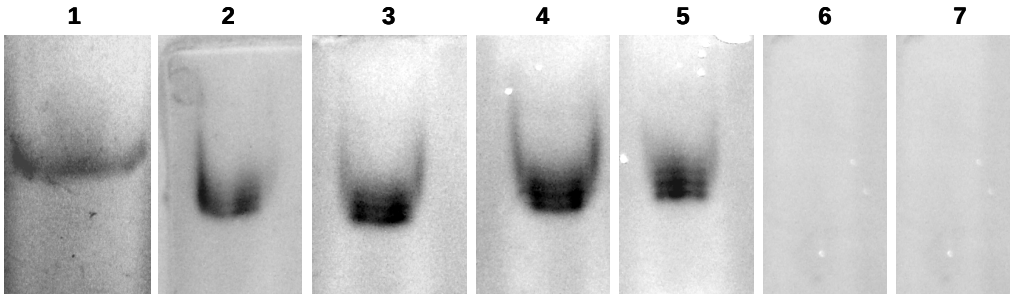
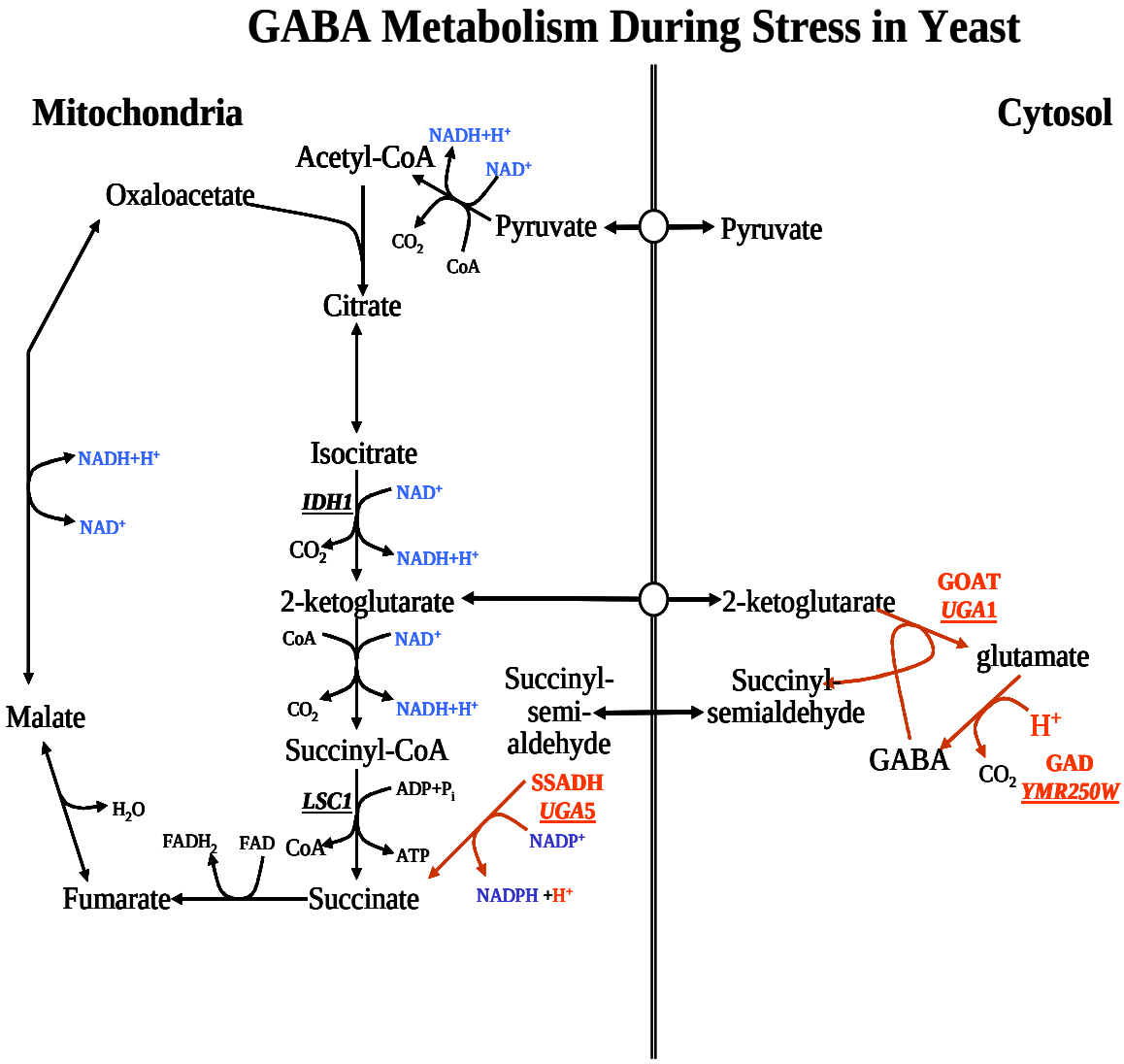


Fig. 2. Zymogram staining analysis for the substrate identification of *Arabidopsis* GABA transaminase. Approximately 100 µg of crude cell extract from 2 week-old *Arabidopsis* seedlings were loaded onto an 8% native polyacrylamide gel. After electrophoresis under native conditions, gels were soaked as described in Fig. 1. The staining solution components vary from each lane: lane 1, pyruvate as substrate instead of α-ketoglutarate; lane 2, α-ketoglutarate as substrate instead of pyruvate; lane 3, α-ketoglutarate as substrate but without SSADH; lane 4, α-ketoglutarate as substrate but without GABA; lane 5, α-ketoglutarate as substrate but without GABA and SSADH; lane 6: α-ketoglutarate as substrate but without NAD^+; lane 7, no α-ketoglutarate as substrate.

APPENDIX I. GABA METABOLISM DURING STRESS IN YEAST



APPENDIX II. GABA METABOLISM DURING STRESS IN PLANTS