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REGULATION OF PROLINE BIOSYNTHESIS IN PLANTS SUBJECTED TO OSMOTIC STRESS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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The Ohio State University
1997

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Approved

Department of Plant Biology
Drought and salinity stresses are the limiting factors for plant growth and productivity. Adaptation of plants to environmental stresses involves morphological, physiological as well as biochemical changes including accumulation of compatible solutes, such as proline, manitol, and betaine. More than 80% of plant species accumulate proline under stress conditions, and proline is thought to play an important role in plant cells for adaptation to osmotic stress. Proline accumulation has shown to increase osmotolerance in bacteria and plants.

In plants, proline is synthesized from both glutamate and ornithine, and the glutamate pathway for proline production is predominant in plants under osmotic stress and nitrogen starvation. Two enzymes, Δ1-pyrroline-5-carboxylate synthetase (P5CS) and Δ1-pyrroline-5-carboxylate reductase (P5CR), are involved in proline biosynthesis from glutamate. The first enzyme of the pathway, P5CS, is the rate-limiting enzyme in the biosynthesis of proline. A P5CS cDNA isolated from Vigna aconitifolia was expressed in E. coli, and the expressed enzyme was purified to homogeneity. Vigna P5CS exhibits two enzymatic activities, γ-glutamyl kinase (γ-GK) and glutamate-5-semialdehyde (GSA) dehydrogenase. The γ-GK activity of the purified P5CS was detected by the hydroxamate assay and by a [14C]glutamate assay. The native molecular mass of the P5CS was found to be 450 kDa with six identical subunits. The
*Vigna* P5CS showed a $K_m$ of 3.6 mM for glutamate while the $K_m$ for ATP was 2.7 mM. The $\gamma$-GK activity of the P5CS is competitively inhibited by proline and ADP while its GSA dehydrogenase activity is insensitive to proline inhibition. In addition, a protein inhibitor of the P5CS was detected in the plant cell. Western blot analysis, using polyclonal antibody raised against *Vigna* P5CS enzyme, showed that the level of the P5CS protein was enhanced in *Vigna* roots treated with NaCl. Two amino acid residues of the P5CS were identified to be involved in proline binding and a single substitution of an alanine for a phenylalanine at position 129 of the P5CS protein resulted in a significant reduction of proline feedback inhibition. The 50% inhibition values of $\gamma$-GK activity of the wild-type and the mutant P5CS were observed at 5 mM and 960 mM of proline, respectively. Other properties of the mutant P5CS remained unchanged. These results may allow genetic manipulation of proline biosynthesis and overproduction of proline in plants for conferring water stress tolerance.

The promoter of *Arabidopsis* P5CS gene was isolated and analyzed using a β-glucuronidase (GUS) reporter gene in transgenic plants. NaCl stress induced GUS activity to the maximum level within 3 hr while dehydration continued to enhance the GUS activity up to 24 hr. The AtP5CS gene promoter was found to contain two transcription start sites, and dehydration stimulated the transcription predominantly from the downstream site. Abscisic acid (ABA) failed to induce AtP5CS gene expression although ABA is known to increase during water stress.
These observations suggest that induction of the AtP5CS gene is the response of plants to salt and water stresses. Although ABA is not directly involved in the AtP5CS gene expression, it is possible that ABA is involved in a post-transcription regulation of the P5CS gene expression and/or regulation of proline dehydrogenase (PDH) gene expression. A reciprocal increase and decrease in the levels of the P5CS and the PDH leads to proline accumulation in plants.
To my wife, Jianxi, and my parents
ACKNOWLEDGMENTS

I am very grateful to my advisor, Desh Pal S. Verma, for his encouragement and enthusiasm in the pursuit of excellence in science, and for his patience in correcting both stylistic and scientific errors. This thesis could not be completed without his expertise and guidance. I would like to thank the members of my dissertation committee, Keith R. Davis, and Zhenbiao Yang for their suggestions and comments on my research project.

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Most of all, my thanks, from the bottom of my heart, go to my wife, Jianxi, for her love, friendship and standing with me through the long period of this endeavor.
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**FIELD OF STUDY**

Plant Biology
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CHAPTER 1

OSMOTIC STRESS AND ACCUMULATION
OF COMPATIBLE SOLUTES

1.1. Introduction

Life, as we know it, occurs in aqueous solution, and water is the most abundant component of living cells. The properties of water are of profound biological significance. Water is a polar molecule, and water molecules associate through hydrogen bonds. The structures of the molecules on which life is based, proteins, nucleic acids, lipid membranes, and complex carbohydrates, result directly from their interactions with their aqueous environment. The combination of solvent properties responsible for the intramolecular and intermolecular associations of these substances is peculiar to water, and no other solvent even closely resembles water in this respect (Voet and Voet, 1990). The liquid status of water at wide range of temperature ensures that the diffusion of solutes is possible; its high thermal capacity per unit mass makes it capable of temperature regulation; its incompressibility results in cell expansion and maintenance of cell volume; and its relative transparency to light enables sunlight to reach chloroplasts for photosynthesis in plants. No other solvent is able to substitute water in supporting life (Taiz and Zeiger, 1991).
Plant cells control the cellular water content by osmosis. Osmosis refers to the diffusion of water, the principal solvent in biological system, through a selectively permeable membrane from a region of higher concentration (of water) to a lower concentration (of water). Osmosis not only controls the water content, but also controls the volume of plant cells by generating turgor pressure. The evolution of higher plants has proceeded from ocean to land, to vascular plants, and to flowering plants. Most of characteristics the higher plants obtained during the entire evolutionary process are either to help plants receive and keep water or to make plants less dependent on water during growth. Not surprisingly, a deficit in water causes severe damage to plant cells.

1.2. Osmotic stress and agricultural production

Jacob Levitt (1972, 1980) proposed a definition of biological stress derived from physical science. Physical stress is any force applied to an object (for example, a steel bar). Levitt suggested that biological stress is any change in environmental conditions that might reduce or adversely change a plant's growth or development. Osmotic stress, normally referring to hyperosmotic effects on plants, is the primary reason for water deficit in plants. Under normal conditions, the dissolved materials in the cytoplasm of plant cells give them a concentration of water lower than that of soil. As a result, water moves by osmosis from soil into plant roots. When soil dries (drought) or contains a large quantity of dissolved salts (high salinity), its concentration of water may be lower than that in plant cells; consequently, water moves out of plant roots to soil. Consequently, most of plants can not survive under these conditions.
Drought was defined as "prolonged dry weather causing a rainfall deficit leads to a soil water deficit and ultimately to a plant water deficit" (Csonka, 1989). Drought stress has frequently caused seriously negative impact to economy and has caused many starvations in the world. Drought has always been the primary reason of famine in Africa. In U. S., drought in 1987 - 1989 cost the government and private sectors $39 billion and affected 70% of the country's population, but it was far less severe compared to the drought in 1934 - 1936 (Riebsame et al., 1991). Boyer (1982) reported that from 1939 to 1978, drought caused over 40 percent of the total insurance indemnities for crop losses in U. S. A. LeRudulier et al. (1984) reported that drought in 1983 alone resulted in more than $10 billion loss in crops.

Water used for irrigation contains various salt elements. When water evaporates, these salts are left behind and thus accumulated. High salinity decreases water potential of soil, leading to an efflux of water from plant cells which is accompanied by a concomitant decrease in the cytoplasmic volume (Csonka, 1989). In California, the irrigated agriculture has less than 100 years of history in most area. However, half of the 8.6 million acres of arable land are now affected by salinity, and the area damaged by salinity is expected to increase to 60% of 8.6 million acres by the turn of this century (Ashraf, 1994).

It is clear that these two types of stress (drought and high salinity) have similar effects on plant cells with respect to reducing the availability of free water in the cell and simultaneously increasing the concentration of intracellular ions. For active metabolism to occur, plants must maintain a balance of intracellular ionic composition, pH, and levels of metabolites. Hence, a decrease in water
potential directly affects cellular metabolisms, growth, and development of the plant.

1.3. Osmotic stress signaling in living organisms

1.3.1. Osmotic stress signaling in bacteria

There are two major proteins, OmpF and OmpC, on the outer membrane of E. coli. These two proteins function as the passive transporters of small hydrophilic molecules across the membrane (Aiba et al, 1989). Expression of the two corresponding genes, *ompF* and *ompC*, is regulated in a reciprocal manner by the medium osmolarity. The *ompC* is preferentially activated with the increase of osmolarity while the *ompF* is preferentially activated with the decrease of osmolarity (van Alphen and Lugtenberg, 1977; Kawaji et al., 1979). This osmoregulation is controlled at the transcriptional level by the other two genes, *ompR* and *env Z*. Hall and Silhavy (1981a and 1981b) proposed a model that explains the role of OmpR and EnvZ proteins in osmoregulation. The essence of the model is that the Env Z responds to changes in the medium osmolarity and then forces OmpR to take one of two alternative structures, which respectively activates the *ompF* and *ompC* genes. It has been shown that OmpR protein binds directly to the promoter region of both *ompF* and *ompC* genes, and it is a positive regulator specifically for *ompF* and *ompC* genes (Inokuchi et al., 1984; Driri et al., 1985; Mizuno and Mizushima, 1986; Ozawa et al., 1987). The EnvZ is a cytoplasmic membrane protein containing two membrane-spanning segments (Mizuno et al., 1982; Forst et al., 1987), and it functions as an osmotic sensor (Hall and Silhavy, 1981a). The results of genetic studies suggested that OmpR
and EnvZ functionally interact with each other (Matsuyama et al., 1986; Slauch et al., 1988). It was demonstrated that EnvZ was autophosphorylated with ATP, and the phosphate group on EnvZ could then be rapidly transferred to OmpR. The phosphorylated OmpR binds to the promoter region of \textit{ompC} and \textit{ompF}, controlling the expression of these genes (Aiba et al., 1989). The phosphorylated OmpR protein was rapidly dephosphorylated in the presence of ATP.

A number of systems have been identified in prokaryotes which can be classified as two-component regulatory systems (Ronson et al., 1987). They each comprise a "sensor" protein detecting environmental stimuli and a "regulator" protein controlling expression of particular genes (Kofoid and Parkinson, 1988). Based on the studies on the EnvZ-OmpR system, NtrB-NtrC system, and CheA-CheY system (Hess et al., 1987; Hess et al., 1988; Oosawa et al., 1988), a common cascade mechanism, \textit{i.e.} the transfer of a phosphate group between the two components, has been suggested to be involved in these regulatory systems (Ronson et al., 1987; Kofoid and Parkinson, 1988; Ninfa et al., 1988).

1.3.2. Osmotic stress signaling in yeast

Yeast (\textit{Sacchromyces cerevisiae}) cells normally accumulate glycerol for adaptation to osmotic stress conditions (Blomberg and Adler, 1992). Glycerol synthesis appears to be particularly important for hyperosmolarity adaptation because mutants defective in \textit{GPD1} (NAD-dependent glycerol-3-phosphate dehydrogenase) can not grow on hyperosmotic media (Albertyn et al., 1994). An osmosensing mechanism in the yeast has been reported recently (Posas et al., 1996), and it involves a two-component signal transducer (Sln1p, Ydp1p, and Ssk1p) and a MAP kinase cascade (Ssk2p/Ssk22p, Pbs2p, and Hog1p). Signals
of osmotic stress activates the HOG1 MAP kinase cascade via the two-component system, resulting in the synthesis of glycerol-3-phosphate dehydrogenase which is the rate-limiting enzyme in the glycerol synthetic pathway (Albertyn, et al., 1994).

The Sln1p is a transmembrane protein containing an extracellular sensor domain, a cytoplasmic histidine kinase domain, and a receiver domain. The Ypd1p is a cytoplasmic protein containing a histidine kinase domain and interacting with both Sln1p and Ssk1p. The Ssk1p contains a receiver domain and activates HOG1 MAP kinase cascade which is composed of three tiers of protein kinase, namely SSK2/SSK22 MAP kinase kinase kinase (MAPKKKs), PBS2 MAP kinase kinase (MAPKKs), and HOG1 MAP kinase (MAPK) (Boguslawiski, 1992; Brewster et al., 1993; Maeda et al., 1995).

It was demonstrated that under normal condition, the Sln1p is autophosphorylated at the histidine residue at the position 576 (His576), and this phosphate group is then sequentially transferred to Sln1p-Asp114, then to Ypd1p-His64, and finally to Ssk1p-Asp554. The phosphorylated Ssk1p is unable to activate the SSK2/SSK22 MAPKKKs and thus inhibits signaling via the HOG1 MAP kinase cascade (Fig. 1). Under osmotic stress conditions (such as 0.4 M NaCl), the Sln1p histidine kinase activity is inhibited, resulting in an accumulation of unphosphorylated Ssk1p, which then interacts with SSK2/SSK22 MAPKKKs to activate the HOG1 MAP kinase cascade (Fig. 1) (Posas et al., 1996). Most prokaryotic two-component systems contain only two elements, a sensor histidine kinase and a response-regulator with a receiver domain, while the yeast osmosensory two-component system is actually composed of three proteins, Sln1p, Ypd1p, and Ssk1p. The transfer of a phosphate group is a positive signal
Arched arrows indicate phosphotransfer reactions, while straight arrows indicate signal flow. A arched arrow or a straight arrow with a cross indicates the inhibition of the reaction or a blocked step in the signal flow. Inactive elements are indicated by dashed boxes. P indicates a phosphorylated form (Posas, et al., 1996).

Figure 1. The current model of the yeast HOG1 pathway
in *E. coli* whereas the transfer of a phosphate group is a negative signal in yeast. In addition, a MAP kinase cascade was found to be involved in stress signaling in yeast.

1.3.3. Osmotic stress signaling in plants

Plants respond to variety of biotic and abiotic signals that influence growth and development. Although the responses of plants to these signals have been extensively studied at the physiological and biochemical levels, the perception and the intracellular transmission mechanisms are largely unknown. Progresses in understanding signal transduction in yeast and animals showed that reversible protein phosphorylation plays a pivotal role in many signaling cascades. Increasing evidence indicates that protein kinase pathways are also involved in signal transduction in plants. A two-component system was proposed to be involved in ethylene signal transduction pathway in *Arabidopsis* (Chang et al., 1993; Hua et al., 1995). A similar phosphorelay regulatory mechanism for a MAP kinase cascade can be predicted for the *Arabidopsis* ethylene response pathway, since a protein kinase of the Raf family (CTR1) has been found downstream of the ETR1 histidine kinase (Kieber et al., 1993). An *Arabidopsis* MAP kinase was suggested to be involved in auxin signal transduction (Mizoguchi et al., 1994). Recently, MAP kinases have been demonstrated to be active upon cutting of leaves (Seo et al., 1995) and exposure of cells to fungal elicitor (Fukuda and Shinshi, 1994). Increased transcriptional levels of genes encoding a MAP kinase module have been taken as evidence for the involvement of a MAP kinase pathway in signaling touch, cold, salt, and water stress (Mizoguchi et al., 1996; Jonak et al., 1996). Taken together, these results imply that
multistep signaling pathways are probably present in stress signal transduction in plants.

1.4. Metabolic responses to osmotic stress in living organisms

Adaptation of living organisms to osmotic stress involves morphological, physiological, as well as biochemical changes including the accumulation of compatible solutes. The term "compatible solute" was first introduced by Brown and Simpson (1972) to describe noninhibitory (inert, nontoxic and harmless) substances accumulated in the cytoplasm of cells at low external water potentials. A compatible solute is an osmotically active compound (osmolyte) that reduces the movement of water out of cells and maintains the cell volume and turgor pressure during osmotic stress.

1.4.1. Accumulation of compatible solutes in bacteria

Many bacteria have learned to use a simple rule of chemistry in a world deficient in available water. They are able to avoid dehydration by taking up or synthesizing molecules that act as compatible solutes. A large number of compounds could serve as compatible solutes, however, bacteria use only a few compounds to fill this need. Figure 2 lists some compatible solutes accumulated in *E. coli* and other enteric bacteria. One of the important chemical features of these molecules is their extreme solubility in water. The solubility of proline, for example, is 1.62 gram per gram of water at 25 °C. There is a positive correlation between the intracellular content of potassium and the ability of bacteria to tolerate
\[(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-\] Glycine Betaine

\[(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}\] Choline

\[(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CHO}\] Betaine aldehyde

\[(\text{CH}_3)_3\text{N}^+\text{(CH}_2)_3\text{COO}^-\] Trimethyl-\(\gamma\)-amino butyrate

\[(\text{CH}_3)_2\text{NCH}_2\text{COO}^-\] Dimethyl glycine

\[
\begin{array}{c}
\text{C} \\
\text{C} \\
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Proline

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\begin{array}{c}
\text{C} \\
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\]
Proline betaine

Figure 2. Structures of compatible solutes generally accumulated in *E. coli* and other enteric bacteria (Le Rudulier et al., 1984).
Christian and Waitho, 1961). Epstein and Schultz (1965) showed that in E. coli, the intracellular concentration of potassium increased from 0.15 to 0.55 M as the osmolarity of the medium was increased from 0.1 to 1.2 osm. It was also reported that the accumulation of $K^+$ was only transient in E. coli exposed to the hyperosmotic media, and 30 minutes after the exposure to hyperosmotic media, the E. coli actively excreted $K^+$ (Dinnbier et al., 1988; Ohwada and Sagisaka, 1988).

Glycine betaine (N,N,N-trimethylglycine) is widely used as a compatible solute by bacteria. Most bacteria are unable to synthesize glycine betaine and depend on the transport of this compound from environment to achieve accumulation (Csonka, 1989). LeRudulier et al. (1984) showed that E. coli grow under completely inhibitory levels of osmotic stress (0.8 M NaCl) when supplemented with glycine betaine (0.001 M) or choline (0.001 M), while glycine added to the above media did not support the growth.

The role of proline as a compatible solute was first described by Christian (1955a; 1955b) who reported that exogenous proline could alleviate the growth inhibition of Salmonella imposed by osmotic stress. It was subsequently demonstrated that a wide variety of bacteria accumulated proline under stress conditions (Measures, 1975). It was reported that proline accumulation in bacteria depends on the presence of exogenous proline (Csonka, 1981; 1988a; 1989). Therefore, it is believed that proline is taken up by an active transport system. A Salmonella mutant, resistant to the toxic proline analog (L-azetidine-2-carboxylic acid), over-produced proline and showed enhanced tolerance to osmotic stress (Csonka, 1981). The mutation was mapped to the proB gene encoding the rate-limiting enzyme, $\gamma$-glutamyl kinase ($\gamma$-GK), in the biosynthetic
pathway of proline (Csonka, 1988b). The γ-GK is feedback inhibited by proline. It was found that an aspartate residue at the position 107 of γ-GK protein was changed to an asparagine, resulting in a mutant enzyme with much less sensitivity to proline inhibition. This mutation did not affect the enzyme activity.

1.4.2. Accumulation of compatible solutes in yeast and animals.

In yeast (*Saccharomyces cerevisiae*), glycerol seems to be the only compatible solute, and specific mechanisms exist to retain the glycerol in the cell (Blomberg and Adler, 1992; Mager and Varela, 1993). Under stress conditions, many yeast genes are induced, including GPD1 which encodes a NADH-dependent glycerol-3-phosphate dehydrogenase involved in glycerol biosynthesis (Larsson *et al.*, 1993; Albertyn *et al.*, 1994). Some algae also accumulate glycerol as a compatible solute (Brown, 1976; 1978).

The common compatible solutes in animals are aresorbitol, glycerophosphocholine (GPC), glycine betaine, myo-inositol, and taurine. The expression of genes involved in the synthesis of above compatible solutes is induced by hyperosmotic stress (Burg, 1994). It was noticed that the accumulation of compatible solutes takes hours to days under stress conditions, but the compatible solutes exit from the cells within minutes via specialized efflux mechanisms when hyperosmotic stresses is removed.
1.4.3. Accumulation of compatible solutes in plants

1.4.3.1. Accumulation of glycine betaine

Glycine betaine has been found to accumulate in some plants under salt or drought stress conditions (Rhodes and Hanson, 1993). These plants include spinach (Coughlan and Wyn Jones, 1982), sugar beet (Hanson and Rhodes, 1983), barley (Wyn Jones and Storey, 1978), and wheat (McDonnell and Wyn Jones, 1988). Glycine betaine is a quaternary ammonium compound (Fig. 2), and properties of glycine betaine make it suitable as a compatible solute. First, betaine is non-toxic and compatible with metabolic functions (Somero, 1986). Second, betaine may protect and stabilize enzymes against the inhibitory effects of osmotic stress (Warr et al., 1988). Third, betaine may serve as a mediator involved in water retention (Grumet and Hanson, 1986).

Glycine betaine is synthesized from choline via two oxidation steps. The enzyme catalyzing the first step was identified as a choline monooxygenase in spinach (Brouquisse et al., 1989), converting choline to betaine aldehyde. The latter is then converted to glycine betaine by betaine aldehyde dehydrogenase (Weretilnyk and Hanson, 1989). The activity of betaine aldehyde dehydrogenase was found to be four-fold higher in sugar beet leaves and roots exposed to high level of salinity (500 mM NaCl) compared to the unstressed plants (Weretilnyk and Hanson, 1990).

1.4.3.2. Over-production of sugar alcohol

The sugar alcohol refers to a group of compounds in which the aldo or keto group of the sugar is reduced to the corresponding hydroxyl group. Thus, a
sugar alcohol molecule has one more alcohol group than its corresponding sugar molecule. Most of the sugar alcohols were first isolated from plants, starting with mannitol in 1860 and sorbitol in 1872 (Lewis and Smith, 1967). Since sugar alcohols have many hydroxyl groups, it was proposed that sugar alcohols might take the place of water in the biopolymers (proteins, DNAs, and so on) of cell cytoplasm and help to maintain activities of enzymes and membranes at a time when water is lost due to osmotic stress (Schobert, 1977; Ahmad et al., 1979). Proteins "hydrated" with sugar alcohols might be more tolerant to high salt concentrations than a conventionally hydrated protein. Most importantly, the sugar alcohols are inert and harmless to enzymes even at high concentrations, and they are one step away from the sugar which are readily synthesized and degraded (Bieleski and Pedgwell, 1980).

Tarczynski et al. (1993) showed that transgenic tobacco carrying a bacterial mtlD gene synthesized and accumulated mannitol. The mtlD gene encodes mannitol-1-phosphate dehydrogenase regulating mannitol synthesis in bacteria, and plants do not normally accumulate mannitol under stress conditions. When the transgenic tobacco and control tobacco were exposed to high level of salinity, the transgenic plants containing mannitol showed an enhanced tolerance to the stress as compared to the control plants. The differences were detected in fresh weight, new roots, leaf formation and flowering.

1.4.3.3. Accumulation of proline

Besides being an amino acid for protein biosynthesis, proline is thought to play an important role as a compatible solute in plants subjected to hyperosmotic stresses, primarily drought and soil salinity. Proline appears to be the most
<table>
<thead>
<tr>
<th>Species</th>
<th>[proline] Increase</th>
<th>Stress Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mesembryanthemum nodiflorum</em> (halophyte) cell suspension culture</td>
<td>7</td>
<td>NaCl: 400 mM</td>
<td>Trichel (1986)</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em> L. (tobacco) cell suspension culture</td>
<td>4.4</td>
<td>NaCl: 428 mM</td>
<td>Binzel <em>et al.</em>, (1988); LaRosa <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>Nicotiana sylvestris</em> (tobacco, salt-resistant strain) cell suspension culture</td>
<td>46</td>
<td>NaCl: 150 mM</td>
<td>Kir’yan and Shevyakova (1985)</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em> L. (tobacco) leaves</td>
<td>20</td>
<td>NaCl: 200 mM</td>
<td>Szoke <em>et al.</em>, (1992)</td>
</tr>
<tr>
<td><em>Spinacia oleracea</em> L. (spinach)</td>
<td>11</td>
<td>-2 mPa</td>
<td>Huang and Cavalieri (1979)</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em> L. (potato) cell suspension culture</td>
<td>9</td>
<td>10% PEG)</td>
<td>Corcuera <em>et al.</em>, (1989)</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em> (tomato) cell suspension culture</td>
<td>319</td>
<td>25% PEG)</td>
<td>Handa <em>et al.</em>, (1983); Rhodes <em>et al.</em>, (1986)</td>
</tr>
<tr>
<td><em>Helianthus tuberosum</em> L. tuber tissue</td>
<td>11</td>
<td>1.0 M sorbitol)</td>
<td>Wrench <em>et al.</em>, (1980)</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>8</td>
<td>NaCl: 120 mM</td>
<td>Chiang and Dandekar (1991)</td>
</tr>
<tr>
<td><em>Medicago sativa</em> L. (alfalfa) Roots</td>
<td>8</td>
<td>NaCl: 150 mM</td>
<td>Fou`gere <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>Vicia faba</em> L. (field bean)</td>
<td>9</td>
<td>two-day drought</td>
<td>Venekamp Koot (1988)</td>
</tr>
<tr>
<td><em>Glycine max</em> L. (soybean) leaves nodules</td>
<td>11</td>
<td>NaCl: 200 mM</td>
<td>Moftah and Michel (1987)</td>
</tr>
<tr>
<td><em>Glycine max</em> L. (soybean) leaves nodules</td>
<td>3</td>
<td>two-day drought</td>
<td>Kohl <em>et al.</em>, (1988)</td>
</tr>
<tr>
<td><em>Oryza sativa</em> (rice)</td>
<td>4</td>
<td>KC: 50 mM</td>
<td>Chou <em>et al.</em>, (1991)</td>
</tr>
<tr>
<td><em>Triticum aestivum</em> L. (wheat) apex and leave</td>
<td>195</td>
<td>-3.6 mPa</td>
<td>Munns <em>et al.</em>, (1979)</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em> (barley)</td>
<td>3</td>
<td>-1.5 mPa</td>
<td>Boggess <em>et al.</em>, (1976b)</td>
</tr>
</tbody>
</table>

Table 1. Induction of proline accumulation in plants under stress conditions.
widely distributed compatible solute accumulated under stress conditions (Table 1). Proline accumulates not only in plants, but also in eubacteria, protozoa, marine invertebrates and algae (McCue and Hanson, 1990; Measures, 1975; Singh et al., 1977; Delauney and Verma, 1993). It has been well documented that there is a positive correlation between proline accumulation and the capacity of survival of plants both in water deficit and high salinity conditions (Taylor, 1996). In addition, free proline accumulates in response to high temperature (Aspinall and Paleg, 1981), low temperature (Zeevaart and Creelman, 1988), heavy metal (Saradhi and Saradhi, 1991), weak acids (Chou et al., 1991), UV light (Saradhi et al., 1995), and abscisic acid application (Mundy and Chua, 1988).

How does proline work? The simplest explanation is that high levels of proline allows the cell to balance the osmotic stress of its cytoplasm with that of its surroundings to prevent a net loss of water. However, there is increasing evidence that proline may interact with crucial macromolecules of the cell to help to modulate their biological activities, such as interacting with membrane systems (Rudolph et al., 1986), regulating cytosolic acidity (Venekamp, 1989), scavenging free radicals (Smirnoff and Cumbes, 1989), balancing the ratio of NADH/NAD+ (Saradhi and Saradhi, 1993), and regulating cellular redox potentials (Kohl et al., 1988). Schobert and Tschesche (1978) found that proline solutions interact with proteins, increasing the solubility of sparingly soluble proteins and reducing the precipitation of soluble proteins by ethanol and (NH4)2SO4. They postulated that the water-binding capacity of proline-protein complex is increased. Studies using M4 lactate dehydrogenase protein also support the important role played by proline in protein structures and functions
(Rajendrakumar et al., 1994). A well defined labile M4-lactate dehydrogenase from rabbit muscle was denatured under freeze-thaw, heat and GuHCl treatment in the presence and absence of proline, and the corresponding structural changes of the enzyme were monitored by fluorescence and CD spectral studies. The results showed that proline formed hydrophilic colloids in aqueous media with a hydrophobic backbone interacting with the protein and providing protection to the structural integrity and enzyme activity (Rajendrakumar et al., 1994).

1.5. Oxidative stress and proline accumulation in plants

Oxidative stress refers to the damage caused by superoxide radicals. The unpaired electrons of superoxides react with many cellular molecules, such as DNAs, proteins, lipids (cell membrane), and other organic molecules with unsaturated bonds, resulting in radical chain processes, crosslinkage, peroxidation, mutations, membrane leakage, and toxic compound production (Fridovich et al., 1978; Davies, 1995). Superoxide radicals can be produced as a by-product during the electron transport where oxygen molecules serve as the terminal acceptor. Lipids degradation also produces superoxides such as H2O2. In addition, autoxidation of hydroquinones, leukoflavins, catecholamines, thiols and tetrahydropterins has shown to generate superoxide radicals (Fridovich et al., 1978). To avoid the toxicity of the free oxygen radicals, aerobic organisms have developed a set of defense systems composed of antioxidant compounds (such as glutathion) and antioxidant enzymes (such as dismutase and peroxidase). However, under stress conditions, the balance between superoxide producing and
removing is broken, and free oxygen radical production exceeds the rate of radical removal.

Many stresses have been demonstrated to be oxidative stress in plants, such as UV radiation, chilling, heavy metal, pollution, pathogen, and mechanical damages (DeKok and Stulen, 1993). Malondialdehyde (MDA) is the major cytotoxic product of lipid peroxidation and serves as an indicator of free radical production and lipid peroxidation (Heath and Pack, 1968; Halliwell and Dutteridge, 1990). By monitoring the production of MDA, Saradhi et al. (1995) found that proline accumulates concomitantly with a increase of MDA content in the shoots of rice seedlings, mustard seedlings, and mung bean seedlings exposed to UV radiations. The level of proline in the seedlings increases significantly with the increase of UV exposure time, and MDA level is higher in the seedling shoots exposed to UV as compared to the controls, suggesting that UV radiation promotes lipid peroxidation and induces proline accumulation. The presence of proline along with linolenic acid micelles during UV exposure causes a considerable reduction in the production of MDA, indicating that proline may protect plant cells against peroxidation processes induced by UV radiation (Saradhi et al., 1995)

1.6. Potential function of proline transporters during flowering and osmotic stress.

Transport processes play an important role in nitrogen allocation in higher plants. Under stress conditions, massive changes in partition of carbon and nitrogen take place. For example, under water stress, delivery of nitrate to shoot
is depressed (Shaner and Boyer, 1976). Carbon and nitrogen reduction are also impaired, and a reduction in phloem transport has been observed (Wardlaw, 1969; Tully et al., 1979). Concomitantly, the stressed plants accumulates compatible solutes, such as sugar, proline, and betaine, in cytosol as a protective mechanism (Bohnert et al., 1995).

In reproductive tissues of Arabidopsis, proline represents 17 to 26% of total free amino acids, whereas in vegetative tissues, proline contributes only 1 to 3% (Chiang and Dandekar, 1995). Moreover, in kiwifruit, the onset of flowering seems to be associated with an accumulation of proline (Walton et al., 1991). Proline is supposed to derive from translocation from leaves to flowers (Savoure et al., 1995). Studies with bean imply that the accumulation of free proline in leaves plays a role in stimulating the production of generative organs due to transfer of proline from leaves to flowers (Venekamp and Koot, 1984).

Two proline transporters, ProT1 and ProT2, have been isolated recently from Arabidopsis by complementation of a yeast mutant with defective shr3 gene which product is required for correct targeting of endogenous amino acid transporters to cell membrane (Rentsch et al., 1996). Normal cytosolic and apoplastic proline concentrations in potato leaves range between 2 to 3 mM (Bussis, 1995). The affinity for proline of both proline transporters with several hundred micromolar is thus suitable for efficient import of proline into cells. Under normal conditions, ProT1 gene expression is much higher in roots, stems, and flowers, where expression was found mainly in the floral phloem supplying the ovules. The elevated expression of ProT1 gene in flowers and stems agrees with an increased accumulation of proline in these organs. Therefore, ProT1
might play a role in floral proline supply and in flower induction and development.

Under normal conditions, ProT2 gene was expressed mainly in roots. Under stress conditions (dehydration or high salinity), the ProT2 gene expression was strongly induced while the ProT1 gene expression remained stable (Rentsch et al., 1996). The authors suggested that ProT2 might be responsible for the increased export of proline though the vascular stem, whereas ProT1 is responsible for import of proline into flowers. In addition, the increase of ProT2 gene expression agrees with the finding that under stress conditions, proline content and transport increase, whereas the export of other amino acid decreases (Tully et al., 1979). Additional experiments using transgenic plants with altered proline transport properties are required to confirm the significance of proline transporter under normal and stress conditions. Under the assumption that proline accumulation is a cell-autonomous effect and a capacity of all cell types, researchers so far have concentrated on biosynthesis and degradation rather than translocation.

1.7. Crassulacean acid metabolism (CAM) in plants

Unlike C3 and C4 plants, CAM plants assimilate atmospheric CO2 into C4 acids predominantly at night and subsequently refix this CO2 into the carbohydrates during the following day. To achieve this nocturnal CO2 uptake, stomata of CAM plants are open at night and closed during most of the day. This strategy allows CO2 uptake to occur when evapotranspiration rates are low and permits daytime photosynthetic carbon fixation by the carbon reduction cycle to
occur behind closed stomata, resulting in minimal water loss and reduced photorespiration. Thus, CAM plants exhibit water use efficiency rates 5 to 10 times higher than C₄ and C₃ plants (Cushman and Bohnert, 1997) resulting in a considerable competitive advantage in water-limiting environments.

The facultative CAM plants, such as *Mesembryanthemum crystallinum*, may shift from C₃ to CAM in response to age or environmental conditions such as photoperiod, high salinity, or water stress. The shift from C₃ photosynthesis to CAM is usually accompanied by dramatic changes in physiology, biochemistry, and gene expression. In facultative CAM plants, activities of glycolytic, gluconeogenic, and C₄ acid metabolism enzymes increase up to 40-fold during the induction of the pathway by environmental stress due at least in part to *de novo* enzyme synthesis (Cushman and Bohnert, 1997; Table 2). Explanation for the basis of CAM shifting range from strictly ontogenetic to solely dependent on environmental conditions (Edwards et al., 1996). Characterization of the expression patterns of the genes involved in CAM pathway of facultative CAM plants has begun to shed light on the molecular mechanisms underlying the evolution and expression of these enzymes (Table 2).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Inducer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ppcl</em></td>
<td>PEPC</td>
<td>NaCl, ABA, drought</td>
<td>Cushman et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thomas et al., 1992</td>
</tr>
<tr>
<td><em>Mdhl</em></td>
<td>NADP-MDH</td>
<td>NaCl</td>
<td>Cushman, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cushman et al., 1992</td>
</tr>
<tr>
<td><em>Modl</em></td>
<td>NADP-ME</td>
<td>NaCl</td>
<td>Cushman, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fisslthaler et al., 1995</td>
</tr>
<tr>
<td><em>Ppdkl</em></td>
<td>PPDK</td>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td><em>Pghl</em></td>
<td>Enolase</td>
<td>NaCl, drought, cold, ABA</td>
<td>Forsthoefel et al., 1995a</td>
</tr>
<tr>
<td><em>Pgml</em></td>
<td>Phosphoglycero-</td>
<td>NaCl, drought, ABA</td>
<td>Forsthoefel et al., 1995b</td>
</tr>
<tr>
<td></td>
<td>mutase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>GapCl</em></td>
<td>GAPDH</td>
<td>NaCl</td>
<td>Ostrem et al., 1990</td>
</tr>
</tbody>
</table>

PEPC, phosphoenolpyruvate carboxylase; NADP-MDH, NADP dependent malate dehydrogenase; NADP-ME, NADP dependent Malic enzyme; PPDK, pyruvate orthophosphate dikinase; Enolase, 2-phospho-D-glycerate hydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Genes and enzymes involved in CAM.
1.8. Proline biosynthesis and degradation in plants

1.8.1. Proline biosynthesis

The proline synthesis pathway was elucidated first in microorganisms by a combination of techniques including radioactive-compound incorporation, isotope competition, accumulation of intermediates in mutants, and demonstration of presence of enzymes in wild type organisms and absence of enzymes in mutants (Vogel and Davis 1952). In bacteria, the conversion of glutamate to proline starts with an ATP-dependent phosphorylation of γ-carboxyl group of L-glutamate by γ-glutamyl kinase (γ-GK; Fig. 3; Table 3, Eq. 1). The resulting γ-glutamyl phosphate (γ-GP) is reduced to γ-glutamyl semialdehyde (GSA) by GSA dehydrogenase (Table 3, Eq. 2). GSA spontaneously cyclizes to Δ^1-pyrrolidine-5-carboxylate (P5C; Table 3, Eq. 3) which is then reduced to proline by P5C reductase (P5CR; Table 3, Eq. 4). In higher plants, a similar pathway has been established where the first two steps are catalyzed by a bifunctional enzyme. A cDNA encoding a P5C synthetase (P5CS; Fig. 3; Table 3, Eq. 2') was isolated from Vigna aconitifolia by functional complementation of an E. coli mutants (Hu et al. 1992). Alignment of the protein sequences between the Vigna P5CS and the E. coli γ-GK and GSA dehydrogenase revealed that the Vigna P5CS is a bifunctional enzyme with activities of both γ-GK and GSA dehydrogenase (Fig. 4). The two enzymatic domains overlap on one peptide, and the reaction catalyzed by the P5CS is equal to the combination of the reactions catalyzed by E. coli γ-GK and GSA dehydrogenase respectively (Fig. 4). Recently, it has been shown that in tomato the P5CS may be encoded be a polycistronic cDNA (Garcia-Rios et al., 1997). The cloning of P5CR cDNAs from plants was achieved earlier.
Enzymes: P5CS, Δ^1-pyrroline-5-carboxylate synthetase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; γ-GK, γ-glutamyl kinase; GSAD, glutamate-5-semialdehyde dehydrogenase

Figure 3. Proline biosynthesis pathway in plants and *E. coli*
Table 3 (continued on next page)

<table>
<thead>
<tr>
<th>Enzymatic step</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>γ-Glutamyl kinase (EC. 2.7.2.11)</strong></td>
<td>glutamate + ATP → γ-glutamyl phosphate + ADP (1)</td>
</tr>
<tr>
<td><strong>γ-Glutamyl phosphate reductase (EC. 1.2.1.41)</strong></td>
<td>γ-glutamyl phosphate + NADPH → glutamyl-5-semialdehyde + NADP⁺ + Pi (2)</td>
</tr>
<tr>
<td><strong>Δ¹-Pyrroline-5-carboxylate synthetase</strong></td>
<td>glutamate + ATP + NADPH → glutamyl-5-semialdehyde + NADP⁺ + ADP + Pi (2')</td>
</tr>
<tr>
<td><strong>Nonenzymatic reaction</strong></td>
<td>glutamyl-5-semialdehyde ↔ Δ¹-pyrroline-5-carboxylate (3)</td>
</tr>
<tr>
<td><strong>Δ¹-Pyrroline-5-carboxylate reductase (EC. 1.5.1.2)</strong></td>
<td>Δ¹-pyrroline-5-carboxylate + NADPH → proline + NADP⁺ (4)</td>
</tr>
<tr>
<td><strong>δ-Ornithine aminotransferase (EC. 2.6.1.13)</strong></td>
<td>ornithine + 2-oxoglutaric acid → glutamyl-5-semialdehyde + glutamate (5)</td>
</tr>
<tr>
<td>The same as Eq. (4)</td>
<td>(6)</td>
</tr>
<tr>
<td><strong>Ornithine α-aminotransferase</strong></td>
<td>ornithine + α-ketoglutaric acid → α-keto-δ-aminovalerate (7)</td>
</tr>
<tr>
<td><strong>Nonenzymatic reaction</strong></td>
<td>α-keto-δ-aminovalerate → Δ¹-pyrroline-2-carboxylate (8)</td>
</tr>
<tr>
<td><strong>Δ¹-Pyrroline-2-carboxylate reductase (EC. 1.5.1.1)</strong></td>
<td>Δ¹-pyrroline-2-carboxylate + NADPH → proline + NADP⁺ (9)</td>
</tr>
<tr>
<td><strong>L-Proline dehydrogenase (EC. 1.5.99.8)</strong></td>
<td>proline + O₂ → Δ¹-pyrroline-5-carboxylate (10)</td>
</tr>
<tr>
<td><strong>Δ¹-Pyrroline-5-carboxylate dehydrogenase (EC. 1.5.1.12)</strong></td>
<td>Δ¹-pyrroline-5-carboxylate + NADP⁺ → glutamate + NADPH (11)</td>
</tr>
</tbody>
</table>
Table 3 (continued)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Enzyme</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA : glutamate N-acetyltransferase (EC. 2.3.1.1)</td>
<td></td>
<td>acetyl-CoA + glutamate → N-acetylglutamate + CoA</td>
</tr>
<tr>
<td>N²-Acetylornithine : glutamate N-acetyltransferase (EC. 2.3.1.35)</td>
<td></td>
<td>N²-acetylornithine + glutamate → N-acetylglutamate + ornithine</td>
</tr>
<tr>
<td>N-Acetylglutamate kinase (EC. 2.7.2.8)</td>
<td></td>
<td>N-acetylglutamate + ATP → N-acetylglutamyl phosphate + ADP</td>
</tr>
<tr>
<td>N-Acetylglutamate-5-semialdehyde oxidoreductase (EC. 1.2.1.38)</td>
<td></td>
<td>N-acetylglutamyl phosphate + NADPH → N-acetylglutamate-5-semialdehyde</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ NADP⁺ + Pi</td>
</tr>
<tr>
<td>N²-Acetylornithine aminotransferase (EC. 2.6.1.11)</td>
<td></td>
<td>glutamate + N-acetylglutamate-5-semialdehyde → 2-oxoglutaric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ N²-acetylornithine</td>
</tr>
<tr>
<td>N²-Acetylornithine decarboxylase (EC. 3.5.1.16)</td>
<td></td>
<td>N²-acetylornithine + H₂O → ornithine + acetic acid</td>
</tr>
</tbody>
</table>

This table lists and numbers the reactions in the text. In the interest of brevity, the designation of the configuration (L) around the α-carbon is omitted in the equations. Abbreviations used are the same as defined in *Journal of Biological Chemistry*.

Table 3. Reactions and enzymes involved in proline metabolism
Amino acid positions: 1 12 128 299 671

*Vigna* P5CS: 
\[ M ------M----------DNDFRD-------M------------------------N \]

*E. coli* γ-GK: 
\[ M----------RADMED---------1----1 \]

*E. coli* GSA dehydrogenase: 
\[ M----------G \]

Enzyme domains in P5CS: \[ \gamma\text{-GK} \] \[ \text{GSA dehydrogenase} \]

Reactions catalyzed: 
\[
\begin{align*}
\text{Glu} & \rightarrow \text{γ-Glu-P} \\
\text{ATP} & \rightarrow \text{ADP} \\
\text{NADPH} & \rightarrow \text{NADP}^+ + \text{Pi}
\end{align*}
\]

Figure 4. Protein sequence comparison between the *Vigna* P5CS and *E. coli* γ-GK and GSA dehydrogenase.
In bacteria, the first two steps (Fig. 3; Table 3, Eqs 1 and 2) for proline synthesis are considered together because of the labile \( \gamma \)-GP intermediate (Vogel and Davis 1952). The purified *E. coli* \( \gamma \)-GK showed no detectable activity by the hydroxamate assay for the production of \( \gamma \)-GP, but the production of \( \gamma \)-GP could be restored by adding purified *E. coli* GSA dehydrogenase (Baich 1969; Hayzer and Leisinger 1981; Smith *et al.* 1984). It has been suggested that *E. coli* \( \gamma \)-GK and GSA dehydrogenase form a complex to afford protection to the labile \( \gamma \)-GP and to directly transfer the intermediate from one enzyme to the other, avoiding equilibration with surrounding medium (Baich 1969; Gamph and Moses 1974). However, such a complex was not detected (Smith *et al.* 1984).

The \( \gamma \)-aldehyde group of glutamyl semialdehyde reacts nonenzymatically and reversibly with the \( \alpha \)-amino group to form a cyclic Schiff base (Table 3, Eq. 3), P5C, that is thought to be the predominant form (Strecker, 1960). The final step in proline synthesis is the reduction of P5C to proline catalyzed by P5CR (Table 3, Eq. 4). Most organisms use both NADH and NADPH as the reductants, but NADPH is preferred in higher plants. The P5CR cDNA from soybean root nodules was isolated by functional complementation of an *E. coli* mutant, and the expressed enzyme was purified. The purified enzyme used both NADH and NADPH as the reductant and its \( K_m \) value for NADH was shown to be 10-fold greater than that for NADPH (Szoke *et al.* 1992). NADP\(^+\) inhibits the P5CR activity while NAD\(^+\) has no effects on the enzyme activity. Expression of the soybean P5CR cDNA in transgenic tobacco plants resulted in a 200-fold increase in the P5CR activity. However, the proline level in the transgenic plants
was not significantly altered (Szoke et al. 1992). These data suggested that the P5CR is not the rate-limiting enzyme in the proline biosynthetic pathway. Similar results were obtained by LaRosa et al. (1991).

Plants also synthesize proline from ornithine. The ornithine biosynthesis is also shown in Figure 5 and Table 3 (Eqs. 12-17). The conversion of glutamate to ornithine starts with an ATP-driven reaction of the glutamate γ-carboxyl group to an aldehyde (Table 3, Eqs. 12-15). Spontaneous cyclization of this intermediate, N-acetylglutamate-5-semialdehyde, is prevented by prior acetylation of its amino group by N-acetylglutamate synthase to form N-acetylglutamate. N-acetylglutamate-5-semialdehyde, in turn, is converted to the corresponding amine by transamination. Hydrolysis of acetyl protecting group yields ornithine (Table 3, Eq. 17). Conversion of ornithine to proline could proceed via two routes (Fig. 5), both involving transamination of ornithine followed by cyclization and reduction. If the α-amino group of ornithine is transaminated, the product would be α-keto-δ-aminocvalerate which cyclizes to Δ1-pyrroline-2-carboxylate (P2C) and is then reduced to proline (Table 3, Eqs 7-9). Alternatively, transamination of the δ-amino group yields GSA which cyclizes to Δ1-pyrroline-5-carboxylate (P5C) and is then reduced to proline (Table 3, Eqs 5, 6, and 4). Delauney et al. (1993) suggested that the P5C is the route taken in plants, although P2C formation may also occur. A ornithine aminotransferase (OAT) cDNA has been cloned from Vigna aconitifolia, and the encoded enzyme was found to be a δ-aminotransferase (Delauney et al. 1993). This result confirmed the formation of P5C from ornithine. The Vigna OAT expressed in E. coli was partially purified, and its $K_m$ values for ornithine and α-ketoglutarate were found to be 2 and 0.75 mM respectively (Delauney et al. 1993). Valine inhibited the OAT activity, but
The enzymes are numbered as shown in Table 3. Intermediates: γ-GP, γ-glutamylphosphate; GSA, γ-glutamyl-5-semialdehyde; P5C, Δ1-pyrroline-5-carboxylate; KVA, α-keto-8-aminovalerate; P2C, Δ1-pyrroline-2-carboxylate.

AcG, N-acetylglutamate; AcGP, N-acetylgutamate-5-phosphate; AcGSA, N-acetylgutamate-5-semialdehyde; AcORN, N-acetylornithine. The thinner arrows indicate the less probable pathway.

Figure 5. Proline and ornithine biosynthesis.
proline had no effect. The *Vigna* OAT does not appear to catalyze the reversible reaction from GSA to ornithine (Delauney *et al.* 1993). It seems that the major role of OAT is to convert ornithine to GSA, contributing to proline biosynthesis. This pathway is, however, subjected to the regulation by external nitrogen levels and stress conditions.

1.8.2. Proline degradation

The degradation of proline as well as its synthesis involves P5C as an intermediate (Fig. 3). Boggess *et al.* (1978) and Huang and Cavalieri (1979) showed that mitochondrial oxidation of proline in higher plants produced glutamate *via* P5C (Table 3, Eqs. 10, 11). The cloning of *Arabidopsis* proline dehydrogenase cDNA (Peng *et al.* 1996; Verbruggen *et al.* 1996; Kiyosue *et al.* 1996) confirmed that the degradation of proline proceeds *via* the reversal of reactions of its biosynthesis.

Recent studies have shown that in Arabidopsis the transcripts of proline dehydrogenase (PDH), the first enzyme in proline degradation pathway (Table 3, Eq. 10), is induced by proline and reduced by osmotic stress (Peng *et al.* 1996; Verbruggen *et al.* 1996; Kiyosue *et al.* 1996). In Arabidopsis plants under stress, the expression of the P5CS gene is enhanced while the expression of PDH gene is reduced. After the removal of stress, PDH gene expression is induced while the P5CS gene expression is reduced. This reciprocal regulation of the levels of P5CS and PDH controls proline levels during and after osmotic stress in plants. These data suggested that accumulation of proline in plants under osmotic stress requires an increase in proline synthesis and a decrease in proline degradation. The above information also suggests that proline accumulation during osmotic
stress is an essential adaptive response to these conditions in plants. Rapid oxidation of proline is an equally important process in recycling the accumulated free proline and providing reducing power, amino nitrogen, and energy in the restoration of cellular homeostasis during recovery from osmotic stress. Thus, proline cycle (Verma and Zhang, 1997; Fig. 6) helps plants in storing and providing necessary energy and nitrogen as needed. In yeast, the level of PDH is induced 50-fold by proline, and PDH gene expression is regulated by the level of free proline pool (Wang and Brandriss 1986). Arabidopsis PDH gene expression is also induced by excess proline in plants. However, this induction does not occur under stress conditions (Peng et al. 1996). Therefore, a mechanism must exist to prevent induction of PDH gene expression by proline in the plant under osmotic stress. The exact nature of this regulatory system is not fully understood.
The enzymes are numbered as shown in Table 3. The thicker arrows represent a metabolism increase under indicated condition. Intermediates: GSA, γ-glutamyl-5-semialdehyde; P5C, Δ¹-pyrroline-5-carboxylate.

Figure 6. The relationship between proline synthesis and degradation as influenced by osmotic stress conditions.
1.9. Proposed research

As indicated above, many plants accumulate free proline under osmotic stress conditions, and high levels of proline play an important role in plants to adapt them to stress conditions. The demonstration that the increase in proline concentration in stressed plants is due primarily to the stimulation of proline biosynthesis (Boggess et al., 1976a, 1976b; Buhl and Stewart, 1983; Rhodes et al., 1986) enhanced the interest in proline synthetic pathways. The cloning and characterization of Vigna P5CS gene (Hu et al., 1992) confirmed the presumed proline biosynthetic pathway in plants. The preliminary data showed that the expressed Vigna P5CS in the crude extract of E. coli was feedback inhibited by proline, and the level of the P5CS transcript was enhanced in Vigna roots treated with NaCl (Hu et al., 1992). Thus, it is likely that the P5CS enzyme is the rate-limiting enzyme in the pathway, and proline levels might be controlled by the P5CS gene expression and P5CS enzyme activity.

The goal of my dissertation research was to investigate the regulation of proline biosynthesis in plants under stress conditions. To achieve this goal, I proposed to study the regulation of proline biosynthesis in two aspects. The first aspect was the study of the kinetic properties of the P5CS enzyme with the focus on the proline feedback inhibition. The information obtained from this study uncovered the factors controlling the enzyme activities, the relationship between the substrate and the inhibitor, and the possibility of removal of proline inhibition. The second aspect was to isolate the promoter of Arabidopsis P5CS gene and determine the promoter activity, using a β-glucuronidase (GUS) reporter gene, in transgenic plants. This study contributed to our understanding of regulation of
the P5CS gene expression in response to changes in environmental conditions. Success in this research may lead to engineering plants resistant to drought and salinity, a major problem in enhancing crop production.
CHAPTER 2

REMOVAL OF FEEDBACK INHIBITION OF Δ¹-PYRROLINE-5-CARBOXYLATE SYNTHETASE, A BIFUNCTIONAL ENZYME CATALYZING THE FIRST TWO STEPS OF PROLINE BIOSYNTHESIS IN PLANTS

2.1. Abstract

Δ¹-Pyrrrole-5-carboxylate synthetase (P5CS) catalyzes the first two steps in proline biosynthesis in plants. The Vigna aconitifolia P5CS cDNA was expressed in Escherichia coli and the enzyme was purified to homogeneity. The Vigna P5CS exhibited two activities, γ-glutamyl kinase (γ-GK) and glutamic-5-semialdehyde (GSA) dehydrogenase. The γ-GK activity of the P5CS was detected by the hydroxamate assay and by a [¹⁴C]glutamate assay. The native molecular mass of the P5CS was found to be about 450 kDa with six identical subunits. The Vigna P5CS showed a $K_m$ of 3.6 mM for glutamate while the $K_m$ for ATP was 2.7 mM. The γ-GK activity of the P5CS was competitively inhibited by proline while its GSA dehydrogenase activity was insensitive to proline. In addition, a protein inhibitor of the P5CS activity was detected in the plant cell. Western blot showed that the level of the P5CS was enhanced in Vigna root under salt stress. Two amino acid residues involved in proline binding of the
P5CS were identified. A single substitution of an alanine for a phenylalanine at amino acid position 129 of the P5CS resulted in a significant reduction of proline feedback inhibition. The 50% inhibition values of γ-GK activity of the wild-type and the mutant P5CS were observed at 5 mM and 960 mM of proline, respectively. The other properties of the mutant P5CS remained unchanged. These results may allow genetic manipulation of proline biosynthesis and overproduction of proline in plants for conferring water stress tolerance.

2.2. Introduction

Proline accumulates in plants under drought and salinity stresses in a number of species and is thought to play an important role in plant cells for adaptation to water stress (Adamas and Frank, 1980; Hanson and Hitz, 1982; Delauney and Verma, 1993). In plants, proline is synthesized from either glutamate or ornithine (Adamas and Frank, 1980; Delauney et al., 1993). We have demonstrated that the glutamate pathway for proline synthesis is predominant under the condition of osmotic stress (Delauney et al., 1993). In *Vigna aconitifolia*, the first two steps of the proline biosynthesis from glutamate are catalyzed by a single bifunctional enzyme, \( \Delta^1 \)-pyrroline-5-carboxylate synthetase (P5CS) with apparent activities of γ-glutamyl kinase (γ-GK) and glutamic-5-semialdehyde (GSA) dehydrogenase (or γ-glutamyl phosphate reductase). In *E. coli*, two separate enzymes, γ-GK and GAS dehydrogenase, are involved in the production of GSA. The purified *E. coli* γ-GK showed no detectable activity, but the addition of the purified *E. coli* GSA dehydrogenase revealed the γ-GK activity (Smith et al., 1984). The product (γ-glutamyl phosphate) of the γ-GK enzyme was suggested to remain the enzyme-bound state.
and was rapidly converted to GSA by GSA dehydrogenase which forms a complex with the γ-GK. The GSA produced by these reactions is spontaneously converted to pyrroline-5-carboxylate (P5C) which is then reduced by P5C reductase (P5CR) to proline. The cDNAs encoding the P5CS and the P5CR have been isolated from plants (Hu et al., 1992; Delauney and Verma, 1990). Expression of the P5CR cDNA in transgenic tobacco resulted in a 200-fold increase in the P5CR activity, but the proline level in transgenic plants was not significantly altered (Szoke et al., 1992). This result indicated that P5CR is not the rate-limiting enzyme in proline biosynthesis in plants. The expressed Vigna P5CS enzyme in the crude extract of E. coli was sensitive to proline inhibition, indicating that the P5CS may be the rate-limiting step in proline pathway in plants (Hu et al., 1992).

It has been demonstrated that proline biosynthesis in bacteria is regulated by the end-product inhibition of the γ-GK activity (Smith et al., 1984). A Salmonella typhimurium mutant resistant to the toxic proline analog, L-azetidine-2-carboxylic acid, over-produced proline and showed enhanced tolerance to osmotic stress (Csonka, 1981). The mutation was due to a change of an aspartate (at position 107) to an asparagine in the γ-GK, resulting in a mutant γ-GK which was much less sensitive to proline inhibition (Csonka et al., 1988; Dandekar and Uratsu, 1988).

Alignment of the protein sequences between the Vigna P5CS and the E. coli γ-GK and GSA dehydrogenase showed (Fig. 4, and Hu et al., 1992) that the two enzymatic domains overlap in the Vigna P5CS protein and the putative amino acid residue implicated in the feedback inhibition of the E. coli γ-GK enzyme at
the position 107 (bold) was found to be conserved in *Vigna* P5CS (at position 128, bold).

We reasoned that site directed mutagenesis of the corresponding feedback inhibition region of γ-GK domain in the *Vigna* P5CS may yield alleles able to retain high levels of the enzyme activity as the concentration of the end product of the pathway, proline, increases. We found that the conserved aspartate residue (at position 128) in the *Vigna* P5CS is not involved in the feedback inhibition, and two other residues were identified to be involved in proline binding. A single substitution of one of the two residues practically eliminated the feedback inhibition of the P5CS by proline.

The P5CS enzyme has not been characterized in plants or animals, and the studies on proline biosynthesis have been limited. In this chapter, we describe the purification, kinetic studies and mutagenesis of the *Vigna* P5CS. It was demonstrated that the γ-GK activity of the P5CS is subjected to feedback inhibition by proline and ADP, respectively. The level of the P5CS was increased in *Vigna* roots treated with NaCl. Removal of the feedback inhibition of the P5CS followed by over-production of the mutant enzyme in transgenic plants is expected to cause high level accumulation of proline. The latter may render plants capable of withstanding osmotic stress imposed by drought or salinity as proline acts as an osmoprotectant.

2.3. Materials and Methods

2.3.1. Bacterial strain and plasmid

*E. coli* strain CSH26 [*ara, Δ(lac proBA), thi*], a proline auxotroph, was
obtained from Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University). The plasmid, pVAB2 (Hu *et al.*, 1992), is a pcDNA II (Invitrogen, San Diego) carrying a full length cDNA encoding *Vigna* P5CS.

### 2.3.2. Purification of *Vigna* P5CS expressed in *E. coli*

The purification procedure is summarized in Figure 7. *E. coli* strain CSH26 carrying pVAB2 was grown for 16 h at 37 °C in LB medium containing 80 µg/ml of ampicillin. The cells were harvested by centrifugation, washed with cold buffer A (30 mM Tris-HCl, pH 7.2, containing 2 mM β-mercaptoethanol) and resuspended in the same buffer. The cells were broken by sonication and centrifuged at 35,000 x g for 20 min. The supernatant was fractionated by 30% saturation of (NH₄)₂SO₄. After centrifugation, the pellet was resuspended in buffer A and applied to a Sephadex G-50 column (Pharmacia). The proteins were eluted with the buffer A and fractions containing the P5CS activity were pooled and applied to a DE-52 column (Whatman). Proteins were eluted by 50 to 300 mM of NaCl linear gradient in buffer A. Fractions with the P5CS activity were combined and applied to a hydroxylapatite column (Bio-Rad) equilibrated with 10 mM of potassium phosphate buffer, pH 7.2, containing 2 mM β-mercaptoethanol. The column was washed with the same buffer and proteins were eluted stepwise with 90 and 180 mM of potassium phosphate in the same buffer. The fractions with the P5CS activity were combined and dialyzed against buffer A containing 100 mM NaCl. The purified P5CS was stored at -80 °C.
Figure 7. An outline of purification procedure of *Vigna* P5CS expressed in *E. coli*
2.3.3. Enzyme assays

2.3.3.1. Determination of γ-GK activity of *Vigna* P5CS using hydroxamate assay

The P5CS activity was assayed first by hydroxamate to detect the γ-GK activity as described by Hayzer and Leisinger (1980). The reaction mixture contained the following in a final volume of 0.1 ml at pH 7.0: 50 mM L-glutamate, 20 mM MgCl₂, 10 mM ATP, 100 mM hydroxamate-HCl, 50 mM Tris and the enzyme plus water. The reaction was started by the addition of the enzyme. After 5 minutes at 37 °C the reaction was terminated by the addition of 0.2 ml of the stop buffer (2.5 g of FeCl₃ and 6.0 g of trichloroacetic acid in a final volume of 100 ml of 2.5 N HCl). Precipitated proteins were removed by centrifugation and the absorbance at 534 nm (A₅₃₄) was recorded against a blank identical to the above but lacking ATP. The amount of γ-glutamyl hydroxamate was determined from the A₅₃₄ by the comparison to a standard curve of γ-glutamyl hydroxamate (Sigma). One unit (U) of γ-GK activity of the P5CS was defined as the amount of enzyme required to produce 1 μmole of γ-glutamyl hydroxamate/min. This assay was used during all steps of purification.

2.3.3.2. Detection of γ-GK activity of *Vigna* P5CS using [¹⁴C]glutamate as the substrate

The hydroxamate assay is not very sensitive. We developed a more sensitive assay for the γ-GK activity of the P5CS using [¹⁴C]glutamate. Root tissue from *Vigna* seedling (5 days old) was homogenized in extraction buffer (50 mM Tris at pH 7.0, 10 mM β-mercaptoethanol, 300 mM sucrose and 5 mM...
MgCl₂). The extract was centrifuged and supernatant was fractionated by 35% (NH₄)₂SO₄ saturation. The pellet was dissolved in the extraction buffer, dialyzed against the same buffer and assayed. The reaction contained the following in a final volume of 20 μl at pH 7.0: 50 mM Tris, 20 mM MgCl₂, 10 mM ATP, 5 mM NADPH, 0.1 μCi [¹⁴C]glutamate (DuPont/NEN) and the enzyme plus water. The reaction mixture was incubated at 37 °C for 10 minutes and then chilled on ice. An aliquot (2 μl) of the reaction mixture was resolved by thin layer chromatography (TLC) on a silica gel (Analtech, Inc.). P5C, glutamine, [¹⁴C]glutamate and [¹⁴C]proline (DuPont/NEN) were used as standards. The P5CR enzyme was purified from a proline mutant of \textit{E. coli} expressing soybean P5CR cDNA (Szoke \textit{et al.}, 1992). The silica gel was developed with a mobile phase (phenol : water : acetic acid, 75 : 25 : 5, W/V/V) containing 0.3% (W/V) ninhydrin in a saturated chamber. After development the gel was dried at 65 °C for 20 minutes, wrapped with Saran Warp and analyzed on a PhosphorImage (Molecular Dynamics) or exposed to a X-ray film.

\subsection{2.3.3.3. Determination of GSA dehydrogenase activity of \textit{Vigna} P5CS}

The GSA dehydrogenase activity of \textit{Vigna} P5CS was assayed as described by Hayzer and Leisinger (1980). The GSA dehydrogenase activity was not possible to detect in the forward (biosynthetic) direction because of the labile γ-glutamyl phosphate (Hayzer and Leisinger, 1980). We measured the reverse reaction by phosphate-dependent reduction of NADP⁺ with glutamic acid-5-semialdehyde (derived from equilibrium with Δ¹-pyrroline-5-carboxylate) as the substrate. The reaction mixture contained the following in a final volume of 0.3
ml at pH 7.0: 2.5 mM P5C prepared as described earlier (Szoke et al., 1992), 1 mM NADP\(^+\), 100 mM KH\(_2\)PO\(_4\), 50 mM imidazole base and the enzyme plus water. The increase in the absorbance at 340 nm was recorded at room temperature against a blank identical to the above but lacking inorganic phosphate. The concentration of P5C was determined with \(\alpha\)-aminobenzaldehyde described by Mezl and Knox (1976).

2.3.4. Molecular mass determination

The native molecular mass of purified \textit{Vigna} P5CS was estimated by gel permeation on a Superose-6 high performance liquid chromatography column (HPLC; 1 x 30 cm, Pharmacia). Protein standards were run simultaneously with the purified enzyme or separately in a second run. The samples were applied to the column equilibrated with buffer A containing 100 mM NaCl. The protein standards (Bio-Rad) used were thyroglobulin (670,000), bovine gamma globulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000) and vitamin B-12 (1,350). The molecular mass of the enzyme was estimated in duplicate by means of a plot of \(K_{av}\) for the standards against the logarithm of the molecular mass (Laurent and Killander, 1964). The subunit molecular mass of the P5CS was estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3.5. Development of Antibodies against \textit{Vigna} P5CS

The purified P5CS was subjected to SDS-PAGE (7.5%) and the protein band was visualized by Coomassie Blue staining (Harlow and Lane, 1988). The band was excised and homogenized in liquid nitrogen. The gel powder was mixed with equal volume of Freund’s adjuvant (GIBCO) and polyclonal
antiserum was prepared in rabbits. Serum obtained 10 days after the third injection was applied to a protein-A column and the IgG fractions were eluted by 100 mM of glycine-HCl, pH 3.0. Following adjustment of the pH to 7.4, the purified IgG was stored at -20°C.

2.3.6. Protein extraction and Western blot analysis

*Vigna* roots (2.5 g, fresh weight, one week old) with or without treatment of NaCl (200 mM, 72 h) were homogenized in liquid nitrogen and resuspended in 2 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride. The resulting slurry was centrifuged at 80,000 x g for 10 min at 4°C. The supernatant was saved and concentrated 10-fold using a Centricon concentrator (*M*_r cut off 10,000, Amicon). The concentrated sample was subjected to SDS-PAGE (7.5%) and protein bands were transferred to nitrocellulose membranes. The P5CS peptide was detected by reacting with the P5CS antibody and a second antibody using ECL procedure (Amersham Corp.).

2.3.7. Alanine scanning mutagenesis of the P5CS

The alanine substitutions were performed using oligonucleotide-directed mutagenesis (Hidchi *et al.*, 1988). The first two PCR reactions produced two overlapping DNA fragments, both bearing the same mutation introduced via primer mismatch in the region of the overlap. The two overlapping fragments were mixed and used as templates for the second PCR reaction with two flanking primers. The fragment (700 bp) produced by the second PCR reaction was purified by agarose gel electrophoresis. The purified fragment was digested with restriction enzyme *Hind*III and subcloned into the pVAB2 (a pcDNA II plasmid
[Invitrogen, San Diego] carrying a full length cDNA of *Vigna* P5CS) from which the corresponding wild-type fragment of the P5CS cDNA had been removed by *Hind*III digestion. The reconstructed pVAB2 carrying a single amino acid substitution was introduced into *E. coli* strain CSH26. Crude extracts were made from the strains harboring the reconstructed pVAB2 and assayed for the γ-GK activity of the P5CS in the presence of proline. DNA sequencing was conducted to confirm the substitution. The P5CS enzymes carrying a single substitution of an alanine for an aspartate at amino acid position 126 and an alanine for a phenylalanine at amino acid position 129 were named as P5CSD126A and P5CSF129A, respectively.

2.4. Results

2.4.1. Purification of *Vigna* P5CS expressed in *E. coli*

The *Vigna* P5CS cDNA was expressed in *E. coli* and the expressed enzyme was purified as summarized in Table 4. 30% saturation of (NH₄)₂SO₄ precipitated the P5CS from the crude extract of *E. coli*. We used a Sephadex G-50 column to remove (NH₄)₂SO₄ quickly because it was found that the expressed P5CS was not stable at 4 °C. This fraction was subjected to DEAE-cellulose chromatography (Fig. 8a) resulted in a 27-fold purification of the enzyme over crude extract and most unwanted proteins were removed by this column, but this step also lost significant amount of the enzyme (Table 4). The enzyme was eluted from the column around 120 mM of NaCl. The hydroxylapatit chromatography (Fig. 8b) gave a final yield of the P5CS of 7% and represented a 54-fold purification over the crude extract (Table 4).
A, DE-52 anion exchange chromatography of *Vigna* P5CS expressed in *E. coli*. The combined fraction from Sephadex G-50 was applied to a DE-52 column and proteins were eluted by 50 - 300 mM of NaCl linear gradient. 

B, Hydroxylapatite chromatography of *Vigna* P5CS expressed in *E. coli*. The combined fraction (28 - 40) from DE-52 column was applied to a hydroxylapatite column, and the proteins were eluted stepwise with 90 (fractions 9-17) and 180 (fractions 18-27) mM of potassium phosphate, pH 7.2, containing 2 mM β-mercaptoethanol. The P5CS was present in the fractions with 180 mM of potassium phosphate. Protein concentration was monitored at 280 nm. Activity = nmole of γ-glutamyl hydroxamate formed/min.

Figure 8. Chromatography of *Vigna* P5CS expressed in *E. coli*
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Pridification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
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<td>79.0</td>
<td>0.2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
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<td>53.0</td>
<td>1.8</td>
<td>9</td>
<td>67</td>
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<tr>
<td>Sephadax G-50</td>
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<td>1.7</td>
<td>9</td>
<td>54</td>
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<td>5.4</td>
<td>10.8</td>
<td>54</td>
<td>7</td>
</tr>
</tbody>
</table>

A 54-fold purification of the P5CS giving almost pure protein suggests that the initial concentration of this enzyme in the crude extract was almost 2% in the IPTG-induced culture.

Table 4. Summary of purification of *Vigna* P5CS expressed in *E. coli*
Lane 1, Protein marker in kilodalton lane 2, crude extract (25 µg) of *E. coli* strain CSH26; lane 3, crude extract (25 µg) of *E. coli* strain CSH26 carrying pVAB2; lane 4, proteins (12 µg) from 30% saturation of (NH₄)₂SO₄ precipitation; lane 5, concentrated fraction (5 µg) from DE-52 column; lane 6, active fraction (2 µg) from hydroxylapatite column.

Figure 9. SDS-PAGE showing the purification of *Vigna* P5CS.
purified enzyme was essentially free of contaminating proteins and appeared as a single band on SDS-PAGE (Fig. 9).

2.4.2. General properties of Vigna P5CS

The γ-GK activity of the purified P5CS displayed a linear response up to 5 minutes with respect to the amount of protein between 2.0 - 3.0 µg per assay. The purified enzyme lost about half and one third of its activity by overnight storage at 4 °C and -20 °C, respectively, but it was stable at -80 °C for up to three weeks. The maximal activity of the γ-GK was obtained at 37 °C with pH optimum between pH 6.5 and pH 7.5 in buffer A (Fig. 10). The specific activity of γ-GK of the purified Vigna P5CS was found to be 10.8 µmole of glutamyl hydroxymate min⁻¹ mg⁻¹, while the specific activity of the GSA dehydrogenase of the Vigna P5CS was found to be 0.8 µmole of NADPH min⁻¹ mg⁻¹. These results thus confirmed at enzymatic level that the Vigna P5CS is a bifunctional enzyme with two separate enzyme activities, γ-GK and GSA dehydrogenase. The native molecular mass of Vigna P5CS was estimated to be 450 kDa as determined by gel filtration on Superose-6 HPLC (Fig. 11). The subunit molecular mass was estimated to be 77 kDa (see discussion), suggesting that Vigna P5CS is a hexamer with six identical subunits. The size of the subunit was confirmed by Western blot analysis using Crude extracts from Vigna roots and root nodules. The P5CS activity was not detected in earlier studies (in vivo), apparently due to the presence of an inhibitor (see below). The purified enzyme allowed us to detect this activity.
The activity was assayed as described in "Materials and Methods". The different pH values were generated by 50 mM Tris-HCl buffer.

Figure 10. The pH effect on the γ-GK activity of the purified P5CS.
Protein markers (see "Materials and Methods")

$\Delta$ Vigna P5CS

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

$V_t$, total volume of the gel bed; $V_o$, void volume of the gel bed; $V_e$, elution volume of a protein.

Figure 11. Determination of native molecular weight of purified Vigna P5CS.
2.4.3. Kinetic properties of *Vigna* P5CS

2.4.3.1. $\gamma$-GK activity of the *Vigna* P5CS is competitively inhibited by proline while ADP is a mixed inhibitor of the P5CS

Plots of $\gamma$-GK activity of the P5CS vs glutamate concentration displayed a typical Michaelis-Menten kinetics. Double-reciprocal plots were used to estimate the $K_m$ and $V_{max}$ values for glutamate, and the values obtained were 3.6 mM and 13.3 $\mu$ mole of $\gamma$-glutamyl hydroxamate min$^{-1}$ mg$^{-1}$. Plots of the $\gamma$-GK activity vs ATP concentration also displayed a typical Michaelis-Menten kinetics, and the $K_m$ for ATP was found to be 2.7 mM. The $\gamma$-GK activity of the P5CS was inhibited by proline and its analog, 3, 4-dehydroproline. A 50% inhibition (in the presence of 50 mM of glutamate) of the $\gamma$-GK was observed in the presence of 5.0 mM of proline or 4.5 mM of 3, 4-dehydroproline. Enzyme kinetics of the $\gamma$-GK at different proline or 3, 4-dehydroproline concentrations indicated that the both are competitive inhibitors, and the estimated $K_i$ for proline was 1.0 mM (Fig. 12a). In addition, the $\gamma$-GK activity of the *Vigna* P5CS is also inhibited by ADP (Fig. 12b) whereas AMP and GMP had no effect (data not shown). ADP was found to be a mixed competitive inhibitor and the estimated $K_i$ for ADP was 6.4 mM.

2.4.3.2. The GSA dehydrogenase activity of the *Vigna* P5CS is not inhibited by proline

The GSA dehydrogenase activity of the *Vigna* P5CS was not inhibited in the presence of 100 mM proline (Fig. 13). Therefore, proline inhibition appears to be limited to the $\gamma$-GK activity of the P5CS only.
3.0 μg of purified P5CS was used in each assay. A, double reciprocal plots of γ-GK activity of the purified P5CS versus glutamate at different concentrations of proline. B, double reciprocal plots of γ-GK activity of the purified P5CS versus ATP at different concentrations of ADP. Note the mixed competitive inhibition of the γ-GK activity by ADP. Activity = nmol of γ-glutamyl hydroxamate formed per min.

Figure 12. The effects of proline and ADP on the γ-GK activity of Vigna P5CS.
3.0 μg of the purified P5CS was used in each assay. The enzyme activity was measured as described in "Materials and Methods". Note that the GSA dehydrogenase activity of the purified P5CS was not affected in the presence of 100 mM proline.

Figure 13. The GSA dehydrogenase activity of *Vigna* P5CS and its sensitivity to proline inhibition.
2.4.3.3. Comparison of some molecular and kinetic properties between the *Vigna* P5CS and *E. coli* γ-GK

The Table 5 lists the major properties of the *Vigna* P5CS and *E. coli* γ-GK. We noticed that the $K_m$ value of the P5CS for glutamate is 10-fold less than the similar value of *E. coli* γ-GK, suggesting that the *Vigna* P5CS is much more sensitive to the change of glutamate concentration, while *E. coli* γ-GK is insensitive to the change of glutamate concentration. The γ-GK activity of purified P5CS can be assayed by hydroxymate while the purified *E. coli* γ-GK showed no activity in the same assay (see discussion).

2.4.4. γ-GK activity of the purified P5CS was inhibited by the crude extract of *Vigna* root.

The P5CS activity has, so far, not been detected in plants. We developed a method using [14C]glutamate as the substrate to detect the P5CS activity in *Vigna* roots. The reaction with purified P5CS showed the accumulation of P5C (Fig. 14, lane 5) and the addition of P5CR to the reaction mixture resulted in the production of proline (Fig. 14, lane 7). No activity of the P5CS was detected in *Vigna* roots (Fig. 14, Lane 3). The root extract added to the purified P5CS inhibited the γ-GK activity, but boiling the root extract prior to the addition removed the inhibition of the P5CS (Fig. 14, lanes 4 and 6). This suggested that there may be a protein inhibitor in the plant extract that inhibits the P5CS activity. The γ-GK activities of the P5CS with and without the addition of the P5CR were similar (data not shown) based on the radioactivity of P5C and proline spots resolved on TLC (Fig. 14, lanes 5 and 7). This data also demonstrated that the
<table>
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<tr>
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<td>(gel filtration)</td>
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<td><em>M</em>&lt;sub&gt;r&lt;/sub&gt; of subunit</td>
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<td><em>K</em>&lt;sub&gt;m&lt;/sub&gt; for glutamate</td>
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<tr>
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<td>pH optimum</td>
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Table 5. Comparison of molecular characteristics between *Vigna P5CS* and *E. coli γ-Glutamyl kinase*
The assay was conducted as described in "Materials and Methods". The positions of the standard amino acids and TLC start and front are indicated on the left side. Lane 1, $[^{14}\text{C}]$ proline; lane 2, $[^{14}\text{C}]$ glutamate; lane 3, the root extract of Vigna (25 μg); lane 4, Vigna root extract (25 μg) plus purified P5CS (0.1 μg); lane 5, purified P5CS (0.1 μg); lane 6, the boiled root extract of Vigna (25 μg) plus purified P5CS (0.1 μg); lane 7, purified P5CS (0.1 μg) plus purified P5CR (0.4 μg); lane 8, purified P5CS (0.1 μg) without ATP in the reaction mixture.

Figure 14. $[^{14}\text{C}]$ glutamate assay of the γ-GK activity of Vigna P5CS.
P5CR is not the rate-limiting step in proline synthesis as a trace amount of it is sufficient for converting P5C to proline.

2.4.5. The level of the P5CS protein was enhanced in *Vigna* roots treated with NaCl

Polyclonal antibodies raised against the purified P5CS were used to detect the native P5CS in *Vigna* roots. The P5CS antibody reacted with a protein band from the extract of stressed roots intensity of which was much higher than that from the extract of unstressed roots (Fig. 15), indicating that the amount of the P5CS in the root was enhanced by salt stress. This band appears at about 75 kD which is similar to the product expressed in *E. coli*. The difference between the subunit sizes of expressed P5CS and the native *Vigna* root P5CS is apparently due to the addition of amino acid residues at the N-terminal of the expressed enzyme from the expression vector in which the P5CS cDNA was fused with the *lac Z* promoter (see Discussion).

2.4.6. A single amino acid substitution of the P5CS reduced proline feedback inhibition significantly, while the other properties of the mutant enzyme remained unchanged.

Eight alanine substitution mutants of the P5CS were created and expressed in *E. coli* strain CSH26. The crude extracts made from the mutants were assayed for the γ-GK activity in the presence of 10 mM of proline. The first mutant, carrying the substitutions of three alanines for the amino acid residues at position 126, 127 and 128 (Fig. 4 and Fig. 16a), showed no inhibition at 10 mM of proline. The second mutant, bearing the substitutions of three alanines for the
amino acid residues at position 129, 130 and 131 (Fig. 4 and Fig. 16a), also showed no inhibition of the activity in the presence of 10 mM of proline. The remaining six mutants were created by the substitution of an alanine for the individual residues at the position from 126 to 131, respectively. Two of the six single substitution mutants, P5CSD126A and P5CSF129A (Fig. 16a), showed significant reduction of proline inhibition while other substitutions showed no effect on proline inhibition (data not shown). The 50% inhibition values of γ-GK of the P5CSF129A (Fig. 16b) and P5CSD126A were observed in the presence of 960 mM and 85 mM of proline, respectively. The $K_i$ of P5CSF129A for proline in the presence of glutamate was 195 mM which is about 200-fold greater than that of the wild-type P5CS (Table 6). The P5CSF129A was expressed in E.coli and purified. Its kinetic characteristics were found to be similar to the wild type P5CS except the feedback inhibition (Table 6). This data suggests that the catalytic domain and feedback inhibition domain of the P5CS are separate.
The Western blot was performed as described in “Materials and Methods”. Lane 1, crude extract (1 μg) of *E. coli* strain CSH26; lane 2, crude extract (1 μg) of *E. coli* strain CSH26 carrying pVAB2; lane 3, purified *Vigna* P5CS (0.1 μg); lane 4, the root extract (65 μg) of *Vigna* treated with 200 mM of NaCl; lane 5, the root extract (65 μg) of *Vigna* without salt stress.

Figure 15. The effect of NaCl on the level of the P5CS in *Vigna* roots.
Amino Acid Position 126 129 131

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<tr>
<td>Asp</td>
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B

A, Amino acid substitutions and their effects on the feedback inhibition of *Vigna* P5CS by proline. The numbers on the top correspond to the amino acid positions in the P5CS protein (aligned by the asterisks, see ref. Hu et al., 1992). All six amino acids were replaced by an alanine individually. The boldface amino acids represent the single substitution in the mutant alleles that reduced proline inhibition. The substitution of the aspartate at position 128, the putative residue involved in proline interaction (Fig. 4 and ref. Hu et al., 1992), had no effect on the feedback inhibition of the enzyme activity. B, The effect of proline on the activities of purified *E. coli* γ-GK, the P5CS and the P5CSF129A. Hydroxamate assay containing 50 mM of glutamate was conducted in the presence of different concentrations of proline. The curve of *E. coli* γ-GK was replotted from the data in the reference (Smith et al., 1984).

Figure 16. Alanine scanning mutagenesis of the P5CS
<table>
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<td>Wild-type P5CS</td>
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<td>Specific activity of $\gamma$-GK for glutamate (U/mg)</td>
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<td>$K_m$ for glutamate</td>
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<td>$K_m$ for ATP</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>$K_i$ for proline in the presence of glutamate</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>50% inhibition of the activity by proline</td>
<td>5.0 mM</td>
</tr>
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<td>$K_i$ for ADP in the presence of ATP</td>
<td>6.4 mM</td>
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<tr>
<td>pH optimum</td>
<td>6.5 - 7.5</td>
</tr>
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Table 6. Comparison of kinetic properties between wild-type P5CS and P5CSF129A.
2.5. Discussion

We described a purification procedure for a bifunctional enzyme, P5CS, catalyzing the first two steps in proline biosynthesis in plants (Hu et al., 1992; Yoshia et al., 1995). The purified *E. coli* γ-K showed no detectable activity using the hydroxamate assay, but the production of γ-glutamyl hydroxamate (γ-GP) could be restored by the addition of purified *E. coli* GSA dehydrogenase (Baich, 1969; Hayzer and Leisinger, 1981). It has been suggested that *E. coli* γ-GK and GSA dehydrogenase form a complex to afford protection to the labile γ-glutamyl phosphate, and to directly transfer the intermediate from one enzyme to the other avoiding equilibration with the surrounding medium (Baich, 1969; Gamper and Moses, 1974). Such a complex has not been detected in *E. coli* (Smith et al., 1984). The *Vigna* P5CS is a fused protein with two separate catalytic domains on a single peptide. The γ-GK activity of the purified P5CS can be detected readily using hydroxamate assay. These results supported the idea that the labile γ-glutamyl phosphate exists in an enzyme-bound state (Baich, 1969) and that GSA dehydrogenase domain interacts with γ-GK, effecting the release of γ-glutamyl phosphate which can be measured as the hydroxamate derivative.

Due to the addition of extra amino acids from the expression vector, the molecular mass of the expressed P5CS subunit was 77 kDa as measured by SDS-PAGE. This value was slightly higher than the molecular mass (73 kDa) deduced from the DNA sequence of the P5CS (Hu et al., 1992). The subunit size of the native P5CS in *Vigna* detected by Western blot was smaller (~ 2 kDa) than that of the P5CS expressed in *E. coli*. (Fig. 15). Therefore the molecular mass of *Vigna* P5CS subunit is likely to be 75 kDa. The native molecular mass of the P5CS is about 450 kDa as determined by gel filtration. These results suggest that *Vigna*
P5CS is a hexamer of six identical subunits. Both γ-GK and GSA dehydrogenase of *E. coli* are also hexamers with six identical subunits (Smith *et al.*, 1984).

The characteristics of *Vigna* P5CS and γ-GK of *E. coli* were compared in Table 4. In *E. coli*, plots of the γ-GK activity vs glutamate concentration was nonhyperbolic and the glutamate concentration yielding half-maximal activity was 37 mM (Smith *et al.*, 1984). This value is about 10-fold greater than the similar value of *Vigna* P5CS, suggesting that plant P5CS has higher affinity for glutamate than *E. coli* γ-GK.

The γ-GK activity of *Vigna* P5CS was inhibited by proline and ADP, but its GSA dehydrogenase activity was not affected, suggesting that the γ-GK is the rate-limiting step in proline biosynthesis in plants. A similar situation was observed in *E. coli* γ-GK but not in yeast γ-GK. The latter is regulated by a general amino acid control system (Li and Brandniss, 1992). Proline decreases the affinity of *Vigna* P5CS enzyme for glutamate, but the inhibition could be partially overcome at higher concentrations of glutamate. ADP, on the other hand, showed a mixed competitive inhibition of γ-GK activity of the P5CS, and it is likely that ADP binds to the same site involved in ATP binding.

The P5CS activity in *Vigna* roots was not detectable. The fractionation of the root extract with (NH₄)₂SO₄ was necessary to separate the P5CS from the glutamine synthetase (GS) activity of which is much higher than the P5CS activity in *Vigna* roots. GS interferes with the P5CS assay. A 35% saturation of (NH₄)₂SO₄ precipitated the P5CS while GS remained in solution (Kishor, *et al.*, 1995). The activity of the purified P5CS was inhibited in the presence of the root extract which was eliminated by boiling the extract, suggesting the presence of an
inhibitor in the plant (Fig. 14, lanes 4, 5 and 6). This may be one of the reasons why the P5CS activity has not been detected in plants so far. The method described here using [14C]glutamate as the substrate is, at least, 50-fold more sensitive than the method of hydroxamate assay for the γ-GK activity of the P5CS.

We have previously shown that the expression of the P5CS mRNA in Vigna roots was enhanced by the treatment of the plant with 200 mM of NaCl (Hu et al., 1992). Compared with unstressed roots, the amount of the P5CS protein in salt-treated roots was found to be enhanced (Fig. 15). Proline biosynthesis in plants, thus, is primarily regulated at the transcriptional level and at the level of enzyme activity by the feedback inhibition. It was also reported that proline degradation is reduced in plants under water stress (Stewart et al., 1977) and the activity of proline dehydrogenase was inhibited by KCl (Rayapati and Stewart, 1991). Therefore, it is possible that the proline accumulation in plants under stress occurs due to the increase in the amount of the P5CS and the decrease of the activity of proline dehydrogenase.

The γ-GK activity of the P5CS is regulated kinetically in three ways. First, the enzyme activity responds to the change of glutamate concentration. We had observed earlier (Delauney et al., 1993) that at a high nitrogen level, the ornithine pathway for the biosynthesis of proline was prominent while the glutamate pathway is reduced. Under the stress conditions (salt and drought) and low nitrogen level, the glutamate pathway for proline biosynthesis was dominant and plants converted more glutamate to proline (Delauney et al., 1993; Boggess et al., 1976). The second control involves the inhibition of the P5CS activity by ADP. Regulation at this level would make proline biosynthesis responsive to
cellular energy level. Finally, γ-GK activity of the P5CS is controlled by the end product of the pathway, proline. This point of control is by far the most important, since this control would ensure that there is no excess proline production when it is not needed. Some earlier experiments had suggested that proline accumulation in plants under stress may involve the loss of feedback regulation (Boggess et al., 1976a; Boggess et al., 1976b). In addition, the presence of an inhibitor we observed may regulate the activity of the P5CS enzyme.

In the γ-GK of E. coli, the change of the aspartate at amino acid residue 107 to an asparagine led to a reduction of proline inhibition (Csonka et al., 1988; Dandekar and Uratsu, 1988). The alignment of protein sequences between Vigna P5CS and E. coli γ-GK showed that the aspartate at position 128 in the P5CS corresponds to the aspartate at the position 107 in E. coli γ-GK (Fig. 4, and ref. Hu et al., 1992). The aspartate (at position 128) in the P5CS was changed to an asparagine, but the mutant P5CS (P5CSD128N) showed no reduction of proline inhibition, suggesting that this aspartate is not involved in proline binding. The alanine scanning of this region resulted in two single substitution mutants of the P5CS, P5CSD126A and P5CSF129A, showing the reduction of proline inhibition. The P5CSF129A exhibited a significant increase of 50% inhibition value by proline (Fig. 16b) while other properties of this enzyme remained unchanged (Table 6). It is likely that the glutamate and proline binding sites are not the same, but may be partially overlapped or are immediately adjacent to each other so that the binding of glutamate affects the binding of proline and vice versa. However, we have no data to exclude the possibility that the substitution caused a change in the conformation of the enzyme so that the enzyme lost its allosteric
properties. Obviously both residues, the aspartate at 126 and the phenylalanine at 129, are involved in proline binding. The phenylalanine is more important with respect to proline binding since the reduction of proline inhibition obtained by the P5CSD126A is only 10% of that obtained by the P5CSF129A. X-ray structure of the P5CS and its mutants may produce interesting results of the mechanism of proline binding. Overexpression of the P5CS in transgenic plants has been demonstrated to produce more proline and render plants less sensitive to osmotic stress (Kishor et al., 1995). Reduction of feedback inhibition of the P5CS may further increase the accumulation of proline in transgenic plants.
CHAPTER 3

CHARACTERIZATION OF Δ¹-PYRROLINE-5-CARBOXYLATE SYNTHETASE GENE PROMOTER IN TRANSGENIC ARABIDOPSIS THALIANA SUBJECTED TO WATER STRESS

3.1. Abstract

Δ¹-pyrroline-5-carboxylate synthetase (P5CS) is the rate limiting enzyme in proline biosynthesis in plants. The promoter of an Arabidopsis P5CS gene was isolated and analyzed using a β-glucuronidase (GUS) reporter gene in transgenic plants. NaCl stress induced GUS activity to the maximum level within 3 hr while dehydration continued to enhance the GUS activity up to 24 hr. The AtP5CS gene promoter was found to contain two transcription start sites, and dehydration stimulated the transcription predominantly from the downstream site. Abscisic acid (ABA) failed to induce the AtP5CS gene expression although ABA is known to increase during water stress. These observations suggest that induction of the P5CS gene is a specific response of plants to salt and water stresses. Since proline accumulation depends on the rate of synthesis and its degradation, a coordinated regulation of the P5CS and PDH gene is important in the final level of proline in a cell both in normal and stress conditions.
3.2. Introduction

Availability of water is important for the growth and development of plants. Drought and salinity stresses are the major limiting factors for plant growth and productivity (Boyer, 1982). Adaptation of plants to water stress involves morphological, physiological as well as biochemical changes including the accumulation of compatible osmolytes such as proline (Hanson and Hitz, 1982; McCue and Hanson, 1990; Delauney and Verma, 1993). The accumulation of proline has been shown to increase osmotolerance in bacteria (Rudulier et al., 1984; Csonka, 1981) and plants (Kishor et al., 1995).

In plants, proline is synthesized from both glutamate and ornithine (Adams and Frank, 1980; Hu et al., 1992), and glutamate pathway is predominant in plants under osmotic stress and nitrogen starvation (Delauney et al., 1993). Glutamate is converted to glutamate semi-aldehyde by a single bifunctional enzyme, Δ1-pyrroline-5-carboxylate synthetase (P5CS). The latter cyclises spontaneously to pyrroline-5-carboxylate which is then reduced to proline by pyrroline-5-carboxylate reductase (P5CR). It has been shown that the first enzyme of the pathway, P5CS, is the rate-limiting enzyme and is feedback inhibited by proline (Zhang et al., 1995). The P5CS cDNA was isolated from Vigna aconitifolia, and it was shown that the level of P5CS transcripts enhanced in plants treated with NaCl (Hu et al., 1992). Similar results were reported in Arabidopsis, where the level of the AtP5CS transcripts were shown to increase in plants treated with NaCl or subjected to dehydration (Yoshiba et al., 1995; Savoure et al., 1995).

The phytohormone abscisic acid (ABA) is shown to play an important role in mediating the responses of plants to environmental stresses, including drought.
and high salinity. ABA was shown to increase the levels of the AtP5CS transcripts and proline in *Arabidopsis* (Yoshida *et al.*, 1995). On the other hand, proline accumulates in a tomato *flacca* mutant which does not have a detectable level of ABA even after osmotic stress. Application of ABA biosynthesis inhibitor to wilted barley leaves did not affect proline accumulation (Stewart and Voetberg, 1987). These observations suggest the possibility of ABA-dependent and ABA-independent pathways regulating the expression of genes in response to osmotic stress.

The relationship between osmotic stress and the P5CS enzyme activity in plants is not clear since the P5CS enzyme activity is not detectable in wild-type plants (Kishor *et al.*, 1995). In order to understand regulation of proline biosynthesis, we have isolated an *Arabidopsis* P5CS gene promoter and investigated the expression of the AtP5CS gene in transgenic plants under stress conditions. Our data showed that the expression of the AtP5CS gene was enhanced by dehydration and external application of NaCl, but not by exogenous ABA. Our observations suggest that the induction of the P5CS gene expression in plants is the response specifically to salt and water stresses.

### 3.3. Materials and Methods

#### 3.3.1. Isolation of *Arabidopsis* P5CS gene promoter

A genomic DNA library of *Arabidopsis thaliana*, ecotype Landsberg (Voytas *et al.*, 1990) was obtained from *Arabidopsis* Resource Center at Ohio State University. The library was screened by plaque hybridization using labeled P5CS cDNA of *Vigna aconitifolia* as a probe. Positive plaques were purified and
the genomic DNA inserts were analyzed by restriction enzyme digestion and DNA blotting. The positive DNA fragments were subcloned into pcDNAII (Stratagene) and sequenced using an automatic DNA sequencer (Applied Biosystems).

3.3.2. Plant transformation

*Arabidopsis thaliana*, ecotype Landsberg and *Nicotiana tabacum* cv. SR1 were used in this study. pBI101 plasmid (Clontech) carrying the AtP5CS promoter region (~1280 bp) was introduced into *Agrobacterium tumefaciens* strain GV3103 for *Arabidopsis* transformation and LBA4404 for tobacco transformation. Transformation of tobacco was performed using the leaf disc system (Horsch et al., 1992). Transformation of *Arabidopsis* was performed using the vacuum infiltration transformation described by Bent et al. (1994). *Arabidopsis* seeds produced by the first generation of transformed plants were screened on MS medium containing sucrose (1%) and kanamycin (50 μg/ml). The positive seedlings (T1) were identified on screening plates and then transferred to pots filled with soil for the production of seeds (T2).

3.3.3. Plant growth and stress treatments

Transgenic *Arabidopsis* seeds (T2) were germinated on MS medium containing sucrose (1%) and kanamycin (50 μg/ml). Ten seedlings of each transgenic line were stained for GUS activity with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Seedlings (25 day old) were removed from agar plates and grown hydroponically in water containing NaCl, ABA, glutamine, and proline respectively for indicated times. Dehydration treatment was performed by drying transgenic *Arabidopsis* plants on a Petri dish at 23 °C and 60% humidity.
under dim light. Dehydration treatment was also carried out on the leaves of transgenic tobacco plants (T1). Leaves (~9 cm^2) were excised and dehydrated as mentioned above. Low temperature (4 °C) treatment was applied by growing transgenic Arabidopsis plants in a cold room for 48 hr. The whole plants were removed from agar plates for GUS activity assay.

3.3.4. GUS activity assay

GUS activity was assayed in plant crude extracts by fluorometric quantification of 4-methylumbelliferone produced from glucuronide precursor using the method described by Jefferson (1987).

3.3.5. Primer extension

Total RNA was isolated from Arabidopsis (Landsberg) prior to or after dehydration. Total RNA (20 μg) was hybridized with a [32p]-labeled oligonucleotide (5'-GAACGCGCGTGAACGATCGATCTCCGTCAT-3'), which annealed to an internal site (Fig. 17, position +1 - +30) of the AtPSCS gene, at 35 °C for 12 hr. Reverse transcription was performed at 40 °C for two hours. Extension products were electrophoresed on an acrylamide gel (6%) and visualized by exposure to a PhosphorImager (Molecular Dynamics) or a X-ray film.

3.3.6. Isolation of 5' end of Arabidopsis P5CS cDNA

The technique of rapid amplification of cDNA ends (RACE; Frohman et al., 1988) was used to clone the 5' end of the P5CS cDNA of Arabidopsis (Landsberg). Total RNA from dehydrated plants and a AtP5CS gene-specific
primer (5'-CTGTTCACAGATAGCTCCTAGACGTCC-3'), annealing to an internal site of the second exon (Fig. 17, position +404 - +430), were used for the synthesis of first-strand AtP5CS cDNA. The cDNA was tailed with dATP and used as a template for polymerase chain reaction (PCR) amplification using a dT17 adapter primer with an Xho I restriction site at the 5' end and a second AtP5CS gene-specific primer (5'-GGATCCGGATCCAGAGCCAATCTTCCACCTTTCCCAGT-3') with two BamH I restriction sites at the 5' end. The second gene specific primer annealed to an internal nested site (Fig. 17, position +377 - +402) of the AtP5CS gene. Following PCR amplification, the products were cloned and sequenced.

3.4. Results

3.4.1. Isolation of A. thaliana P5CS gene

A genomic DNA library of A. thaliana (Landsberg) was screened using Vigna P5CS cDNA as a probe. Fourteen positive clones were obtained after screening 200,000 plaques. Restriction enzyme digestion showed that although the insert length of most of these clones varied, all contained the same Sac I - Xho I fragment (~7.5 kb) with respect to the polymorphism of restriction enzyme sites. This fragment hybridized to the Vigna P5CS cDNA, and was partially sequenced. The homology between the P5CS genes of A. thaliana, ecotype Landsberg (this work) and A. thaliana, ecotype Columbia (Yoshiba et al., 1995; Savoure et al., 1995) is about 90% in exon regions in terms of both nucleotide sequence and deduced amino acid sequence. No homology was found between the two genes.
The intron regions are indicated with lowercase letters. The promoter and exon regions are shown in uppercase letters. The AtP5CS gene has two transcription start sites mapped by primer extension (see "Materials and Methods"). The putative upstream transcription start site is indicated by an arrow (↓) while the downstream transcription site is indicated by an open triangle (▲). The first nucleotide of the AtP5CS cDNA, obtained by 5' end RACE, is indicated by a filled triangle (●). The first nucleotide of coding region of the AtP5CS gene is designated as the nucleotide +1. The upstream start site starts about 14 bases upstream of the first nucleotide of the P5CS cDNA while the downstream start site starts 62 bases downstream of the first nucleotide of the cloned cDNA. A TATA-like sequence is underlined. The nucleotide sequence alignment of the first two exons and the first intron of the P5CS genes is also shown in this figure. The comparison includes the P5CS gene (AtP5CS, this work) isolated from A. thaliana, ecotype Landsberg, the P5CS gene (p5csB) of A. thaliana, ecotype Columbia, reported by Strizhov et al. (unpublished, GenBank, Accession number: X86778), and the P5CS gene (AtP5S) of A. thaliana, ecotype Columbia, isolated by Savoure et al. (1995). The dash lines indicate identical nucleotides. The asterisks indicate the absence of nucleotides.

Figure 17. Nucleotide sequence of the part of the promoter region, first two exons, and first intron of the P5CS gene of A. thaliana (Landsberg).
in intron regions (Fig. 17), suggesting that the AtP5CS gene characterized here is
different from that described previously by Yoshiba et al. (1995) and Savoure et
al. (1995). The nucleotide sequence of the P5CS promoter region, the first two
exons, and first intron is shown in figure 17.

3.4.2. AtP5CS gene has two transcription start sites

To determine the transcription start site of the AtP5CS gene the 5' end of
Arabidopsis (Landsberg) P5CS cDNA was cloned using 5' end RACE, and the
primer extension was performed. The cloned cDNA showed 100% identity with
its genomic DNA in exon regions. Interestingly, the first primer extension
reaction indicated that the primary transcription site is inside the untranslated
region downstream of the first nucleotide of the AtP5CS cDNA (Fig. 17, Fig.
18A). In the second primer extension reaction, total RNA from dehydrated plants
was used, and the gel was analyzed by a PhosphorImager (which has a higher
sensitivity than X-ray film). The result demonstrated that the AtP5CS gene has
two transcription start sites (Fig. 18B). One site starts about 14 bases upstream
of the first nucleotide of the AtP5CS cDNA obtained by 5' end RACE while the
other starts 62 bases downstream of the first nucleotide of the cloned cDNA (Fig.
17 and Fig. 18B). Dehydration stimulated the transcription from both sites, but
the induced transcription was much stronger from the downstream site than the
upstream site (Fig. 18B).
A, total RNA (20 μg) isolated from *A. thaliana* (Left lane) was used for primer extension. The primer extension product, indicated by an arrow on the left, corresponds to the transcription start site indicated by an open triangle in Figure 17. The sequence ladder (Lanes a, c, g, t) is from a sequencing reaction with the same primer as that used in the primer extension. The nucleotide "A", which is at the same position as the primer extension product, corresponds to the nucleotide "T" indicated by an open triangle in Figure 17. B, PhosphoImage analysis of primer extension products. Total RNA (20 μg) isolated from water-stressed *A. thaliana* (Lane 1) or control plants (Lane 2) was used for primer extension. The bottom arrow on the left points at the extension product from the downstream transcription start site indicated by an open triangle (V) in Figure 17. The top arrow on the left points at the extension product corresponding to the upstream transcription start site indicated by an arrow (↓) in Figure 17. The size (bp) of the DNA markers (Lane 3) is indicated on the right.

Figure 18. Mapping of the transcription start site of *Arabidopsis P5CS* gene.
3.4.3. Induction of AtP5CS gene expression by dehydration, NaCl, and other factors

To investigate the expression of the AtP5CS gene in plants under stress conditions a chimeric gene construct carrying the AtP5CS promoter was fused to a GUS reporter gene and introduced into *Arabidopsis* and tobacco. Stable transgenic lines were obtained and analyzed. The GUS activity increased rapidly in 6 hr and continued to increase up to 24 hr in both transgenic *Arabidopsis* and tobacco subjected to dehydration (Fig. 19A). The transgenic *Arabidopsis* showed much higher GUS activity than transgenic tobacco, but the trends of induction of the gene expression in both plants under dehydration were very similar. Figure 19B presents the GUS activity of five independent transgenic lines of *Arabidopsis* (T2) and three independent transgenic lines of tobacco (T1) dehydrated for 12 hours. A 2- to 4-fold increase in GUS activity was observed in both transgenic plants.

The effects of NaCl stress on AtP5CS gene expression were studied in transgenic *Arabidopsis* plants treated with 250 mM NaCl by measuring GUS activity before and after the stress. The GUS activity increased rapidly within 3 hr after the treatment with NaCl. Five independent transgenic lines of *Arabidopsis* (T2) were used in the assay, and all of them showed induction by NaCl (Fig. 20). The enhanced level of GUS activity by NaCl is similar to that by dehydration.

We have earlier demonstrated that plants under drought stress accumulated more proline in nitrogen-rich soil than in nitrogen-poor soil (Kishor et al., 1995), and the P5CS enzyme is feed-back inhibited by proline (Zhang et al., 1995). To test whether the nitrogen level has any effects on AtP5CS gene expression, the
The leaves of transgenic tobacco (T1) and transgenic *Arabidopsis* plants (T2) were used in these experiments. Dehydration treatment was performed by drying transgenic *Arabidopsis* plants or tobacco leaves in a Petri dish at 23 °C and 60% humidity under dim light. A, Time course of GUS activity in dehydrated transgenic plants. *Arabidopsis* plants of T2-2 transgenic line and tobacco leaves of T1-2 transgenic line were used in this experiment. The Y axis on the left represents the GUS activity of transgenic tobacco while the Y axis on the right represents the GUS activity of transgenic *Arabidopsis*. B, Five independent transgenic lines of *Arabidopsis* (T2) and tobacco leaves (~ 9 cm²) of three independent transgenic lines (T1) were dehydrated for 12 hr. The measurement of GUS activity for a individual plant or leaf was repeated 2-3 times. The GUS activity for each transgenic line is the average of three individual *Arabidopsis* plants or leaves from three transgenic tobacco plants. Error bars represent the standard deviation.

Figure 19. Induction of the AtP5CS gene expression by dehydration.
Five independent transgenic lines of *Arabidopsis* plants (T2) were treated with 250 mM NaCl for three hr, and GUS activity was measured as the way mentioned in the legend of figure 19. The data for each transgenic line is the average of three individual *Arabidopsis* plants. Error bars represent the standard deviation.

Figure 20. Induction of the AtP5CS gene expression by NaCl.
transgenic *Arabidopsis* plants were treated with 5 mM of glutamine for 24 hours. The result showed that glutamine has no effects on the gene expression (data not shown). Transgenic *Arabidopsis* plants treated with 10 mM of proline for 24 hours showed no decrease of GUS activity (data not shown). GUS activity of transgenic *Arabidopsis* growing under low temperature (4 °C) for 48 hours was also measured, but no induction of the AtP5CS gene expression was observed.

### 3.4.4. The expression of AtP5CS gene is not affected by external ABA

It has been reported that P5CS transcripts and proline accumulated in *Arabidopsis* plants treated with external ABA (Yoshiba *et al.*, 1995; Savoure *et al.*, 1997). To investigate the ABA effects on the gene expression, we monitored GUS activity in transgenic *Arabidopsis* plants treated with external ABA ranging from 0.1 to 1.0 mM. Figure 21 presents a typical result of ABA treatment. Exogenous ABA failed to show any induction of the AtP5CS gene expression.
The plants of three independent transgenic lines of *Arabidopsis* (T2) were grown hydroponically in water with or without 1 mM ABA for indicated times. GUS activity was measured using crude extracts. The data of each transgenic line is the average of four plants. ABA showed no effects on the AtP5CS gene expression.

Figure 21. Effects of ABA on the induction of the AtP5CS gene expression.

3.5. Discussion

We isolated a P5CS gene from a genomic DNA library of *A. thaliana*. All 14 clones isolated from the library contained the same *Sac I - Xho I* fragment, although the lengths of the inserts varied. It was reported that there is only one copy of the P5CS gene in *Arabidopsis* (Yoshiba *et al.*, 1995; Savoure *et al.*, 1995), but as documented by Strizhov *et al.*, a second P5CS gene is present in *Arabidopsis* (unpublished, GenBank, Accession number: X86778). The second P5CS gene, isolated from *A. thaliana*, ecotype Columbia, showed about 90% homology to the first one (Savoure *et al.*, 1995) in the exon regions and no homology in the intron regions. However, the second P5CS gene cloned by
Strizhov showed more than 98% homology to the AtP5CS gene from Landsburg (this work) in terms of nucleotide sequence including the exon and intron regions (Fig. 17). Thus, the P5CS gene we have isolated is a different P5CS gene from that characterized by Savoure et al. (1995).

There is no apparent TATA box near any of the transcription start sites although a TATA-like sequence is found at 447 bp upstream of the nucleotide +1 (Fig. 17). Several TATA-less promoter for class II genes have been reported earlier (Smale and Baltimore, 1989; Blackshear, 1993), but there does not appear to be any common features shared among these genes. One of the most intriguing features of the AtP5CS gene is that it has two transcription start sites as mapped by primer extension (Fig. 18B). Since the P5CS cDNA sequence, amplified and isolated directly from total RNA of *Arabidopsis*, matches perfectly with that of genomic clone, it is unlikely that the cDNA sequence is artifactual due to some sort of DNA rearrangement during cloning. Although there are two copies of the P5CS gene in *Arabidopsis*, we believe that the two primer extension products in figure 18B correspond to the two transcription start sites of the same P5CS gene since the 5' end cDNA sequences (two longer and one shorter ones, this work) showed 100% identity with this gene and both P5CS genes, reported earlier (Savoure et al., 1995) and isolated in this work, have no homology in over 100 bp of untranslated nucleotide at the 5' end of the cDNAs. A carrot gene encoding a hydroxyproline-rich glycoprotein was found to have two transcription start sites (Chen and Vaener, 1985). Wounding induced transcription from both sites. A barley gene, HVA22, was also shown to have two transcription start sites; the downstream one was inside the 5' untranslated region of the cDNA and was used frequently while the upstream one was very rarely used (Shen et al., 1993). It is
clear that the AtP5CS gene expression was induced by dehydration from both transcription start sites, but the downstream one was frequently used (Fig. 18B).

To investigate the expression of the AtP5CS gene in plants under stress conditions, the AtP5CS promoter region was fused with the GUS reporter gene, and the chimeric construct was introduced into *Arabidopsis* and tobacco. The GUS activity was enhanced in transgenic plants dehydrated for 24 hours. A 2- to 4-fold increase of GUS activity was observed in both transgenic *Arabidopsis* and transgenic tobacco (Fig. 19). These results are consistent with the primer extension results (Fig. 16B) and the RNA blotting results reported earlier that the level of the P5CS transcripts is enhanced in *Arabidopsis* plants following water stress (Yoshiba *et al.*, 1995). Similar results were observed by NaCl stress (Fig. 20). These results showed that the P5CS gene expression is regulated at the level of transcription by water stress. Glutamine, proline and low temperature showed no induction or depression of the AtP5CS gene expression. Proline regulates its own biosynthesis only at the enzyme level. The above results suggested that the induction of the AtP5CS gene expression is the response of plants specifically to water and salt stresses.

Recent studies have shown that in *Arabidopsis* the proline dehydrogenase (PDH) gene expression is induced by proline and reduced by osmotic stress (Peng *et al.* 1996; Verbruggen *et al.* 1996; Kiyosue *et al.* 1996). In *Arabidopsis* plants under stress, the P5CS gene transcripts is enhanced while the PDH gene transcripts is reduced. After the removal of the stress, the PDH gene expression is induced while the P5CS gene expression is reduced. This reciprocal regulation of the levels of the P5CS and PDH controls proline levels during and after osmotic stress in plants. These data suggested that accumulation of proline in
plants under osmotic stress needs an increase in proline synthesis and a decrease in proline degradation. The above information also suggests that proline accumulation during osmotic stress is an essential adaptive response to these conditions in plants. Rapid oxidation of proline is an equally important process in recycling the accumulated free proline and providing reducing power, amino nitrogen, and energy in the restoration of cellular homeostasis during recovery from osmotic stress. Thus, proline cycle (Verma and Zhang, 1997; Fig. 6) helps plants in storing and providing necessary energy and nitrogen as needed.

ABA has been proposed to be a mediator of plant responses to drought (Cohen and Bray, 1990; Gost et al., 1995; Iwasaki et al., 1995), salt (Yoshiba et al., 1995; Bostock and Quatrano, 1982) and cold (Lang and Palva, 1992; Nordin et al., 1993; Nordin et al., 1991). Many genes (rab) responsive to ABA and stress are controlled at the transcriptional levels. However, the studies have shown that the expression of rab genes is also controlled by a variety of other processes and that the changes of ABA levels are not always related to the changes in gene expression (Nordin et al., 1991; Espelund et al., 1992; Vilardell et al., 1994). Kirch et al. reported that the promoter activity of a cold and ABA responsive gene, cit, of potato was not induced in transgenic potato by cold, although the cit transcripts were induced in response to cold, drought, and ABA (Kirch et al., 1997). The authors suggested that the cold-induced expression of the cit gene in potato may be accounted for by post-transcriptional control mechanisms (Kirch et al., 1997). It has been reported that the levels of the P5CS transcript and proline in Arabidopsis were enhanced by the application of exogenous ABA (Yoshiba et al., 1995; Savoure et al., 1997). In our studies, the transgenic Arabidopsis plants treated with external ABA from 0.1 to 1.0 mM...
failed to show any increase of GUS activity compared with untreated plants (Fig. 21). However, we have no date to exclude the possibility that some regulatory elements may not be included in the cloned promoter region. It appears that both P5CS genes in *Arabidopsis* are not inducible by ABA as shown by studies in the reference (Savoure *et al.*, 1997) and our results presented here. The characterization of ABA-biosynthetic mutant under stress conditions revealed that the induction of a given gene by external ABA does not necessarily imply that this gene is regulated by endogenous ABA (Giraudat *et al.*, 1994). It is likely that the *AtP5CS* gene expression is not mediated directly by ABA, but ABA might be involved in post-transcriptional regulation of the *AtP5CS* gene expression and accumulation of proline as proposed by Savoure *et al.* (1997), suggesting the presence of ABA-independent and ABA-dependent regulation of the *AtP5CS* gene expression. Moreover, the accumulation of proline depends on the reciprocal regulation of the P5CS and proline dehydrogenase genes (Peng *et al.*, 1996; Kiyosue *et al.*, 1996; Verbruggen *et al.*, 1996). The latter may be regulated by ABA.
CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

Proline is thought to play a cardinal role as an osmoregulatory solute in plants subjected to hyperosmotic stress, primarily drought and soil salinity. Indeed, the accumulation of this imino acid might be part of a general adaptation to adverse environmental conditions, as documented in many studies (Delauney and Verma, 1993). The question remains whether proline accumulation provides an adaptive advantage to plant cells under stress conditions or is merely a secondary response effected by stress-induced changes in metabolism. The evidence that proline accumulation in stressed plants is the result of a increase in proline synthesis trigged the intense studies of proline pathway. The isolation of various genes involved in proline pathways provided a clear picture of proline metabolism, but the regulation of proline synthesis is not uncovered, and factors controlling the gene expression and the enzyme activity need to be explored.

The aim of my dissertation research was to understand the regulation of the gene expression and activity of the key enzyme, the P5CS, in proline biosynthetic pathway. The reason of choosing the P5CS is based on the preliminary data created in Dr. Verma's group that the P5CS is involved in the primary pathway of proline synthesis, and it might be the rate-limiting enzyme
controlling the rate of proline synthesis. The up-regulation and down-regulation
of the P5CS gene expression and enzyme activity may be corresponding to the
changes of environmental conditions, reflecting the functions of proline
accumulation in plants under stress conditions.

I studied the regulation of proline biosynthesis in both the P5CS gene
expression and the P5CS enzyme activities. The following results from this study
represent major progresses. The γ-GK activity of *Vigna* P5CS is feedback
inhibited by proline and ADP, while the GSA dehydrogenase activity is not
inhibited by proline. Thus, the γ-GK domain of the P5CS is the rate-limiting step
in proline synthesis. The *Km* values of the P5CS for glutamate is similar to that
of GS, but 10-fold less than that of *E. coli* γ-GK (Smith *et al.*, 1984), suggesting
that proline synthesis in plants is much more sensitive to the change of glutamate
concentration and further to the change of nitrogen level in soil. The level of the
P5CS protein was enhanced in *Vigna* roots treated with NaCl. Two amino acid
residues involved in proline binding of the P5CS were identified, and a
substitution (P5CSF129A) of one of these two residues greatly reduced the
proline inhibition, while other properties of the enzyme remained unchanged. The
P5CS gene expression was enhanced by water stress and salt stresses.

The previous results obtained in our lab showed that in plants subjected to
stress or nitrogen starvation (Delauney *et al.*, 1993), the OAT transcript level was
significantly depressed concomitant with the elevation of the P5CS transcript
level. Conversely, in plants under normal condition or excess nitrogen condition,
the P5CS transcript was significantly depressed concomitant with the elevation of
the OAT transcript level (Delauney *et al.*, 1993). Several recent studies have
shown that in *Arabidopsis* the transcripts of proline dehydrogenase (PDH), the
first enzyme in proline degradation pathway (Table 3, Eq. 10), is induced by proline and reduced by osmotic stress (Peng et al. 1996; Verbruggen et al. 1996; Kiyosue et al. 1996). In Arabidopsis plants under stress, the expression of the P5CS gene is enhanced while the expression of the PDH gene is reduced. After the removal of the stress, the PDH gene expression is induced while the P5CS gene expression is reduced. This reciprocal regulation of the levels of the P5CS and the PDH controls proline levels during and after osmotic stress in plants.

Taken all together, the above information strongly supports the idea that the proline accumulation in stressed plants provides an adaptive advantage to plant cells. In plants, glutamate pathway for proline synthesis can be considered to reflect the major biosynthetic pathway since it is controlled by the end product, proline, but ornithine pathway for proline synthesis is not regulated by proline. The P5CS is the rate-limiting enzyme, and its production and activity are regulated kinetically in three ways. First, under normal physiological condition and excess nitrogen condition, low level expression of the P5CS gene results in a trace amount of the enzyme leading to a reduced competition between the P5CS and the GS for glutamate (the $K_m$ value of P5CS for glutamate is similar to that of GS), and most of glutamate is converted to glutamine for the purpose of nitrogen assimilation. The demand for proline is low. The increase of OAT gene expression under above conditions may be the results of a large pool of ornithine which is mainly converted to arginine for nitrogen storage (Fig. 22). The formation of ornithine is controlled by arginine. Under stress conditions (high salinity and low water), increase of the P5CS gene expression is needed for more P5CS enzyme, resulting in accumulation of proline. It is likely that nitrogen level regulates the cross-talk between arginine and proline biosynthetic pathways. The
second control involves the inhibition of γ-GK activity of the P5CS by ADP. Regulation at this level would make proline synthesis responsive to cellular energy level. Finally, the P5CS activity is controlled by the end-product, proline. This point of control is the most important since this control ensures that no excess proline production occurs, if not needed. Some earlier experiments suggested that proline accumulation in plants under stress may involve the loss of feedback regulation due to the conformation change of the P5CS (Boggess et al. 1976a; 1976b). Our results of P5CS mutagenesis showed that the reduction of proline inhibition can be achieved without changing the normal catalytic properties of the enzyme.

The thicker arrows represent a increase of metabolism of indicated pathways.

Figure 22. The relationship between proline synthesis and nitrogen assimilation in plants under different conditions.
By monitoring the content of MDA, Peng (1997) has been demonstrated that NaCl stress causes substantial lipid peroxidation in the tobacco cell culture. Therefore, osmotic stress, like many other stress, causes oxidative damages and triggers proline accumulation in plants, and it is possible that free radicals are the agents which induce the proline accumulation. In vitro experiments have shown that proline scavenges free radicals (Smirnoff and Cumbes, 1989; Trelstad et al., 1981)
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