

Abstract

Raffinose family oligosaccharides (RFO), raffinose and stachyose, accumulating during seed development are thought to play some roles in desiccation tolerance of seeds. However, functions of RFO in drought tolerance of plants have not been elucidated. Here, I examined the functions of RFO in *Arabidopsis thaliana* plants under drought and cold stress conditions based on the analyses of function and expression of genes involved in RFO biosynthesis. Sugar analysis showed that drought-, high-salinity- and cold-treated *Arabidopsis* plants accumulate a large amount of raffinose and galactinol, but not stachyose. Raffinose and galactinol were not detected in unstressed plants. This suggests that raffinose and galactinol are involved in the tolerance to drought, high-salinity and cold stresses. Galactinol synthase (Gols) catalyzes the first step in the biosynthesis of RFO from UDP-galactose. I identified three stress-responsive *Gols* genes (*AtGols1*, 2 and 3) among 7 *Arabidopsis Gols* genes. *AtGols1* and 2 were induced by drought and high salinity stresses but not by cold stress. By contrast, *AtGols3* was induced by cold stress but not by drought or salt stress. All the GST fusion proteins of GST-*AtGols1*, 2 and 3 expressed in *Escherichia.coli* had galactinol synthase activities. Overexpression of *AtGols2* in transgenic *Arabidopsis* caused an increase in endogenous galactinol and raffinose, and showed reduced transpiration from leaves to improve drought tolerance. These results show that stress-inducible galactinol synthase plays a key role in the accumulation of galactinol and raffinose under abiotic stress conditions, and that galactinol and raffinose may function as osmoprotectants in drought-stress tolerance of plants.

Introduction

Plant growth is greatly affected by environmental abiotic stresses, such as drought, high salinity and low temperature. Plants respond and adapt to these stresses in order to survive under stress conditions. Among these abiotic stresses, drought or water deficit is the most severe limiting factor against plant growth and crop production.

Water deficit stress induces various biochemical and physiological responses in plants. It is known that higher plants have sophisticated mechanism for adaptation against drought and salt stress such as stomata closure, accumulation of salt into vacuole, succulent and cuticularization of leaf to protect cells from these stresses. Various osmoprotectants and antioxidants accumulates during water stress in plant cells. Recently, a number of genes have been shown to be induced to drought at the transcriptional level (Ingram and Bartels, 1996, Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1996, 1997 and 1999). Their gene products are thought to function in stress tolerance and response. Recently, stress-inducible genes were used to improve stress tolerance of plants by gene transfer. I think it is important to analyze functions of stress-inducible genes not only for further understanding of molecular mechanisms of stress tolerance and response of higher plants but also for improvement of stress tolerance of crops by gene manipulation.

The plant hormone abscisic acid (ABA) is produced under water deficit conditions and plays important roles in response and tolerance to dehydration. Most of the genes that have been studied to date are also induced by ABA (Giraudat *et al.*, 1994). It appears that dehydration triggers the production of ABA, which, in turn, induces various genes. Several reports have described genes that are induced by dehydration but are not responsive to exogenous ABA treatment. These findings suggest the existence of ABA-independent as

well as ABA-dependent signal-transduction cascades between the initial signal of drought stress and the expression of specific genes (Ingram and Bartels, 1996, Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1996, 1997 and 1999). To understand the molecular mechanisms of gene expression in response to drought stress, *cis*- and *trans*-acting elements that function in ABA-independent and ABA-responsive gene expression by drought stress have been precisely analyzed. A variety of transcription factors are involved in stress responsive gene expression, which suggests the involvement of complex regulatory systems in molecular responses to drought stress.

Various genes respond to drought stress in various species, and functions of their gene products have been predicted from sequence homology with known proteins. Many drought-inducible genes are also induced by salt stress and low temperature, which suggests the existence of similar mechanisms of stress responses. Genes induced during drought-stress conditions are thought to function not only in protecting cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the drought stress response (Ingram and Bartels, 1996, Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1997 and 1999). Thus, these gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance, such as chaperones, LEA (late embryogenesis abundant) proteins, osmotin, antifreeze proteins, mRNA binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes and various proteases. The second group contains protein factors involving in further regulation of signal transduction and gene expression that probably function in stress response: protein

kinase, transcription factors and enzymes in phospholipid metabolism (Bohnert *et al.*, 1995, Ingram and Bartels, 1996, Bray, 1997, Shinozaki and Yamaguchi-Shinozaki, 1997 and 1999).

Recently, several different approaches were attempted to improve stress tolerance of plants by gene transfer of stress-inducible genes (Holmberg and Bulow, 1998). Stress-inducible genes for functional proteins such as key enzymes for osmolyte biosynthesis, LEA proteins and detoxification enzymes were overexpressed in transgenic plants to produce stress tolerant phenotype of the plants, which indicates that their gene products really function in stress tolerance. Among numerous stress-responsive traits in higher plants, many scientists have been interested in accumulation of osmolytes, the organic osmotic solutes, in response to drought and salinity. The accumulation of osmolytes during osmotic stress due to drought and salinity is a ubiquitous biochemical mechanism in all organisms from bacteria, fungi and algae to vascular plants and animals. The accumulated osmolytes include amino acid, their derivatives (proline, glycine betain, beta-alanine betain, proline betain), tertialy amines, sulfonium compounds (choline o-sulfate, dimethylsulfoniopropionate), sugars and polyols (glycerol, mannitol, sorbitol, trehalose, fructans, and methylated inositol) (Bohnert and Jensen, 1996a, Bohnert and Jensen, 1996b, Hanson *et al.*, 1994, McCue and Hanson, 1990, Trossat *et al.*, 1996). Advantages of these organic osmolytes are, (1) a compatibility with macromolecular structure and function at high or variable (or both) osmolyte concentrations, (2) little effect on various proteins to function in concentrated intracellular solutions. Recently, osmolytes are thought to function not only in osmotic adjustment but also in protection of cells and macromolecules, such as

maintaining membrane integrity, preventing protein denaturation, protection against oxidative damage by scavenging free radicals and lowering of T_m value of the nucleic acid (Smirnoff and Cumbes, 1989, Shen *et al.*, 1997, Crowe *et al.*, 1987, Incharoensakdi *et al.*, 1986, Nomura *et al.*, 1995, Nomura *et al.*, 1998, Rajendrakumar *et al.*, 1997, Schobert and Tschesche, 1978). Furthermore, glycine, betaine, mannitol, ononitol, trehalose, fructan and proline have been shown to be important for the improvement of stress tolerance in plants by the manipulation of genes encoding key enzymes of osmolyte synthesis or degradation pathway (Hayashi *et al.*, 1997, Sakamoto *et al.*, 1998, Lilius *et al.*, 1996, Takabe *et al.*, 1998, Nuccio *et al.*, 1998, Tarczynski *et al.*, 1993, Thomas *et al.*, 1995, Sheveleva *et al.*, 1998, Holmström *et al.*, 1996, Pilon-Smits *et al.*, 1995, Kavi Koshor *et al.*, 1995, Nanjo *et al.*, 1999a, Nanjo *et al.*, 1999b).

Recently, it has been suggested that raffinose family oligosaccharides (RFO), raffinose and stachyose, have the similar function as osmolyte. RFO accumulate during seed development and are thought to play some roles in desiccation tolerance of seeds. Concretely, RFO accumulate at the late stage of maturation and desiccation process of soybean seeds (Saravitz *et al.*, 1987, Castillo *et al.*, 1990). In maize, raffinose accumulates during the seed desiccation process and is thought to function in stress tolerance, whereas sucrose accumulates independently of desiccation tolerance. Desiccation tolerance of seeds is not achieved in the absence of raffinose accumulation (Brenac *et al.*, 1997). These results suggest that the ratio of sucrose to RFO is critical for desiccation tolerance of seeds rather than the total amount of sugars. Though young excised soybean seeds are not tolerant to desiccation, but slow dehydration induces stress tolerance, which is strongly

correlated with a significant increase in stachyose content (Blackman *et al.*, 1992).

Figure 1a shows the metabolic pathway of RFO in plants. Galactinol synthase (GolS) catalyzes the first committed step in the biosynthesis of RFO and plays a key regulatory role in carbon partitioning between sucrose and RFO (Saravitz *et al.*, 1987). Therefore, GolS potentially catalyzes a metabolic key step and its gene provides an experimental tool to manipulate the level of RFO in seeds or vegetative tissues to analyze the function of RFO as osmoprotectants. The GolS activity in kidney bean seeds increases upon exposure of plants to cold, and the expression of *GolS* genes is induced by cold stress in *Arabidopsis* and *Ajuga reptans* plants (Liu, J. J. *et al.*, 1998, Sprenger and Keller, 2000). During cold acclimation, RFO accumulate in leaves of *Ajuga reptans* (Bachmann *et al.*, 1994). However, functions of RFO in stress tolerance and roles of GolS in RFO biosynthesis have not been elucidated.

At least 7 *GolS* related genes are found in the *Arabidopsis* genome, but little is known about their roles in the accumulation of galactinol and raffinose in plants under water-deficit conditions. In the present study, I report that three *Arabidopsis GolS* genes, *AtGolS1*, 2 and 3, are differentially induced by drought, low temperature, high-salinity and ABA. I showed that their gene products have galactinol synthase activity. Furthermore, transgenic plants that overexpressed the *AtGolS* cDNA showed improved drought tolerance. This study provides a direct evidence that the stress-inducible *GolS* gene controls the level of RFO, and that galactinol and raffinose play important roles in drought-stress tolerance.