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Proline Accumulation and Methylation to Proline Betaine in *Citrus*: Implications for Genetic Engineering of Stress Resistance

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Abstract. Proline and various betaines can function as osmoprotectants and cryoprotectants when accumulated in the cytoplasm of cells. Genetic engineering can raise levels of these compounds and thereby improve stress resistance; *Citrus* species are potential candidates for this. Before attempting such engineering, it is necessary to characterize the natural osmoprotectants of *Citrus* and related genera. We therefore surveyed 55 cultivated and wild species of the Aurantioideae, analyzing proline and betaines in leaves of mature trees. Some citrus relatives accumulated proline alone; others accumulated proline and proline betaine, as did all *Citrus* species studied. The levels of these two compounds ranged from about 20 to 100 μmol·g^{-1} dry mass, and were significantly inversely correlated. Proline betaine is known to be synthesized from proline and to be a better osmoprotectant. Because *Citrus* species all have more proline than proline betaine, there is scope for engineering more of the latter. Many species had small amounts of hydroxyproline betaine; other betaines were essentially absent. The lack of other betaines means that it would also be rational to engineer the accumulation of glycine betaine or similar compounds.

Cultivated citrus species are cold tender and salt sensitive, and citrus breeding has long sought to alleviate these defects (Maas; 1993; Yelenosky, 1985). While there are sources of cold and salt tolerance within the citrus gene pool, incorporating these traits into scion or rootstock cultivars is slow and difficult by conventional breeding methods (Grosser and Gmitter, 1990; Yelenosky, 1985; Young et al., 1982). The various obstacles to conventional citrus breeding make genetic engineering an attractive alternative; it is now practical because methods for genetic transformation of citrus have been developed (Moore et al., 1993).

One promising engineering approach to salt and freeze tolerance is to increase the cytoplasmic levels of small molecules with osmoprotectant properties (Bartels and Nelson, 1994; Bohnert and Jensen, 1996). These osmoprotectants—also termed compatible solutes—include polyols, proline, betaines (fully *N*-methylated amino acid derivatives), and the sulfonium compound 3-dimethylsulfoniopropionate (DMSP) (Yancey, 1994). Figure 1 shows the structures of some of these. Engineering the accumulation of mannitol, proline, or glycine betaine has been reported to increase salt tolerance in tobacco (*Nicotianum tabacum* L.) (Kavi Kishor et al., 1995; Lilius et al., 1996; Tarczynski et al., 1993) and in various model microorganisms (e.g., Csonka, 1989; Nomura et al., 1995). The impact of engineered accumulation of these compounds on freeze hardiness has not been assessed, but all osmoprotectants have cryoprotectant activity and so can reduce damage from freeze-induced dehydration (Carpenter and Crowe, 1988; Withers and King, 1979).

To plan a genetic engineering strategy for osmoprotectants in citrus, it is essential to know the types and amounts of such compounds that are present naturally. It is also instructive to know whether citrus relatives—especially stress-tolerant ones—differ from commercial cultivars in types or amounts of osmoprotectants. While the literature on citrus osmoprotectants is limited to just a few cultivars, it clearly establishes the importance of nitrogenous compounds. Thus, many studies have shown that unstressed citrus leaves have high levels of free proline, and that cold, salinity, or

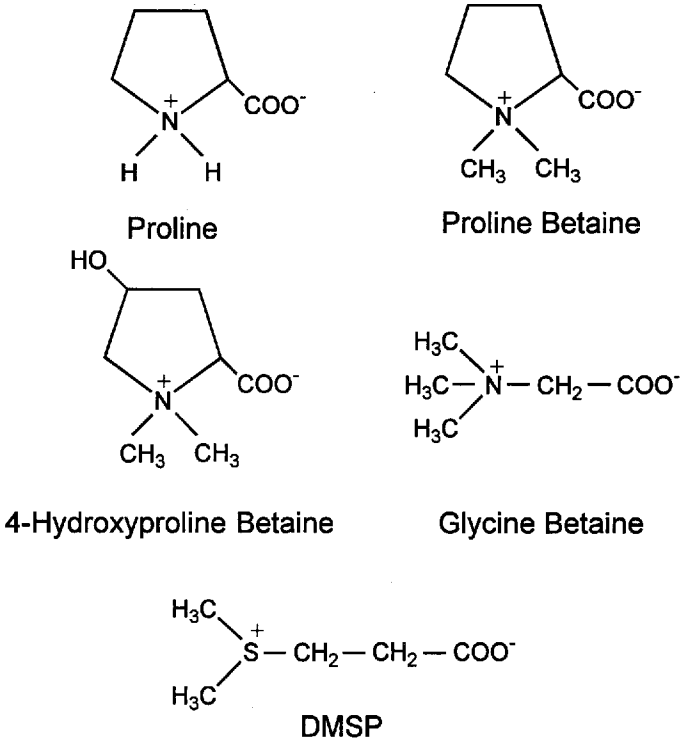

Fig. 1. Structures of some osmoprotectants found in higher plants.

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Table 1. Distribution of the accumulation of proline, proline betaine (ProBet) and hydroxyproline betaine (HypBet) in the subfamily Aurantioideae and other species of Rutaceae.

Subfamily	Tribe	Subtribe	Genus and species[z]	Proline[y]	ProBet[x]	HypBet[x]
Rutoideae	Xanthoxyleae	Evodiinae	*Zanthoxylum fagara* f	+	–	–
			Zanthoxylum clava-herculis f	+	–	–
Toddalioideae	Toddalieae	Toddaliinae	*Casimiroa edulis* f	–	–	–
			Casimiroa tetrameria f	+	–	–
			Skimmia japonica n	+	–	–
		Amyridinae	*Amyris balsamifera* f	–	–	–
Aurantioideae	Clausenae	Micromelinae	*Micromelum minutum* n	+	–	–
		Clauseninae	*Glycosmis pentaphylla* f	+	–	–
			Clausena lansium f	+	+	–
			Murraya paniculata f	+	–	–
			Murraya koenigii f	+	–	–
		Merilliinae	*Merillia caloxylon* n	+	–	–
	Citreae	Triphasiinae	*Wenzelia dolichophylla* n	–	–	–
			Monanthocitrus cornuta h	–	–	–
			Merope angulata h	+	–	–
			Triphasia trifolia f	+	+	+
			Pamburus missionis f	+	–	–
			Luvunga scandens h	–	–	–
			Paramignya scandens n	+	–	–
			Paramignya lobata n	–	–	–
		Citrinae	*Severinia buxifolia* f	+	–	–
			Severinia disticha f	+	–	–
			Pleiospermium latialatum n	+	–	–
			Pleiospermium sp. n	+	–	–
			Burkillanthus malaccensis h	–	–	–
			Limnocitrus littoralis n	+	+	+
			Hesperethusa crenulata f	+	–	–
			Citropsis gilletiana f	+	–	–
			Atalantia sp. f	+	–	–
			Fortunella japonica f	+	+	+
			Fortunella margarita f	+	+	+
			Fortunella hindsii f	+	+	+
			Eremocitrus glauca f	+	+	+
			Poncirus trifoliata f	+	+	+
			Clymenia polyandra n	+	+	–
			Microcitrus australasica f	+	+	–
			Microcitrus australis f	+	+	+
			Microcitrus warburgiana f	+	+	+
			Citrus medica f	+	+	+
			Citrus limon f	+	+	+
			Citrus aurantifolia f	+	+	+
			Citrus aurantium f	+	+	+
			Citrus sinensis f	+	+	+
			Citrus reticulata f	+	+	+
			Citrus grandis f	+	+	+
			Citrus paradisi f	+	+	+
			Citrus indica f	+	+	+
			Citrus tachibana f	+	+	+
			Citrus ichangensis f	+	+	+
			Citrus latipes n	+	+	–
			Citrus micrantha n	+	+	–
			Citrus celebica n	+	+	–
			Citrus macroptera f	+	+	–
			Citrus hystrix f	+	+	–
		Balsamocitrinae	*Swinglea glutinosa* f	+	–	–
			Aegle marmelos f	+	+	+
			Afraegle paniculata f	+	+	–
			Afraegle gabonensis f	+	+	+
			Balsamocitrus dawei n	+	+	+
			Feronia limonia f	+	–	–
			Feroniella oblata n	+	+	–

[z]Of scion. Binomials only are given; authorities are according to Swingle and Reece (1967). n = National Clonal Germplasm Repository; f = Florida Citrus Arboretum; h = herbaria.
[y]Free proline levels ≥8 μmol·g^{-1} dry mass.
[x]Levels ≥0.5 μmol·g^{-1} dry mass.

drought promote further accumulation (e.g., Bañuls and Primo-Millo, 1992; Yelenosky, 1985). It has also been shown that leaves of several species or hybrids accumulate proline betaine, and that proline betaine levels increase in response to salinity or water deficit and vary among genotypes (Duke et al., 1986; Honda, 1990; Lloyd et al., 1989, 1990). Other betaines have not been found (Lloyd et al., 1989).

The occurrence of varying amounts of proline betaine is especially noteworthy because evidence from bacteria shows that it is a superior osmoprotectant to proline (Amin et al., 1995; Hanson et al., 1994; LeRudulier et al., 1984) and because it is synthesized from proline in just two enzymatic steps (successive *N*-methylations) (Essery et al., 1962). Also, chemosystematic evidence from the Plumbaginaceae indicates that the evolution of proline conversion to proline betaine was associated with an advance in stress tolerance (Hanson et al., 1994).

The above considerations led us to focus on nitrogenous osmoprotectants and to survey the levels of proline, proline betaine, and other betaines in *Citrus* species and in representative species from almost all the other genera of the orange subfamily (Aurantioideae) of the Rutaceae. Fast atom bombardment mass spectrometry (FABMS) was used to analyze betaines because this method is highly sensitive and selective for these compounds (Rhodes et al., 1987).

Materials and Methods

PLANT MATERIALS. Our nomenclature and taxonomy follow those of Swingle and Reece (1967). The trees sampled were from the Florida Citrus Arboretum (Winter Haven) and the National Clonal Germplasm Repository for Citrus (Riverside, Calif.). *Citrus* species from California were greenhouse grown; all other species were field grown. Irrigation and fertilization were according to standard management practices. Trees were 4 to 20 years old, except for *Limnocitrus littoralis*, *Wenzelia dolichophylla*, *Skimmia japonica*, and *Citropsis gilletiana*, which were 2 to 3 years old. Commercial species were on various rootstocks (most often Cleopatra mandarin or Milam rough lemon), whereas most citrus relatives were on their own roots; a list of stock/scion combinations is available from the authors. Sampling dates were February and October 1995 in Florida and April 1995 in California. Several young, fully expanded leaves were taken from single trees of each species or cultivar, frozen in dry ice, and lyophilized. The dry leaves were then milled to pass mesh size 40 and stored at –20 °C until analysis. When live trees were not available, leaves were taken from herbarium specimens; as betaines and proline are stable compounds, they can be analyzed reliably in this way (Blunden et al., 1996; Hanson et al., 1994). Herbarium specimens and sources were as follows: *Merope angulata*, National Arboretum (Washington, D.C.); *Luvunga scandens* and *Burkillanthus malaccensis*, New York Botanical Garden (New York); *Monanthocitrus cornuta*, Royal Botanic Gardens (Kew, U.K.).

EXTRACTION AND ANALYSIS OF BETAINES. Portions (usually 100 mg) of milled leaf material were extracted with a methanol/chloroform/water procedure and fractionated by ion exchange chromatography as described (Hanson et al., 1991). The *n*-butyl esters of betaines were prepared and analyzed by FABMS using the methods of Rhodes et al. (1987). Betaines were quantified relative to an internal standard (448 nmol) of deuterated glycine betaine (Rhodes et al., 1987) using response factors determined with authentic standard compounds. Authentic proline betaine was from Extra-Synthèse (Lyon, France). Hydroxyproline betaine (*trans*-4-hydroxy-L-proline form) was synthesized by the method of Musich and Rapoport (1977).

Table 2. Effect of rootstock on proline and proline betaine levels (μg·g^{-1} dry mass) in scion leaves.

Scion	Rootstock	Proline	Proline betaine
Triphasia trifolia	Own	82	26
Wenzelia dolichophylla	Own	2	<0.5
Wenzelia dolichophylla	*Triphasia trifolia*	3	<0.5
Citrus reticulata[z]	Own	93	63
Citrus taxa[y]	Own	86 ± 5	33 ± 5
Citrus taxa[y]	*Citrus reticulata*[z]	99 ±3	27 ± 1

[z]'Cleopatra'.
[y]Mean values ±SD for *Citrus aurantium, Citrus indica,* and *Fortunella* sp.

PROLINE AND DMSP ASSAYS. Proline was extracted from 30-mg portions of milled material by heating in 5 mL water at 100 °C for 30 min and determined colorimetrically as described (Hanson et al., 1977), except that the ion-exchange step was omitted. DMSP was estimated using the dimethylsulfide release assay described by Paquet et al. (1994).

Results

TAXONOMIC DISTRIBUTION PATTERNS. The distribution of proline accumulation, proline betaine, and hydroxyproline betaine is summarized in Table 1, in which taxa are arranged according to Swingle and Reece (1967). In distinguishing proline accumulators from nonaccumulators, we adopted a threshold of 8 μmol·g^{-1} dry mass. This was based on a literature survey indicating that proline levels in leaves of unstressed plants are usually less than half this

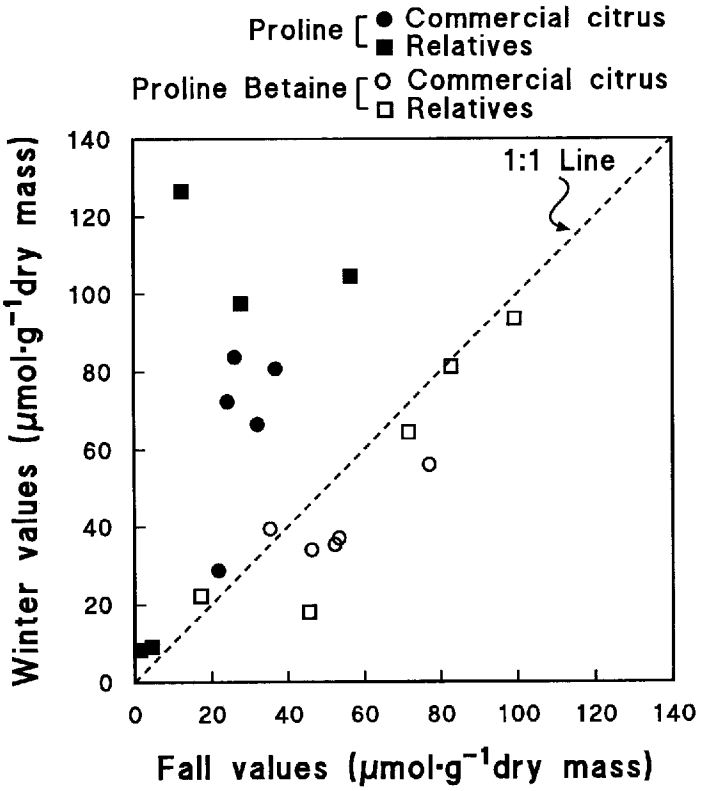

Fig. 2. Relationship between early fall (6 Oct.) and winter (1 Feb.) levels of proline and proline betaine in leaves of commercial citrus species and citrus relatives. A single tree of each species was sampled on both dates. Commercial species: *Citrus sinensis, C. paradisi, C. reticulata, C. medica, C. grandis.* Relatives: *C. hystrix, C. ichangensis, C. macroptera, Aegle marmelos, Afraegle paniculata.*

value (Poljakoff-Mayber et al., 1987; Treichel et al., 1984; see also citations in Delauney and Verma, 1993; Hanson and Hitz, 1982). By this criterion, proline accumulation occurs in almost all the Aurantioideae as well as in two other subfamilies (from which selected species were tested for comparison). The accumulation can be taken to be constitutive as none of the trees analyzed was salt or drought stressed and many were not cold hardened. Constitutive proline accumulation has been found in only a few angiosperms (Poljakoff-Mayber et al., 1987; Treichel et al., 1984). It may result from weak feedback regulation of proline synthesis (Delauney and Verma, 1993).

The principal betaine detected was proline betaine. Most species with proline betaine also had small amounts of hydroxyproline betaine (about 3% to 15% of the proline betaine level). The 3- and 4-hydroxy isomers of this compound are not distinguished by FABMS, so the position of the hydroxyl group remains to be determined. No species had more than a trace (≈1 μmol·g^{-1} dry mass) of glycine betaine, and none had detectable DMSP (<0.03 μmol·g^{-1} dry mass). Small amounts of trigonelline (up to ≈2 μmol·g^{-1} dry mass) occurred sporadically, as in other families (Blunden et al., 1996; Rhodes and Hanson, 1993).

The distribution of proline betaine broadly paralleled the taxonomic scheme of Swingle and Reece (1967), although there were significant discrepancies. Thus, except *Clausena*, all the proline betaine-accumulating genera were from the tribe Citreae. Within the Citreae, proline betaine occurred in only one (*Triphasia*) of seven genera from the subtribe Triphasiinae, but in four of six genera from the Balsamocitrinae, and in seven of thirteen genera from the Citrinae. Of these seven, all save one (*Limnocitrus*), belong to the true citrus fruit tree group defined by Swingle and Reece (1967). The inexact coincidence between proline betaine distribution and the taxonomy of Swingle and Reece is not surprising in the light of other chemosystematic data indicating that this arrangement is to some extent artificial (da Silva et al., 1988). Overall, the distribution of proline betaine suggests it is a specialized condition that arose within some subgroups of the Aurantioideae, and so presumably evolved later than proline accumulation.

Rootstock effects. Rootstocks may (Yelenosky, 1979) or may not (Lloyd et al., 1989, 1990) have consistent influences on proline and proline betaine levels in scion leaves of 1-year-old trees. To assess the importance of rootstock in our study, which involved mainly mature trees, we compared proline and proline betaine levels in stock/scion combinations in which the partners differed (Table 2). *Wenzelia dolichophylla* had little proline and no proline betaine whether or not it was on a rootstock of *Triphasia trifolia*, a species with leaves rich in both compounds. Similarly, three species with moderate proline betaine levels did not have more of this compound when combined with a rootstock (Cleopatra mandarin) whose leaves had a much higher level. Taken with the

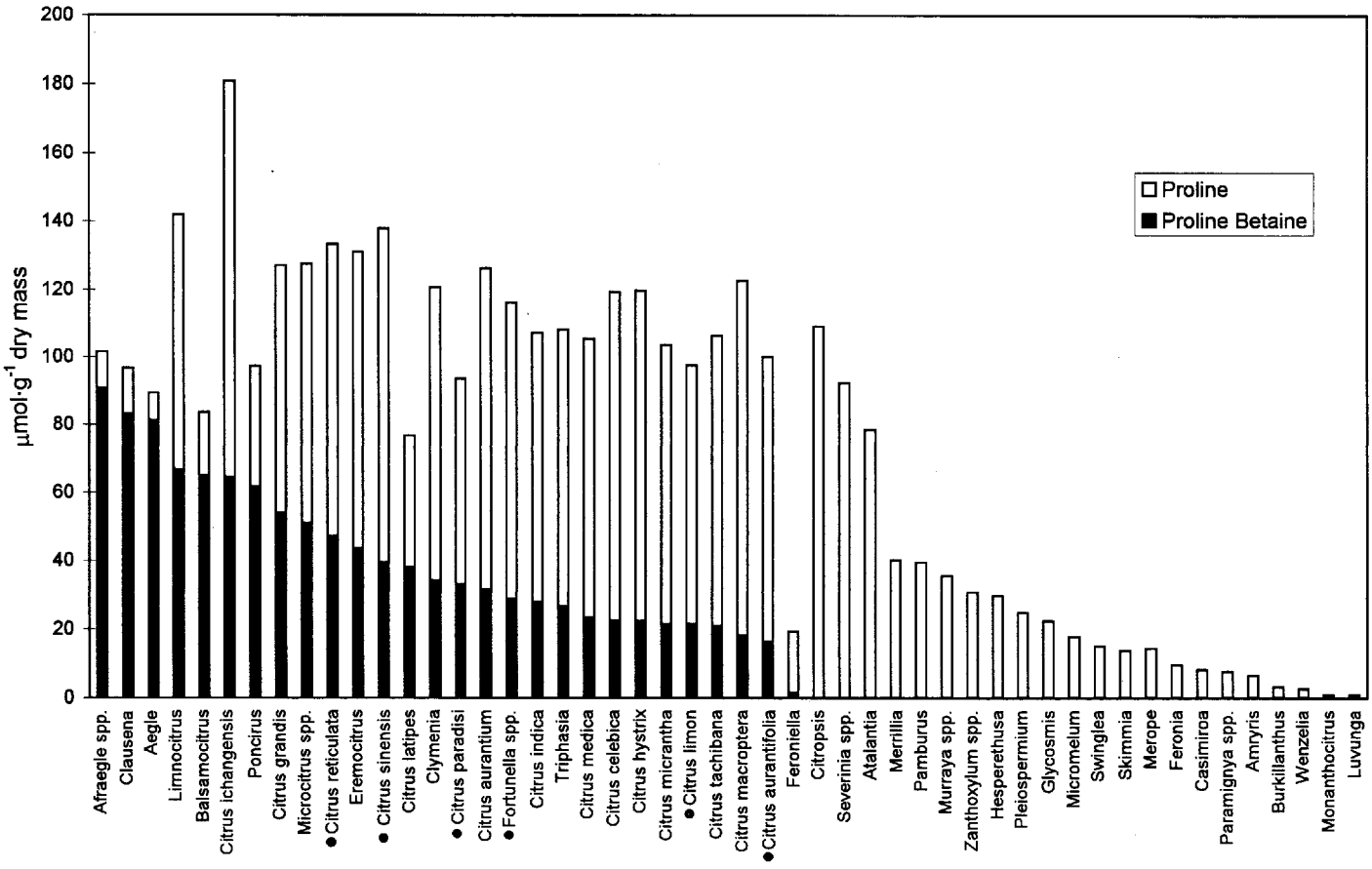

Fig. 3. Proline and proline betaine levels of taxa from the orange subfamily (Aurantioideae), ranked in descending order with respect to proline betaine, then to proline. The species were those of Table 1. Major commercial species are marked with a bullet. When a single species of a genus was analyzed, only the genus name is shown. Data for cultivated *Citrus* species are means for two or three cultivars, and data for *Afraegle, Fortunella, Microcitrus, Murraya, Paramignya, Pleiospermium, Severinia,* and *Zanthoxylum* are means for the species listed in Table 1. The extent of variation within these taxa was sufficiently small that ranking each cultivar or species separately gave a pattern little different from that shown. The deciduous species *Feronia limonia, Poncirus trifoliata,* and *Zanthoxylum clava-herculis* were sampled in October; other species were sampled in February or April.

physiological evidence that the leaf is the main site of proline synthesis in citrus (Kato, 1986), these results suggest that the type of rootstock did not have a major influence on proline or proline betaine levels in the trees we tested.

SEASONAL CHANGES. Proline levels in leaves of commercial citrus are known to rise during the winter (Yelenosky, 1985; Syvertsen and Smith, 1983). We therefore compared the proline and proline betaine levels among a representative group of species, sampling at dates expected to give minimum and maximum values for proline (Fig. 2). As expected, proline levels in general rose substantially in winter in the commercial citrus species, and also in the citrus relatives. In contrast, proline betaine levels remained the same or fell moderately. The increase in proline and near-stasis in proline betaine is interesting from an engineering standpoint as it emphasizes that a large proline pool may be available for conversion to proline betaine.

RELATIONSHIP BETWEEN PROLINE AND PROLINE BETAINE. Figure 3 presents quantitative data on proline and proline betaine, with the taxa ranked with respect to the levels of these compounds. It should be noted that, except for three deciduous species, the values in Fig. 3 are for trees sampled in winter or early spring, when proline levels were probably near maximal (Syvertsen and Smith, 1983). Three features of Fig. 3 are relevant to genetic engineering. First, the levels of proline and proline betaine are inversely correlated. For the 30 taxa with combined proline plus proline betaine contents exceeding 75 $\mu mol \cdot g^{-1}$ dry mass, the coefficient of linear correlation (–0.693) was statistically significant at $P = 0.001$. Second, the major commercial citrus species have intermediate or low levels of proline betaine and all have more proline than proline betaine. Last, five of the ten taxa richest in proline betaine are considered very resistant to stresses (Swingle and Reece, 1967; Yelenosky, 1985); these are the freeze-hardy evergreen *Citrus ichangensis* and *Citrus reticulata*, the freeze-hardy deciduous *Poncirus trifoliata* and *Aegle marmelos*, and the salt-tolerant *Limnocitrus littoralis*. The remaining 40 taxa include only a few that are reported to be highly stress resistant (e.g., the freeze-hardy *Fortunella* species, the salt-tolerant *Merope angulata*, and the drought-tolerant *Eremocitrus glauca* (Swingle and Reece, 1967; Yelenosky, 1985).

Discussion

Freeze hardiness and salt tolerance are complex multigenic traits. Genetic engineering of single characters such as osmoprotectant accumulation therefore cannot be expected to give spectacular advances in these traits. This does not mean that engineering should not be explored, because sometimes a small advance is all that is needed. Citrus freeze hardiness is a case in point: it has been estimated that a 1 to 2 °C increase in hardiness compared to current cultivars would cut tree and fruit losses by 10% to 20% in freeze-prone citrus production regions (Yelenosky, 1985). Salt tolerance presents a similar case because citrus is among the most salt sensitive of crops, and the decline in yield as salinity increases is very steep (Maas, 1993).

This study aimed to provide the background on nitrogenous osmoprotectants needed to devise genetic engineering approaches to citrus stress tolerance. Two strategies suggested by our findings are discussed below, both requiring the insertion of just one or two genes. These strategies involve modifying osmoprotectant levels in leaves, which raises the issue of their relevance to practical citriculture, where damage to woody tissues and fruit is critical. This issue cannot yet be resolved, but evidence from citrus and from other plants shows that proline, proline betaine, and glycine betaine made in leaves can be translocated to, and accumulated by, other organs (Essery et al., 1962; Hanson and Hitz, 1982; Purvis and Yelenosky, 1983).

MODIFYING THE PROLINE BETAINE/PROLINE RATIO. Because proline betaine is biosynthesized from proline (Essery et al., 1962), the data of Fig. 3 show that citrus and many relatives produce similar amounts of proline ($\approx 100\ \mu mol \cdot g^{-1}$ dry mass), but they vary greatly in how much of it they convert to proline betaine (from <20% to >90%). Moreover, whereas proline levels increase during cold hardening, proline betaine levels do not (Fig. 2). Proline betaine is a more potent protectant than proline (Amin et al., 1995; Hanson et al., 1994; LeRudulier et al., 1984), and commercial citrus species have less proline betaine than several more stress-resistant taxa (Fig. 3). Taken together, these observations make it reasonable to attempt to engineer the proline betaine/proline ratio in citrus to determine whether increasing it improves stress tolerance and decreasing it has the opposite effect. Genes for the enzymes catalyzing the two-step methylation of proline to proline betaine (Essery et al., 1962) would be needed to implement this strategy. These are not yet available but should be straightforward to clone.

INTRODUCING NON-NATIVE BETAINES. Glycine betaine is the most common betaine in flowering plants, and it can occur within the same family and even the same species as proline betaine (Blunden et al., 1996; Hanson et al., 1994; Rhodes and Hanson, 1993; Wood et al., 1991). It is nontoxic to humans and occurs in many food crops. As glycine betaine was not found in significant amounts in species from the Aurantioideae, it would be rational to insert genes for its synthesis into citrus. Glycine betaine is made from the primary metabolite choline by a two-step oxidation (Rhodes and Hanson, 1993). Single bacterial genes encoding enzymes that catalyze both steps can be used to engineer glycine betaine synthesis in plants (Deshnium et al., 1995; Lilius et al., 1996). Alternatively, two plant genes that encode separate enzymes for each step could be introduced. Genes for the plant enzyme mediating the second step have been available for some time (Ishitani et al., 1995; McCue and Hanson, 1992), and a gene for the first enzyme was recently cloned in our laboratory (B. Rathinasabapathi and A.D. Hanson, unpublished data).

Because the sulfonium betaine DMSP is absent from citrus, it is appropriate to consider engineering the accumulation of this compound also. In plants, DMSP is probably made in two steps from the ubiquitous intermediate *S*-methylmethionine (Hanson and Gage, 1996). The enzymes and genes involved have not yet been isolated but in principle certainly could be. It would be particularly valuable to obtain these genes because DMSP appears to have exceptionally strong cryoprotectant properties (Karsten et al., 1996).

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