ACCUMULATION OF ASPARAGINE IN NaCl-STRESSED BARLEY SEEDLINGS

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INTRODUCTION

Within the leaves of many plants subjected to moderate or severe water- or salt stress, one striking change in nitrogen metabolism is the accumulation of free proline (Hanson and Hitz 1982, Tully et al. 1979, WynJones and Storey 1978). Recent observations with tomato suspension cultures adapted to water stress also showed the importance of proline in osmotic adjustment, since the cells adapted to water stress showed a 300-fold increase in proline pool which represents over 50% of the total free amino acids (Rhodes et al. 1986). The elevated proline pool is primarily the consequence of a 10-fold elevated rate of proline synthesis via the glutamate pathway (Rhodes et al. 1987). Although the function of the adaptive increase in the proline pool is not yet clear, it could be an osmoticum, an enzyme/membrane protectant, or a nontoxic store of reduced nitrogen (Hanson and Hitz 1982, Rhodes et al. 1986).

Metabolic changes in other free amino acids, such as increase in GABA, alanine, valine (Hanower and Brzozowska 1975, Rhodes et al. 1986), have been shown under the water/salt stressed conditions, but these changes are small compared to the changes in the proline pool. However, we found that the asparagine pool is increased markedly in barley seedlings at the primary leaf stage when the plants were treated with 50mM NaCl in a hydroponic culture. Herein, we discuss the physiological function of asparagine accumulated by the NaCl-stress.

MATERIALS AND METHODS

Plant Materials

Seeds of barley (*Hordeum vulgare* L. cv. Kikaihadaka) were germinated in aerated water for 12h at room temperature. The germinated seeds were planted on a plastic net in a 6*l* plastic container with 4*l* of a

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half strength nutrient solution containing 2mM KNO_3 and 0.5mM $(NH_4)_2SO_4$ as the nitrogen source, as described previously (Matsumoto et al. 1980). The seedlings were grown for about 10 days in a Tabai refrigerated incubator LNC-130 at 24°C. The seedlings were illuminated with fluorescent lamps giving about 100μmol/m^2·s during a 12-h photoperiod.

Extraction and isolation of amino acids

Barley roots and leaf blades (0.5g fresh weight) were incubated in 20ml of an 80% ethanol solution for a few days at room temperature for the extraction of amino acids. The tissues were then homogenized in the ethanol solution with a mortar and pestle, filtered through a Toyo filter paper No. 6, washed with 10ml of 80% ethanol, and then the filtrate was evaporated to dryness in a rotary evaporator under vacuum at 40°C. The dried extract was dissolved in 1ml of water and then applied to a Dowex 50W H^+ (200-400 mesh) ion-exchange column (0.6×4cm) equilibrated with water. The column was washed with 30ml of water to remove sugars and organic acids. The amino acids were eluted with 20ml of 2N NH_4OH. Each sample was rotary evaporated to dryness, dissolved in 0.5ml of water, and centrifuged at 16,000rpm with a Sakuma M-150 microcentrifuge. The supernatant fraction was used for derivatization of amino acids.

Isolation of amino acids from xylem sap

The leaf sheath of barley seedlings was cut approximately 10mm above the endosperm with a sharp razor blade. The exudate from the cut end was collected with a Pasteur pipet during a 1.5-h period and designated as a xylem sap. The volume of the xylem sap was measured with a micro-pipet and then ethanol (to give a final concentration of 75%) was added. Amino acids were isolated from the ethanol extract as described above.

Analysis of amino acids

Amino acids isolated from the ethanol extracts were first derivatized with phenylisothiocyanate to synthesize phenylthiocarbamy-amino acid derivatives and the derivatives were then analyzed with a reversed-phase high performance liquid chromatography (HPLC) as described elsewhere (Yamaya and Matsumoto 1988).

Isolation of vacuole from leaf-blade protoplasts

Protoplasts were isolated from barley leaf blades and purified as described previously (Yamaya and Oask 1988).

Vacuoles were isolated by the methods of Foster et al. (1987) with minor modifications. The purified protoplasts (about 1mg chlorophyll) were pelleted by centrifugation at 300g for 5min. Vacuoles were released from these protoplasts by the addition of 0.9ml 0.1M K_2HPO_4-HCl (pH 7.5) and 0.1ml of mannitol medium consisting of 0.5M mannitol, 2mM dithiothreitol (DTT), 2mM ethylenediaminetetraacetate (EDTA), 2mM $CaCl_2$, 0.2% bovine serum albumin (BSA), 25mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes)-KOH (pH 7.5). After 5min at 25°C, 1ml of the mannitol medium was added and loaded onto Ficoll gradients consisting of 4ml steps of 1, 5, 10% (w/v) Ficoll-400 in 25mM Hepes-KOH (pH 7.5) containing 0.4M mannitol, 1mM $CaCl_2$, 1mM DTT, 1mM EDTA, and 0.1% BSA. The gradients were centrifuged at 26,000rpm (100,000g) for 45min at 4°C in a SW-27.1 rotor in a Beckman model L5-75 ultracentrifuge. Purified vacuoles at the 1/5% Ficoll interface were removed with a Pasteur pipet.

Other assays

Chlorophyll content (Arnon 1949) and α-mannosidase (Boller and Kende 1979) were determined based on published procedures.

RESULTS AND DISCUSSION

Amino acid pool sizes in roots, xylem sap, and leaf blades of barley seedlings under NaCl stress

The major amino acids in the roots without NaCl stress were glutamate, asparagine and glutamine ; they occupied about a half of the total free amino acids (Table 1). In a 50mM NaCl stress condition, the asparagine pool was increased about 4-fold within a day and the aspartate pool was increased 4.4 to 6-fold when compared to the 0 time control (Table 1). Other amino acids, such as glutamate, alanine, and serine, were also increased a few-fold.

Analysis of the amino acid pool size in xylem sap obtained from unstressed barley revealed that the glutamine was the largest pool accounting for 32% of the total amino acids detected in the sap, followed by asparagine which accounted for about 20% (Table 2). This indicates that these amides were the main amino acids as the form of transport from root to top of the barley seedlings. By the NaCl treatment, the asparagine pool was increased 2.8 to 4.5-fold within two days to represent about a half of the total amino acids. On the other hand, the glutamine pool was rather decreased after the stress treatment. Some other minor amino acids, such as alanine, ornithine, and proline, were also increased. It is worthy to note that glutamate, one of the major amino acids in root tissues, and aspartate, increased several fold by the

TABLE 1 Effect of 50mM NaCl on pool size of free amino acids in roots

Amino acid		0 Time	Day 1	Day 2
		(μmol/g fresh weight root)		
Aspartate	(Asp)	0.168	0.743(4.42)	1.008(6.00)
Glutamate	(Glu)	0.687	2.178(3.17)	1.342(1.95)
Serine	(Ser)	0.312	0.697(2.24)	0.641(2.06)
Asparagine	(Asn)	0.623	2.610(4.19)	2.381(3.82)
Glycine	(Gly)	0.058	ND	ND
Glutamine	(Gln)	0.360	0.659(1.83)	0.547(1.52)
Homoserine	(Hser)	0.030	0.080(2.70)	0.040(1.34)
Histidine	(His)	0.035	0.062(1.75)	0.063(1.76)
γ-amino butyrate	(GABA)	0.023	0.145(6.39)	0.061(2.25)
Threonine	(Thr)	0.152	0.341(2.23)	0.344(2.26)
Alanine	(Ala)	0.146	0.914(6.26)	0.685(4.69)
Arginine	(Arg)	0.021	0.102(4.83)	0.091(4.33)
Proline	(Pro)	0.029	0.068(2.35)	0.065(2.22)
Tyrosine	(Tyr)	0.057	0.095(1.66)	0.098(1.71)
Valine	(Val)	0.231	0.407(1.76)	0.361(1.56)
Methionine	(Met)	ND	0.012	0.012
Isoleucine	(Ileu)	0.180	0.230(1.28)	0.247(1.38)
Leucine	(Leu)	0.168	0.200(1.19)	0.245(1.46)
Phenylalanine	(Phe)	0.064	0.103(1.61)	0.105(1.63)
Orinithine	(Orn)	0.063	0.076(1.21)	0.072(1.15)
Lysine	(Lys)	0.094	0.139(1.49)	0.159(1.70)

ND : not detected
Parentheses indicate ratio of pool size compared to 0 time.

stress in roots, but were hardly detected in xylem sap obtained either from control or NaCl-stressed samples. Thus, these acidic amino acids were probably not transported directly from roots through xylem flux. It should be noted that NaCl treatment strongly inhibited the exudation from the xylem, and no sap can be collected after a 5-day treatment with 50mM NaCl or after 2 days with 200mM NaCl.

In leaf blades without NaCl stress, the major amino acids were glutamate, asparagine, and alanine (Table 3). Unlike in roots or xylem sap, the leaf sheath contained a rather small glutamine pool, which suggests rapid metabolism of glutamine transported from roots. As was seen in roots and in xylem sap, the asparagine pool in leaf blades was also expanded about 2-3 fold by the NaCl stress to represent about one third of the total amino acids within one or two days. By the treatment, free pools of serine, alanine, glutamine, and GABA were also increased but not as much as the accumulation of asparagine. The aspartate and glutamate pools had essentially the same size before and after the treatment. The proline pool was also unchanged by the treatments.

TABLE 2 Effect of 50mM NaCl on pool size of free amino acids in xylem sap

Amino acid	0 Time	Day 1	Day 2
	(pmol/μl of xylem sap)		
Asp	27	8(0.30)	94(3.42)
Glu	80	98(1.23)	178(2.22)
Ser	630	935(1.49)	1,200(1.91)
Asn	1,709	4,790(2.80)	7,707(4.51)
Gly	ND	ND	ND
Gln	2,850	1,850(0.65)	2,494(0.88)
Hser	41	60(1.46)	43(1.04)
His	84	104(1.24)	138(1.65)
GABA	68	50(0.74)	99(1.44)
Thr	519	429(0.83)	507(0.98)
Ala	189	619(3.28)	441(2.34)
Arg	173	389(2.25)	374(2.16)
Pro	22	63(2.86)	72(3.25)
Tyr	113	71(0.62)	120(1.06)
Val	786	675(0.86)	949(1.21)
Met	17	23(1.37)	27(1.59)
Ileu	398	232(0.58)	341(0.86)
Leu	286	156(0.54)	206(0.72)
Phe	151	77(0.51)	123(0.81)
Orn	57	181(3.20)	255(4.50)
Lys	776	590(0.76)	776(1.00)

ND : not detected
Parentheses indicate ratio of pool size compared to 0 time.

When the barley seedlings were grown in 0.5mM $CaCl_2$ alone (without a nitrogen source), the pool size of asparagine was increased 1.9- and 0.8-fold in roots and in leaf blades, respectively both in the presence and absence of 200mM NaCl (data not shown). Thus, the supply of nitrogen into the medium caused the large accumulation of asparagine in roots and leaf blades.

Amino acid composition in protoplasts and vacuoles

Compartmentation of amino acids was investigated by measuring their pool sizes in protoplasts and in isolated vacuoles, which can be considered to be a storage pool, from barley leaf blades with or without NaCl stress. To determine the distribution of each amino acid in the vacuole, the actual amount of amino acids was divided by α-mannosidase activity which is the marker enzyme of the vacuole (Boller and Kende 1979, Foster et al. 1987).However, since the recovery of the vacuole based on the yield of α-mannosidase activity from the protoplast was only 5-7%, the results may not represent a true compartmentation.

TABLE 3 Effect of 50mM NaCl on pool size of free amino acids in leaf blades

Amino acid	0 Time	Day 1	Day 2
	(μmol/g fresh weight leaf)		
Asp	0.367	0.184(0.50)	0.375(1.02)
Glu	1.921	1.769(0.92)	1.634(0.85)
Ser	0.639	1.076(1.68)	1.312(2.05)
Asn	1.281	2.605(2.03)	3.933(3.07)
Gly	0.175	0.670(3.83)	0.019(0.11)
Gln	0.243	0.423(1.74)	0.417(1.71)
Hser	0.045	0.019(0.41)	0.042(0.93)
His	ND	ND	ND
GABA	0.191	0.266(1.39)	0.402(2.11)
Thr	0.328	0.372(1.14)	0.639(1.95)
Ala	0.951	0.990(1.04)	1.151(1.21)
Arg	0.052	0.063(1.20)	0.116(2.21)
Pro	0.052	0.046(0.89)	0.082(1.59)
Tyr	0.081	0.102(1.26)	0.158(1.96)
Val	0.352	0.404(1.15)	0.598(1.70)
Met	ND	0.021	ND
Ileu	0.217	0.243(1.12)	0.376(1.74)
Leu	0.143	0.140(0.98)	0.231(1.62)
Phe	0.119	0.152(1.27)	0.252(2.12)
Orn	0.048	0.037(0.76)	0.085(1.76)
Lys	0.276	0.265(0.96)	0.341(1.24)

ND : not detected
Parentheses indicate ratio of pool size compared to 0 time.

As shown in Table 4, asparagine, aspartate, glutamine, isoleucine and leucine were mostly located in the vacuole. The value of over 100% probably means further metabolism of these amino acids during the isolation of the vacuole. On the other hand, proline, alanine, GABA, glutamate, serine, and ornithine were detected in small amounts in the vacuole, which indicates that these amino acids were located in the cytosol and other compartments, such as chloroplasts and mitochondria.

At the primary leaf stage of barley seedlings, NaCl stress first enhanced the asparagine pool in roots, xylem sap and leaf blades, but not proline pool which has been reported by many groups (Hanson and Hitz 1982, Rhodes et al. 1986, Tully et al. 1979, WynJones and Storey 1978). Assimilation of inorganic nitrogen is not affected by a short period of salt or water stress (Hanson and Hitz 1982). Our data are in agreement with their finding, since the total amino acid pool in roots, xylem sap, and leaf blades was increased by the NaCl treatment. At this stage material in the endosperm of the barley seedlings is still left over, i. e., growth of the plants is heterotrophic in the carbon status. A continuous

TABLE 4 Localization of amino acids in vacuole isolated from leaf blade protoplasts of barley seedings treated with or without NaCl stress

Amino acid	Control	200mM NaCl
	(% in vacuole isolated from protoplasts)	
Asp	206	171
Glu	29	85
Ser	75	58
Asn	166	77
Bly	ND	58
Gln	104	128
Hser	88	ND
GABA	27	39
Thr	77	ND
Ala	25	61
Arg	109	53
Pro	38	68
Tyr	62	ND
Val	157	80
Ileu	143	101
Leu	88	169
Phe	81	38
Orn	38	26
Lys	135	55

ND : not detected in either protoplast or vacuole
His and Met were not detected. Relative distribution of each amino acid in vacuoles was obtained by comparing its amount in protoplasts on a basis of α-monnosidase activity.

supply of inorganic nitrogen and sufficient amounts of carbon skeleton could allow the synthesis of amino acids in roots. Because strong inhibition of xylem exudation was observed in the plants treated with NaCl, excess amounts of amino acids synthesized by the stressed plant could be converted to asparagine as a storage form of nitrogen in roots. This asparagine was then transported to the top of the plants through the xylem stream. Less accumulation of asparagine with no exogenous supply of nitrogen and its vacuolar localization in the leaf blades also support our consideration on the function of increased asparagine as a storage form of nitrogen.

Large accumulation of proline was not observed in our experiment when barley seedlings at the primary leaf stage were tested under the salt stressed conditions. Tomato suspension cultures adapted to water stress, which are also heterotrophic growth conditions similar to that in our stage of barley seedlings accumulate proline more than a half of total amino acid pools (Rhode et al. 1986). This large proline pool is suggested to work as an osmoregulator, but proline synthesis was not

stimulated by NaCl stress in our plants. The barley seedling under stress at this stage could not survive. The severe inhibition of xylem flux and the lack of osmoregulator synthesis could be the main reason for the poor survival of our plants.

SUMMARY

Free pools of amino acids in roots, xylem sap, and leaf blades of barley (*Hordeum vulgare* L. cv. Kikaihadaka) seedling at the primary leaf-stage, treated with or without 50mM NaCl, were determined by a reversed-phase high performance liquid chromatography. The NaCl-stress caused a marked increase in asparagine pool which accounted for 28, 48, and 32% of total free amino acids in roots, xylem sap, and leaf blades, respectively, when three-week old seedlings were treated for 2 days. Accumulation of proline was small compared to that of asparagine in the seedings. More than 75% of the asparagine was detected in vacuoles isolated from protoplasts of the leaf blades.

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