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NUCLEOTIDE EXTRACTION AND QUANTITATION FROM TOMATO ROOTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INDRANI DUTTA, GERARD A. O'DONOVAN* and DON W. SMITH
Department of Biological Sciences, University of North Texas, Denton, TX 76203 (U.S.A.)

SUMMARY

The chromatographic separation and quantitation of nucleotides in tomato root tips are seriously impaired by the presence of phenols, which greatly increase in the absence of boron. However, when polyvinylpolypyrolidone is added to the 6% trichloroacetic acid homogenizing solution and the mixture is shaken on ice for 30 min, there is a decrease in phenol concentration such that nucleotides can be separated and quantified on anion-exchange Partisil SAX10 columns after neutralization with freon–amine. This treatment extends column life to more than 70 analyses per cartridge. Such a technique is unique in that it can be used with less than 500 mg of root tip tissue and allows quantitation of plant nucleotides in the picomol range.

INTRODUCTION

Boron is not required for the growth of animals and bacteria but is an essential micronutrient for vascular plants[1,2]. The primary role and mode of action of boron remains obscure[3], but there is increasing evidence to suggest that several aspects of nucleic acid metabolism are affected in boron deficient plants[4,5]. One way to monitor changes in nucleic acid metabolism is to compare the concentration of ribo- and deoxyribonucleotides in boron-sufficient and boron-deficient plants. High-performance liquid chromatography (HPLC) is the method of choice for monitoring such changes in nucleotide content in bacteria and animal systems[7]. However, although HPLC can be readily used for quantifying plant nucleotides in boron-sufficient plants[8–11], there is considerable difficulty in the quantitation of nucleotides in boron-deficient plants. This is due to the high concentration of phenols and other secondary metabolities which accumulate in boron-deficient plants.

The success of nucleotide separation by HPLC lies in proper extraction and purification. Numerous extraction procedures are available for measuring free nucleotides in biological samples of different living organisms[12]. However, the chromatographic separation and UV determination of nucleotides in plant extracts are seriously impaired, and in tomato roots prevented, by the presence of phenols[13,14]. Phenols strongly absorb UV below 400 nm. Thus, in plant extracts, they can prevent detection, measurement, and characterization of nucleotides by UV absorbance. Phenols and nucleotides have not been separated from each other satisfactorily by dif-

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ferential extraction or solvent-partitioning procedures, nor by conventional gel filtration, sorption chromatography, or ion-exchange chromatography[8].

Chemically, the plant phenolic compounds are extremely heterogenous, ranging from simple monomers to very large polymers. Phenols are selectively sorbed by several water-insoluble polymers having hydrogen-binding capability, like polyvinylpolypyrrolidone (PVPP)[15,16]. The addition of PVPP decreases the phenol concentration of plant extracts sufficiently to improve the resolution of nucleotides on anion-exchange columns. Moreover, PVPP has been used[9], along with Amberlite XAD-2 and charcoal, to purify nucleotides, but this method required 15 g of plant tissue as starting material. A solid-phase extraction technique, in which phenylsilane-bonded silica gel is used for the separation of phenols from nucleotides in tobacco has also been devised[10,11] but this technique is not applicable to root tips of tomato plants, as the nucleoside triphosphates are lost completely (data not shown) and the Partisil SAX10 column is irreversibly destroyed after ten runs.

Since never more than 500 mg of root tips were available, a new extraction procedure for the accurate and reproducible quantitation of endogenous nucleotide levels had to be devised. In this paper, we describe a method which affords detection of nanomolar concentrations of nucleotide pools in tomato root tips by HPLC.

EXPERIMENTAL

Chemicals and reagents

Nucleotides, trichloroacetic acid, PVPP and tri-*n*-octylamine were from Sigma (St. Louis, MO, U.S.A.); 1,1,2-trichloro-1,2,2-trifluoroethane (freon) and monobasic ammonium phosphate were from Mallinckrodt (Paris, KY, U.S.A.); and potassium chloride was from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Plant material

Tomato seeds (*Lycopersicon esculentum* Mill., cv. Improved Summertime) were obtained from the Texas A & M University Experiment Research Station (College Station, TX, U.S.A.). Seeds were germinated in plastic petri plates (9 cm in diameter) after a disc of Whatman No. 1 filter paper was inserted into each dish. The filter paper was moistened with water, deionized with a Milli Q water system (Millipore, Bedford, MA, U.S.A.). Petri dishes were incubated for five to seven days under UV radiation at 26 ± 2°C until the first true leaf pairs emerged. Seedlings about 5 cm in length with two or three small leaves, were transplanted to an aerated, full-strength complete nutrient solution[17]. The pH of the nutrient solution was adjusted to pH 4.7 by either 0.1 *M* sodium hydroxide or 0.1 *M* hydrochloric acid. Plants were hydroponically grown in the greenhouse in 250-ml soft-glass jars, prewashed with 6 *M* hydrochloric acid. Two plants were mounted in a two-holed No. 6 rubber stopper. Fresh nutrient solutions were supplied after every five days. When the plants were 20 cm in height (measured from the cotyledons to the apical meristems), they were used as experimental materials. At the desired time-interval of treatment, the roots were measured, and the tips were harvested for nucleotide analysis or returned to the solution for later measurement and analyses.

Extraction of nucleotides

Excised root tips typically at 250 mg per sample, were washed, weighed, and frozen in liquid nitrogen within 30 s. The frozen root tissue was kept at −80°C until ready for purification, when it was powdered in a mortar precooled by liquid nitrogen. The powdered root tissue was homogenized in chilled 6% (w/v) trichloroacetic acid, containing PVPP (0.5 g/10 ml), and incubated on ice in a gyrating shaker for 30 min. before centrifugation at 14 000 *g* for 12 min. The acid extract, containing nucleotides, was neutralized with an equal volume of ice-cold freon–amine[18] solution. The freon–amine mixture was shaken on a vortex mixer for 1 min and then allowed to separate while standing at 4°C for 10 min. The top aqueous layer, which contains the nucleotide pool extract, was removed with a 5-ml syringe, filtered through a 0.45-μm ACRO LC13 filter (Gelman, Ann Arbor, MI, U.S.A.) and frozen at −20°C until ready for use.

Chromatographic apparatus and conditions

The HPLC equipment (Waters, Milford, MA, U.S.A.) consisted of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector and a Model 481 LC spectrophotometer. Nucleotides were detected by monitoring the column effluent at 254 nm with sensitivity fixed at 1 V a.u.f.s. Separations were performed on a Waters Radial Pak Partisil SAX10 cartridge (10 cm × 0.8 cm I.D.) using a Waters radial compression Z-module system. The elution buffer system used consisted of eluent A, 7 m*M* ammonium dihydrogenphosphate (pH 3.8), and eluent B, 250 m*M* ammonium dihydrogenphosphate (pH 4.5) with 500 m*M* potassium chloride[6,7].

Nucleotide samples (100 μl), obtained from plant root tips, were injected into the column. A linear gradient of eluent A to eluent B was applied for 20 min., followed by an isocratic period of 10 min with eluent B[6]. The column was regenerated by washing with 30 ml of eluent A buffer. The flow-rate was maintained at 4 ml/min; analyses were performed at ambient temperature. Peaks were integrated either manually on a Cole Palmer (Chicago, IL, U.S.A.) strip-chart recorder or on a Waters 740 data module. Individual components of the acid-soluble nucleotide pools were identified, using retention time relative to known standards and by injecting known internal standards. The recoveries of nucleotides from solutions of nucleotide standards were determined by measuring peak heights before and after the extraction procedures.

CALCULATIONS

The concentration of the sample was calculated by comparing its peak height with the standard for which the concentration was known (1 m*M*). The nanomol value per gram fresh weight for all nucleotides was computed as follows:

$$\frac{S_a}{S_t} C \frac{V_{st}}{V_{sa}} \frac{V}{FW}$$

where S_a is the peak height of the sample, S_t the peak height of the standard, C is the amount of compound (gram) in the standard divided by the molecular weight of

the compound, V is the total volume of the sample, V_{sa} the volume of sample injected, V_{st} the volume of standard injected and FW the fresh weight.

RESULTS AND DISCUSSION

One of the major problems associated with determining the level of nucleotide in plant tissues arises from severe interference by low-molecular-weight material, such as phenolic compounds and precursors or degradation products of chlorophyll, which are extracted along with the nucleotides[8]. In this experiment, the small quantity of available root tissue, typically less than 500 mg, was an additional obstacle. Formic acid[19] at 1 M, perchloric acid[10] at 0.4 M and various concentrations (6, 8, 10 and 12%) of trichloroacetic acid were initially used for the extraction of nucleotides. Since the most reproducible results were obtained with 6% TCA, we used this concentration throughout. Grinding of the frozen tissues in a precooled mortar proved to be a very efficient and convenient method of homogenizing[10].

Tomato plants are especially rich in phenols, and this makes nucleotide determinations very difficult under any circumstance. This was further complicated by the fact that the phenol content of plant tissues increased enormously on boron deprivation[20]. In order to remove phenols, PVPP was added to trichloroacetic acid (6%) and used as a homogenization buffer. It is critical that the samples after extraction be shaken on ice for at least 30 min before centrifugation. In this procedure all the phenols are adsorbed and the recovery of nucleotides is increased. Samples prepared by this technique did not affect column life. After this preparation, it was possible to quantitate more than 70 samples on the same Partisil SAX10 column without any loss in sensitivity.

There is a previous report[10] which shows that freon–amine neutralization leads to lower recovery. In our case, neutralization with potassium hydroxide, followed by removal of the potassium chloride precipitate by centrifugation resulted in a distortion of peaks and decreased column life. Accordingly, the freon–amine neutralization step[18] was used throughout the experiments, despite the 3 ± 0.5% loss in recovery.

Fig. 1a shows the elution profile of a mixture of the twelve known nucleotide standards. In order to avoid errors in identification due to variations in retention times, a mixed standard was injected between every sample run. Regardless of individual variations, retention times always followed the pattern: monophosphates < diphosphates < triphosphates. As seen in Fig. 1, the twelve ribonucleoside mono-, di- and triphosphates were separated. Fig. 1b–d presents typical sample chromatograms of each experimental condition while representative HPLC data are presented in Table I. Extracts were prepared from the roots of boron-sufficient (+B, positive control) and boron-deficient (+B, negative control) tomato plants. As can be seen from Table I, in the boron-deficient plants there was a significant decrease in the levels of all ribonucleotides. When boron-sufficient plants were starved for boron longer than two days, their roots did not recover, and the plants died. Referring again to Table I (column 3), when boron was provided to plants starved for boron for two days, recovery, as reflected in nucleotide levels, was not at all certain. Though the plants seemed to survive, they were unable to replenish their nucleotide pools to the boron-sufficient levels. It is noteworthy that addition of the pyrimidine base, uracil, to boron-deficient nutrient solution[17] restored the nucleotide pools to their boron-sufficient level (Fig. 1d; Table I).

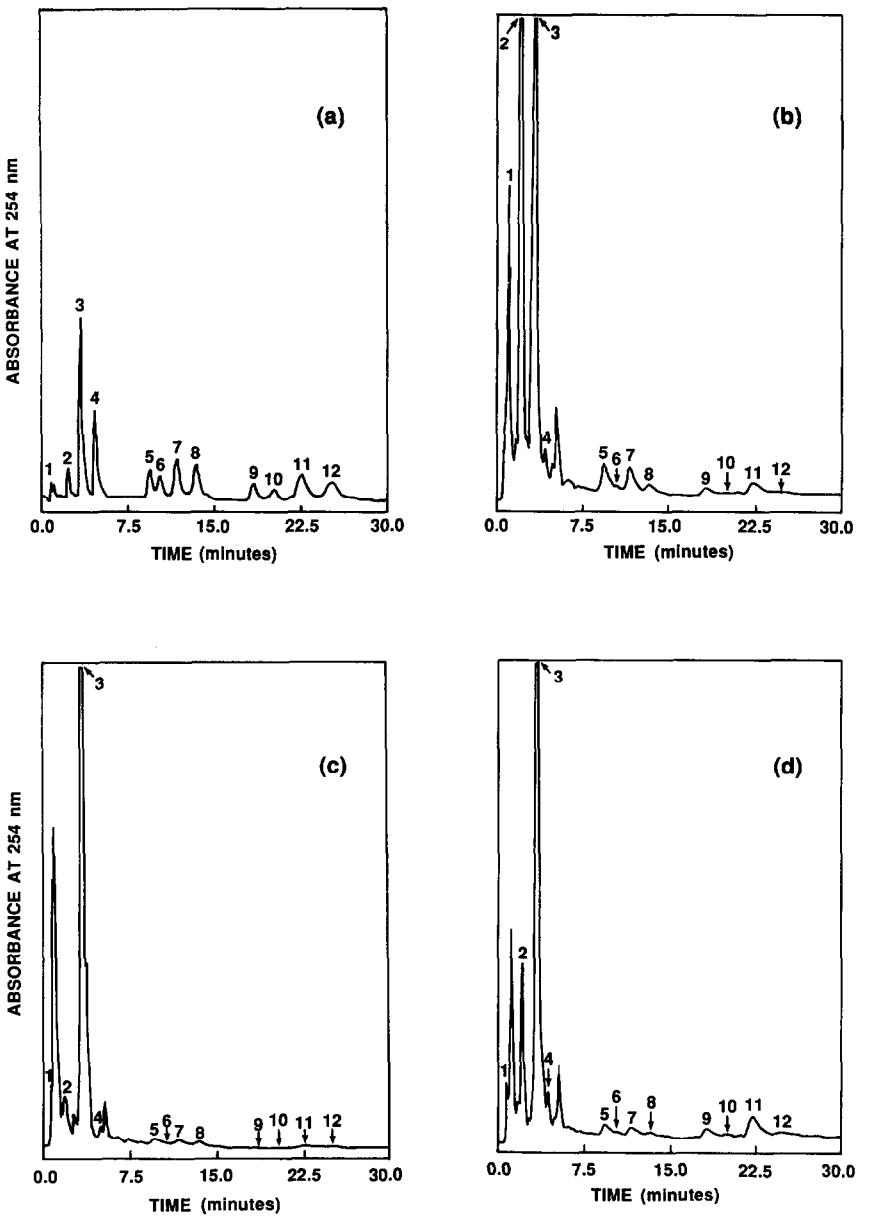

Fig. 1. Representative anion-exchange chromatograms of nucleotides obtained with a Radial Pak Partisil SAX cartridge. (a) Standard ribonucleoside mixture (100 μl), consisting of 10^{-5} M of each nucleotide. Sample (100 μl) from tomato plant root tip tissue, grown in boron-sufficient (b); in boron-deficient (c); and in uracil-containing, boron-deficient (d) nutrient media respectively. Abbreviations used: 1, CMP; 2, AMP; 3, UMP; 4, GMP; 5, UDP; 6, CDP; 7, ADP; 8, GDP; 9, UTP; 10, CTP; 11, ATP; 12, GTP. Under these conditions it was possible to detect 0.1 nmol of nucleotide.

TABLE I

YIELD OF NUCLEOSIDE MONO-, DI- AND TRIPHOSPHATES FROM TOMATO PLANT ROOT TIPS (nmol/g FRESH WEIGHT)

All values are the averages of two separate analyses. +B = Positive control; −B = negative control. Under the conditions described in the footnotes it was possible to detect 0.1 nanomol of nucleotide.

Nucleotides	*+B*	*−B**	*−B +B***	*−B +U**	*−B +U***
UMP	55.00	53.00	41.00	48.00	37.70
CMP	31.00	15.79	10.34	25.59	13.00
AMP	11.20	11.20	4.00	8.60	10.00
GMP	6.00	2.20	0.70	1.20	2.90
UDP	3.40	0.49	0.24	2.20	9.00
CDP	2.70	0.49	0.24	1.24	1.20
ADP	3.50	0.93	0.46	1.10	5.60
GDP	1.50	1.30	0.45	0.16	2.20
UTP	2.89	0.80	0.41	0.61	3.00
CTP	2.69	0.62	0.62	0.82	1.60
ATP	6.53	0.70	0.54	0.90	3.00
GTP	2.86	0.57	0.38	0.38	1.30

* Plants were grown in a medium without boron, to which uracil was added at 0.1 m*M* precisely when boron removed.

** Plants were grown in a medium without boron for two days and then given boron–uracil for two additional days.

An important aspect of studies on metabolite pools is that the pool levels remain unaffected by the technical manipulations involved. In this study, the level of ATP obtained by rapidly extracting root tip cells with 6% (w/v) TCA was similar to the reported earlier[10,11]. These observations suggest that the pool levels remain relatively constant and resist turnover for several minutes. Harvesting and washing of eukaryotic (unlike prokaryotic) cells do not reduce their adenosine nucleotide content. Moreover, throughout these studies, using TCA and freon–amine, the recovery of nucleotides was always at 97 ± 0.5%.

The following general statements can be made regarding nucleotide measurements from the roots of tomato plants. (i) Nucleotide pool measurements always paralleled the root elongation data. (ii) The four monophosphates could be quantitated accurately in boron-sufficient plants. They ranged in values from 55 nmol/g fresh weight for UMP to 6.0 nmol/g fresh weight for GMP. These values are in agreement with values reported previously[11]. (iii) The four diphosphates were reproducibly quantitated in the boron-sufficient samples and appeared to be particularly sensitive to boron deprivation. (iv) The values for the four triphosphates were lower than the values for the mono- and diphosphates, regardless of how samples were prepared. As has always been observed for other systems[6], the ATP value was greatest of the four triphosphates, but its value in the root was only 6.53 nmol/g fresh weight. To check the accuracy of the column in these estimations, two internal control tests were carried out routinely. (a) A sample prepared from *Escherichia coli*, was tested for adenosine triphosphate levels, which are known to be 2–4 m*M*. A

value within that range was found. (b) Adenosine was added to the boron-sufficient plant medium and adenylate values were measured. Since adenylate concentrations are known to increase when adenosine is added to bacterial cultures, the same result was anticipated in plants. We found this to be the case. This technique should be of value in plant physiology experiments where nucleotide concentrations are to be measured.

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