Plant Molecular Biology **33:** 811–820, 1997.

Overexpression of D-*myo*-inositol-3-phosphate synthase leads to elevated levels of inositol in *Arabidopsis*

Cheryl C. Smart* and Susan Flores
*Institute of Plant Sciences, Plant Biochemistry and Physiology, Swiss Federal Institute of Technology (ETH) Zürich, Universitätsstrasse 2, CH-8092 Zürich, Switzerland (*author for correspondence)*

Received 9 August 1996; accepted in revised form 29 November 1996

Key words: Spirodela polyrrhiza, D-*myo*-inositol-3-phosphate synthase, inositol, overproduction, *Arabidopsis thaliana*, salt stress

Abstract

In this paper, we report on the generation of transgenic *Arabidopsis* plants containing elevated levels of the gene product encoding the enzyme catalysing the first committed step in inositol biosynthesis, D-*myo*-inositol-3-phosphate (Ins3P) synthase. These plants exhibit both an increase in Ins3P synthase activity and an increase in the level of free inositol of over four-fold compared to wild-type plants. Despite these changes, we could detect no significant difference in phenotype in the transgenic plants for a number of characteristics linked with putative functions of inositol and inositol-derived metabolites. Our results indicate that the proposed engineering of inositol metabolism to generate specific plant phenotypes (e.g. salt tolerance) may require the manipulation of several genes, and that Ins3P synthase activity can be manipulated to increase the pool size of free inositol.

Introduction

Inositol is an essential plant metabolite required for normal cell growth and other critical functions. In addition to incorporation into structural elements of the cell (phosphoinositides in membranes and pectic polysaccharides in the cell wall [25]), inositol metabolites play an important role in intracellular signal transduction (inositol phosphates [3]), and in the synthesis of potential osmoprotectants (e.g. D-pinitol [8]). Inositol hexakisphosphate may also be important in phosphate storage at particular stages of plant development [12]. Despite this complex metabolism, inositol synthesis occurs via a single defined pathway. The first committed step in inositol synthesis involves the synthesis of D-*myo*-inositol-3-phosphate (Ins3P) from D-glucose-6-phosphate (G6P) which is catalysed by the enzyme D-*myo*-inositol-3-phosphate synthase (Ins3P synthase) [24]. The Ins3P generated is then dephosphorylated by inositol monophosphatase (IMP) to release free inositol. The inositol generated by this two-stage process from G6P can enter a number of different pathways and cellular compartments.

As a result of previous work, we isolated a cDNA (*TUR1*) encoding Ins3P synthase from the water plant *Spirodela polyrrhiza* [32]. Interestingly, we could show that the mRNA transcript level and enzyme activity were up-regulated by the hormone abscisic acid (ABA), which acts in *S. polyrrhiza* to induce dormant buds (turions) which have an elevated resistance to environmental stress [22]. Turion formation is associated with a number of molecular and physiological events which could theoretically be linked to an altered inositol metabolism, such as decreased cell elongation via changes in cell wall inositol-derived metabolites [27, 32] and ABA-linked signal transduction via inositol phosphates [16]. In order to examine the role of increased Ins3P synthase levels in the plant's response to ABA and in dormant bud formation we have overexpressed the *Spirodela* Ins3P synthase in the model plant *Arabidopsis thaliana* (*S. polyrrhiza* being not yet transformable).

Inositol produced by Ins3P synthase activity can follow a number of metabolic fates. The end products of these different pathways may have various effects on the plant in terms of both its development and its

response to the environment. Since reduction of cellular inositol levels has been linked to inhibition of cell division in plant cell cultures [5], overproduction of inositol might show whether inositol plays any role in limiting cell division in the intact plant. Changes in inositol-derived cell wall components may change the extensibility of the cell wall [27]. Thus plants with an increased inositol synthesis may display an altered cell wall extension leading to either dwarf or elongated phenotypes. Alternatively, changes in the contents of inositol-derived membrane components may affect the plant's ability to withstand stress conditions like low temperature [28].

In addition, bearing in mind the recent literature suggesting that inositol [31] and inositol-derived metabolites [7, 8] can play a role in salt stress resistance, and the long standing association of ABA with general plant responses to environmental stress [2, 10, 11], we hypothesised that an elevated Ins3P synthase activity might be required for an increased flux to inositol during the plant's response to stress, and indeed might even be sufficient to induce some element of salt tolerance. The generation of transgenic plants overexpressing the Ins3P synthase cDNA provides a means of testing this theory.

In this paper, we report the generation of transgenic *Arabidopsis* plants overexpressing the Ins3P synthase *TUR1* cDNA from *S. polyrrhiza*. We show that the plants obtained have an elevated level of Ins3P synthase activity, and that concomitantly there is an about fourfold increase in the endogenous level of free inositol. In addition, we present an analysis of the effect of this altered inositol metabolism on plant phenotype and response to low temperature and salt stress.

Materials and methods

Plant material

Arabidopsis thaliana ecotype C24 plants were used for all transformation experiments and subsequent tissue analysis. Plants were either grown in soil under long day conditions of 16 h light at 21 °C and 8 h darkness at 17 °C or aseptically on germination medium (half-strength Murashige and Skoog salts (Sigma M5524) pH 5.7 with KOH, 1% sucrose, 0.8% agar) under continuous light at 23 °C. For analysis of *in vitro* seedling root growth plates were incubated vertically.

Plasmid construction, transformation and regeneration of plants

The *Spirodela polyrrhiza TUR1* cDNA lacking its 5′-untranslated region was inserted into the expression vector pRT104 (courtesy of Dr Reinhard Töpfer, MPI, Köln) which contains the 35S promoter and polyadenylation signal of the cauliflower mosaic virus (CAMV) [35]. A 300 bp PCR fragment was synthesised from pCS 30.3.1 [32] which corresponded to the region coding for the second amino acid of TUR1 to a region 82 bp downstream of a unique *Sal*I site (Fig. 1A). The forward primer was 5′-TTCATCGAGAAGTTCCGGGTG-3′ and the reverse primer was 5′-AGTAGTTGGCCTGCTGGACC-3′. The PCR fragment was phosphorylated with polynucleotide kinase and digested with *Sal*I. The rest of the *TUR1* cDNA was isolated as a 1.5 kb *Sal*I-*Eco*RI fragment. The pRT104 vector was restricted with *Nco*I and this site was filled in with Klenow to give a blunt end which conserved the first ATG after the CaMV 35S promoter. The vector was then restricted with *Eco*RI and dephosphorylated with calf intestinal phosphatase and ligated to the 218 bp PCR fragment and the 1.5 kb *Sal*I-*Eco*RI fragment to obtain the plasmid pRT104-TUR1. The 2.5 kb expression cassette of pRT104-TUR1 was excised using *Hind*III and was ligated to the *Hind*III restricted dephosphorylated pBIN19 *Agrobacterium* vector [4] to give the plasmid pBIN104-TUR1 (Fig. 1B). As a control we used pBIN19 containing the expression cassette from the plasmid pSH4GUS [19] containing the *GUSINT* gene [37] under the control of the CaMV 35S promoter which was a kind gift from Dr Sönke Holtorf (ETH, Zürich). These plasmids were used to transform *A. tumefaciens* strain LBA4404 containing the helper plasmid pLBA4404 (Clontech) [18].

Callus induced from roots of *Arabidopsis thaliana* was transformed by cocultivation with *A. tumefaciens* LBA4404 bearing the plasmid pBIN104-TUR1 [20]. Primary transformants (T_0) were obtained after regeneration on kanamycin-containing medium [36] and plants were allowed to set seed in aseptic culture. These seeds (T_1) were germinated on kanamycin, and kanamycin-resistant seedlings were grown to maturity and selfed to produce T_2 seeds. This step was repeated once to obtain the T_3 generation seeds. T_3 seeds were germinated on kanamycin and scored for kanamycin resistance ratios. Samples of T_3 seedlings all showing the Kan^R phenotype and presumably homozygous were further analysed by PCR for presence of the *TUR1*

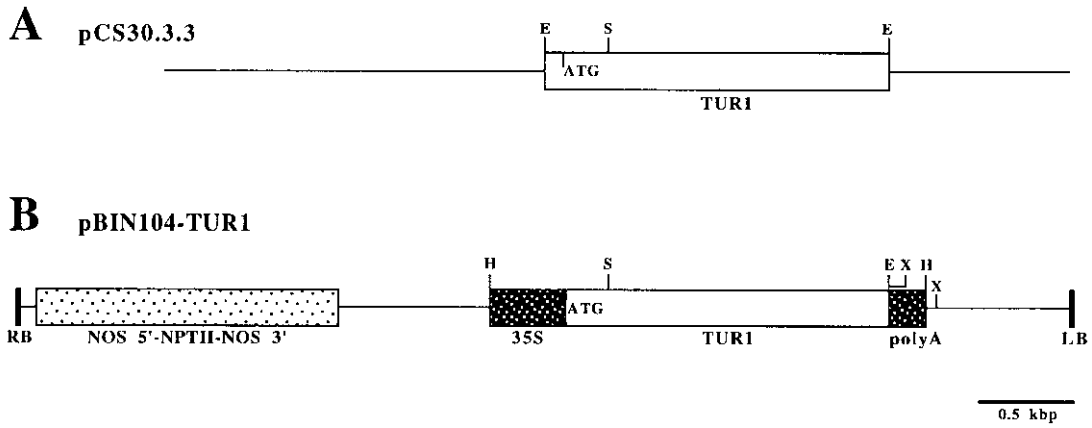

Figure 1. Construction of the *Spirodela polyrrhiza TUR1* cDNA expression vector pBIN104-TUR1. **A.** Plasmid pCS30.3.3 contains the 1.84 kb *TUR1* cDNA (TUR1) inserted into the *Eco*RI site (E) of the vector pBluescript SK–. The translation initiation site of *TUR1* is indicated by ATG and a unique *Sal*I site by S. The coding region and 3′-untranslated region of the *TUR1* cDNA was inserted between the CAMV 35S promoter and polyadenylation signal in the vector pRT104 to give the plasmid pRT104-TUR1 (not shown separately). **B.** The T-DNA region of pBIN104-TUR1 contains the expression cassette from pRT104-TUR1 inserted into the *Hind*III site (H) between the right (RB) and left (LB) borders of the T-DNA region of the *Agrobacterium* vector pBIN19. The CAMV 35S promoter (35S) and polyadenylation signal (poly(A)) are indicated by dark-stippled boxes. The *TUR1* cDNA coding region and 3′-untranslated region is indicated by a hollow box (TUR1), and the light-stippled box represents the chimaeric selectable marker gene (NOS 5′-NPTII-NOS 3′) composed of the regulatory sequences of the nopaline synthase gene (NOS 5′ and NOS 3′) and the coding sequence of the bacterial neomycin phosphotransferase II gene (NPTII). Restriction sites for *Xba*I used for genomic Southern blot analysis are indicated (X). All constructs are drawn to the scale shown.

transgene, by Southern blotting for integration of the transgene and by northern blotting for analysis of its expression. Plants transformed with the *GUSINT* gene and mock-transformed kanamycin-sensitive regenerants were used as controls.

PCR analysis of transgenic plants

Leaf extracts [40] of the kanamycin resistant T_3 plants were analysed by PCR for the presence of the *TUR1* transgene using the forward primer 5′-CCAAGAGCAACGTAGTTGACG-3′ and the reverse primer 5′-GGGAAAAGATTCATGCCACGC-3′ which were specifically designed to amplify the 3′ third of the *Spirodela TUR1* coding sequence and part of its 3′ untranslated region and not the homologous *Arabidopsis* gene [23].

Southern and northern analysis of transgenic plants

Genomic DNA [30] was digested with *Xba*I and 1 μg was used per lane. After electrophoresis in 0.8% agarose and blotting onto nylon membranes (Boehringer) it was hybridised at 68 °C with the 1.84 kbp *TUR1* cDNA labelled with digoxigenin (DIG) using the DIG DNA labelling kit (Boehringer). Hybridisation, subsequent high stringency washes and DIG-detection with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl) phenyl phosphate (CSPD) were performed according to the manufacturer's protocols.

Total RNA was extracted from *Arabidopsis* seedlings [26] and *Spirodela* fronds [17], and 10 μg fractionated on 1.1% agarose-formaldehyde gels and transferred onto Hybond membranes (Amersham). The 1.84 kb *TUR1* cDNA was labelled with [α-^{32}P]dCTP by the random-priming method (Pharmacia) and hybridised to the RNA blots at 65 °C according to the filter manufacturer's instructions. Blots were exposed to Hyperfilm MP (Amersham) at –80 °C with an intensifying screen for 24 h.

Measurement of Ins3P synthase activity

Frozen plant tissue was homogenised in 3 vol. of 250 mM sucrose, 5 mM Tris-acetate pH 8.0, 2 mM dithiothreitol (DTT), 0.1 mM EDTA. After centrifugation (20 min, 15 000$\times g$) the supernatant was dialysed for 1 h against 5 mM Tris-acetate pH 8.0, 2 mM DTT and for a further hour against 5 mM Tris-acetate, pH 8.0. The protein concentration was determined [9] and the Ins3P synthase activity in the protein extract was measured by a modification of a previously described

method [32]. Although this assay was optimised for the introduced *Spirodela* Ins3P synthase, it is unlikely to grossly underestimate the *Arabidopsis* enzyme. Each assay consisted of 2 mM [1-^{14}C]G-6-P (457 Bq/nmol) (New England Nuclear), 14 mM NH_4Cl, 0.8 mM NAD^+, 100 mM Tris-acetate pH 8.0 and 20–30 μg protein in a reaction volume of 20 μl. Enzyme assays were incubated for 1 h at 30 °C and the reaction stopped by boiling for 3 min. Alkaline phosphatase (1.2 units) was added and the reaction incubated at 37 °C for 2 h to dephosphorylate the remaining substrate to glucose and the product of the reaction to inositol. The reaction was stopped again by boiling and the extract was clarified by centrifugation. An internal standard of [^{3}H]inositol was added to correct for recoveries. Inositol was then separated from glucose by HPlc on a Partisphere PAC column (4.6 mm × 250 mm, Whatman) [29] using acetonitrile water (80:20, v/v) as the mobile phase, at a flow rate of 1 ml/min. Separation was monitored using the UV absorbance of added unlabelled standards at 192 nm. Under these conditions, glucose and inositol have retention times of ca. 8 min and 13 min, respectively. Fractions (0.5 ml) were collected and mixed with 2.5 ml of Ultima Gold MV (Packard) for liquid scintillation counting.

Measurement of myo-inositol levels

myo-Inositol levels were determined by a modification of the method of Fry [14]. Briefly, frozen tissue was ground to a powder in liquid nitrogen and further homogenised in chloroform methanol (1:1, v/v), after addition of *scyllo*-inositol as internal standard [13]. Water was added, causing two phases to form, and the mixture centrifuged for 10 min at 4000×*g*. The upper phase was removed from the organic phase and pelleted cell debris, and H_2SO_4 added to a final concentration of 0.1 M. The extract was then heated 20 min in a boiling water bath to oxidise monosaccharides. After cooling, solid CO_2 was added until the pH of the solution was 5.8–6.0. $BaCO_3$ and $BaSO_4$ were removed by centrifugation. The supernatant was deionised over an ion-exchange column (either 1 ml BioRad AG 501-X8, or a triple-layer column consisting of 0.25 ml each of Serdolit Blue (HCO_3^-) and Polyclar AT, and 0.4 ml Dowex 50W X8 (H^+) (all Serva) [1]). The eluate was dried under reduced vacuum, and dissolved in 100 μl DMSO for derivatisation. Alditol acetates were prepared using acetic anhydride and 1-methylimidazole as catalyst [6]. The alditol acetates were separated by GC on Silar 10-C (3% on GasChromQ, 1.8 m × 3.2 mm, Alltech) at a column temperature of 225 °C, using nitrogen as carrier gas, at a flow rate of 25 ml/min. Quantitation was based on the relative areas of the *myo*- and *scyllo*-inositol peaks. It had previously been established that *Arabidopsis* has no detectable components eluting with the retention time of *scyllo*-inositol.

Results

Production of transformed plants

Forty-three primary transformants (T_0) were obtained after regeneration on kanamycin-containing medium and 32 of these plants set viable seed in sterile culture. These seeds (T_1) were germinated on kanamycin. From each of 18 lines, several kanamycin-resistant seedlings (sublines) were further grown either in sterile culture without kanamycin or in soil, and selfed to produce T_2 seeds. Several T_2 seeds of each subline were planted in soil, grown to maturity and selfed to obtain the T_3 generation seeds. From each plant about 500 T_3 seeds were germinated on kanamycin and scored for kanamycin resistance ratios. Samples of kanamycin resistant T_3 seedlings were further analysed by PCR for presence of the *TUR1* transgene, by Southern blotting for integration of the transgene and by northern blotting for analysis of its expression.

Molecular and biochemical analysis of plants overexpressing Ins3P synthase

While all of the transgenic lines analysed contained the *TUR1* transgene as indicated by PCR, ratios of Kan^R to Kan^S seedlings of the T_3 generation indicated that some of the transgenic lines showed either patchy kanamycin resistance or complex kanamycin resistance segregation patterns. Upon further analysis these lines showed multiple insertions of the *TUR1* transgene in Southern blots, and showed no expression of the *TUR1* transcript in northern blots (data not shown). From the remaining transgenic lines a transformant was isolated, designated R3, which displayed a simple (3 Kan^R:1 Kan^S) kanamycin resistance segregation pattern in its progeny indicating a single locus insertion. On the basis of the expression of kanamycin resistance, homozygous sub-lines of R3 were isolated and the presence of a single *TUR1* integration event was confirmed by Southern analysis of the *Arabidopsis* genomic DNA using the *Spirodela TUR1* cDNA as probe (Fig. 2). The *TUR1* transgene was integrated into a 9 kb *Xba*I

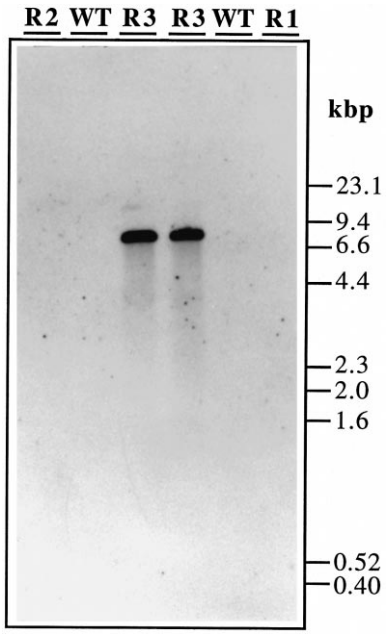

Figure 2. Genomic Southern blot analysis of *Arabidopsis* seedling DNA showing integration of the *TUR1* cDNA from *S. polyrrhiza*. Genomic DNA was isolated from *in vitro* grown wild-type (WT) and mock-transformed seedlings (R1) in the absence of kanamycin, and from seedlings transformed with the *GUSINT* gene (R2) and *TUR1*-transformed seedlings from two homozygous R3 sublines (R3E1-3 and R3A4-7) grown on kanamycin. Fragments of genomic DNA (1 μg) digested with *Xba*I were separated by gel electrophoresis, blotted, and hybridised with the 1.84 kb DIG-labelled *TUR1* cDNA as probe. The size in kb of DNA markers is indicated.

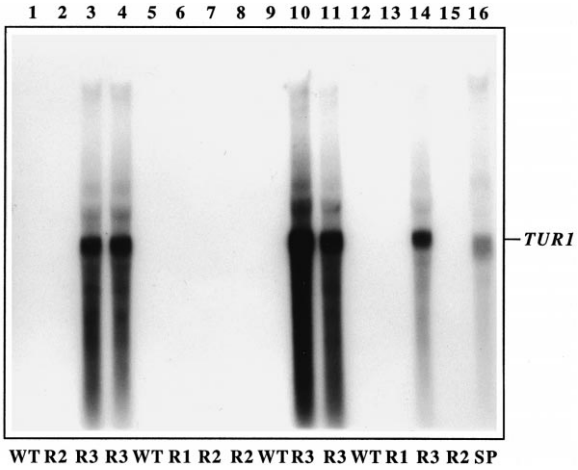

Figure 3. Northern blot analysis of *Arabidopsis* seedling RNA showing expression of the *TUR1* mRNA from *S. polyrrhiza*. Total RNA was isolated from *in vitro* grown wild-type (WT; lanes 1, 5, 9, 12) and mock-transformed seedlings (R1; lanes 6, 13) in the absence of kanamycin, and from seedlings transformed with the *GUSINT* gene (R2; lanes 2, 7, 8, 15) and *TUR1*-transformed seedlings from four homozygous R3 sublines (R3B1-7, R3A5-3, R3E1-3, R3A4-7; lanes 3, 4, 10, 11, respectively) and 1 heterozygous subline (R3B6-4; lane 14) grown on kanamycin. RNA was also isolated from *S. polyrrhiza* fronds which had been treated with 250 nm ABA for 2 h (SP; lane 16). RNA samples (10 μg) were separated by denaturing gel electrophoresis, blotted, and hybridized with the ^{32}P-labelled 1.84 kb *TUR1* cDNA as probe.

fragment of the *Arabidopsis* genome. Genomic DNA from control plants (wild-type, *GUSINT*-transformed and mock-transformed) showed no signal corresponding to the endogenous *Arabidopsis* gene under the high stringency conditions used.

Northern analysis of total seedling RNA from several R3 homozygous sub-lines and one heterozygous subline showed that the expression of the *TUR1* transgene was extremely high (Fig. 3) and the transcript level was estimated by eye to be at least 6 times higher than the ABA-induced transcript level in *Spirodela*. While all the R3 sub-lines showed greatly elevated levels of the *TUR1* transcript there were small differences in signal intensity shown by the individual sublines which were not due to differences in total RNA as shown by methylene blue staining. Seedlings from the one heterozygous subline examined showed the lowest level of *TUR1* expression. No cross-hybridisation of the *TUR1* probe was seen with RNA from control plants (wild-type, *GUSINT*-transformed and mock-transformed) either at high stringency (Fig. 3) or at low stringency (data not shown) indicating that the high levels of expression of the *TUR1* transcript were solely due to the presence of the *TUR1* transgene.

To determine whether transgenic plants which highly express the *TUR1* cDNA also express increased activity of the Ins3P synthase enzyme, rosette leaves from individual 4-week-old soil-grown plants of the homozygous sub-line R3E1-3 were tested for Ins3P synthase activity and compared to wild-type plants. The Ins3P synthase activity of wild-type *Arabidopsis* was found to be at an average level of 1.83 nmol per mg protein per hour, whereas the R3 transgenic plants contained an average level of 9.69 nmol/mg protein/h, an increase of 5.3-fold. The activity of Ins3P synthase in individual *Arabidopsis* wild-type and R3E1-3 transgenic plants is shown in Fig. 4A.

Rosette leaf extracts of the R3 Ins3P synthase overproducers were then tested for *myo*-inositol levels and were found to contain on average 3.4 μmol inositol per g fresh weight which is ca. 4-fold higher than in wild-type plants. The levels of inositol in individual

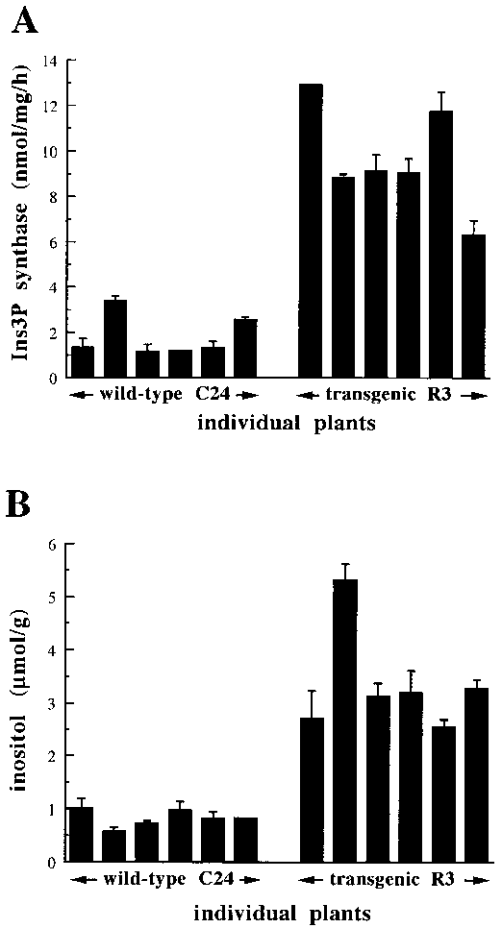

Figure 4. Ins3P synthase activity and inositol levels in wild-type and *TUR1*-transgenic R3 plants. **A.** Ins3P synthase activity in wild-type and *TUR1*-transgenic R3 plants. Rosette leaf protein extracts from individual 4-week-old soil-grown wild-type plants or plants of the homozygous sub-line R3E1-3 were assayed for Ins3P synthase activity using [^{14}C]G6P as substrate and HPLC separation of the products as described in Materials and methods. Ins3P synthase activity is expressed as nmol per mg protein per hour and indicates the average of two determinations on plant extracts from 6 individual wild-type and 6 individual transgenic plants. The error bars show the range of the measurements for each plant. **B.** Inositol levels in wild-type and *TUR1*-transgenic R3 plants. Rosette leaf protein extracts from individual 4-week-old soil-grown wild-type plants or plants of the homozygous sub-line R3E1-3 were assayed for inositol levels by GC as described in Materials and methods. Inositol levels are expressed as μmol per g fresh weight and indicate the average of 3–7 GC determinations on plant extracts from 6 individual wild-type and 6 individual transgenic plants. The error bars show the standard deviation of the GC measurements for each plant.

Arabidopsis wild-type and R3E1-3 transgenic plants are shown in Fig. 4B.

Phenotypical analysis of plants overexpressing Ins3P synthase

Seeds of the T_4 generation of the homozygous R3 sub-lines were sown in soil and compared to wild-type *Arabidopsis* with respect to various phenotypical traits throughout their germination, growth and development. There were no significant differences between the R3 transgenic sub-lines and the results were therefore pooled (Table 1). Plants were scored for germination, general phenotype, number of rosette leaves, bolting, first and second internode length and final plant height and stem diameter. Seeds were also sown on germination medium in order to study early seedling root growth. No significant effect on the phenotype of the R3 transgenic *Arabidopsis* plants at the gross morphological level was observed. Small differences between the transgenic plants and the wild-type control plants were observed with the transgenic plants having somewhat slower and poorer soil germination, shorter roots *in vitro*, fewer rosette leaves, later bolting, shorter first internode extension (due to later bolting) and a smaller stem diameter. However, these differences were very small and probably the result of an overall decrease in 'general fitness' of the transgenic plants due to somaclonal variation since these differences were also observed in control plants either transformed with the *GUSINT* gene or mock-transformed plants (data not shown).

Physiological analysis of plants overexpressing Ins3P synthase

In view of the often postulated role of cyclitols in conferring salt tolerance [2, 7, 31], we asked whether the transgenic *Arabidopsis* were more tolerant of NaCl than wild-type plants. We first investigated the effect of high salt (200 mM NaCl) and/or low temperature (11 °C) on the germination rate of wild-type controls, R1 mock-transformed controls, R2 *GUSINT*-transgenic controls and R3E1-3 *TUR1* transgenic seeds. Sterilised seeds were plated onto germination medium and scored each day for germination for a period of 11 days. A seed was considered germinated when the seedling radicle had emerged and the cotyledons had greened. The results are expressed as the germination index and are shown in Table 2.

These results can be summarized as follows:

1. The germination rates of all control and transgenic seeds were similar under normal medium and temperature conditions.

Table 1. Phenotypical analysis of Ins3P synthase-overproducing transgenic *Arabidopsis* plants. Seeds of the T_4 generation from 4 homozygous sublines of the R3 transgenic line (R3A4-7, R3A5-3, R3B1-7, R3E1-3) and wild-type controls (C-24) were sown either aseptically on germination medium for analysis of seedling root growth or in soil for analysis of seed germination and plant growth and development. No differences were observed between the individual R3 sublines and the results were pooled for comparison to the wild-type controls. Mean values are followed by the standard deviation of the mean and the sample number. Statistical analysis was performed using Student's *t*-test and the statistical significance of the differences indicated at the probability levels $^{*} < 0.05$, $^{**} < 0.02$, $^{***} < 0.002$.

	C-24 controls	R3 (*TUR1*) transgenics
% Germination day 3	86	55
% Germination day 6	93	80
% Germination day 10	93	82
Root growth in vitro (cm) day 11	1.71±0.04 (*n*=52)	1.55±0.03 (*n*=217)**
Phenotype day 17	normal seedlings	normal seedlings
Phenotype day 25	normal plants	normal plants
Rosette leaf number day 27	25.3±1.1 (*n*=14)	22.7±0.9 (*n*=54)*
% Bolting day 33	21	26
% Bolting day 35	79	56
1st internode length (cm) 39 days	4.08±0.50 (*n*=14)	3.03±0.33 (*n*=54)*
2nd internode length (cm) 39 days	2.62±0.53 (*n*=14)	1.92±0.25 (*n*=54)
Plant height (cm) day 47	36.6±1.1 (*n*=14)	36.9±1.5 (*n*=54)
Stem diameter (micrometer units) day 47	12.57±0.23 (*n*=14)	11.65±0.19 (*n*=54)***

Table 2. Effect of high salt and/or low temperature on the germination index of Ins3P synthase-overproducing (*TUR1*) transgenic *Arabidopsis* plants. Seeds of the homozygous *TUR1* transgenic subline R3E1-3 were compared to wild-type C24 seeds, mock-transformed R1 seeds, and *GUSINT* transformed R2 seeds for their germination kinetics at 23 °C and 11 °C in the presence or absence of 200 mM NaCl. For each seed type and treatment 46 seeds were sown aseptically on germination medium and scored each day for 11 days for the presence of green unfolded cotyledons as the criterion of germination. Germination is expressed as the germination index after 11 days which gives maximum weight to seeds that germinate first and less weight to those that germinate later using the formula:

$$\text{germination index} = 11 \times n_1 + 10 \times n_2 + \ldots\ldots\ldots\ldots + 1 \times n_{11}$$

where n_1, n_2, $\ldots n_{11}$ are the number of seeds germinated on the first, second and subsequent days until the 11th day; 11, 10, . . . , and 1 are the weights given to the number germinated on the first, second and subsequent days.

Temperature, °C	Salt	C24 (wild-type)	R1 (mock)	R2 (*GUSINT*)	R3 (*TUR1*)
23	–	366	368	368	368
23	+	352	287	343	326
11	–	193	145	170	170
11	+	118	134	129	119

2. Low temperature decreases the germination rate in all control and transgenic seeds to a similar extent, although the wild-type controls were the least affected and the R1 mock-transformed controls the most affected.
3. There was no dramatic difference seen between the salt-sensitivity of germination in the various lines, although again wild-type control seeds were least affected and R1 mock-transformed controls the most affected.
4. The presence of the overproduced *TUR1*-encoded Ins3P synthase and inositol confer no protection to the inhibiting effect of salt and/or low temperature on seed germination.

We then compared the salt-tolerance of the R3E1-3 *TUR1* transgenic subline to wild-type C24 plants grown in soil. Rosette-stage plants (22 days old) were watered either with tap water or with 200 mM NaCl twice weekly for 16 days. The height, and fresh and dry weights of the aerial parts were measured. Under these conditions, growth of both wild-type and transgenic plants was inhibited by salt treatment: the fresh weights were 33% and 40% of control values, respectively. However, wild-type plants grew consistently slightly better than the transgenics under both watering regimes. There was also no significant difference in water content. In a further experiment, treatment of salt was started earlier, when the plants were only 11 days old (first leaf stage), and 250 mM NaCl was used. Whereas in the previous experiment no visible symptoms were evident, the more severe salt treatment led to leaf necrosis and eventual death of the plants, before flowering. Again, no advantage of increased inositol biosynthesis in the transgenic plants was seen.

Discussion

Increased Ins3P synthase activity leads to elevated levels of inositol

The introduction into *Arabidopsis* of the *S. polyrrhiza TUR1* cDNA encoding Ins3P synthase under the control of the CAMV 35S promoter led to an accumulation of the mRNA transcript and an increase in the respective enzyme activity. At the same time, the endogenous pool of free inositol rose by a factor of over four times relative to the level in wild type plants. The simplest interpretation of these data is that the elevated level of Ins3P synthase activity in the transgenic plants led to an increased flux of G6P into the inositol biosynthetic pathway and thus to an accumulation of inositol. We cannot preclude the possibility that as a result of this primary change other pleitropic effects on inositol metabolism occurred, but whatever the precise response of the entire inositol metabolic network, the manipulation of Ins3P synthase enzyme activity reported here led to an accumulation of inositol. Whether a further increase in inositol levels would be possible by simultaneously overexpressing inositol monophosphatase (IMP) (which catalyses the synthesis of inositol from Ins3P) is unclear, but the recent cloning of this gene should allow this hypothesis to be tested [15]. The generation of plants with an increased pool of free inositol allows the analysis of various questions relating to the purported roles of inositol and inositol-derived metabolites in plants.

Increased inositol levels do not affect plant growth or stress response

The initial basis of our investigation was the finding that the formation of ABA-induced dormant buds (turions) in *S. polyrrhiza* was associated with the induction of Ins3P synthase. Turion formation is associated with a number of molecular and physiological events which could theoretically be linked to an altered inositol metabolism. These include changes in cell extension [27, 32], ABA-linked signal transduction [16], and increased resistance to environmental stress [22]. In our analysis of the transgenic *Arabidopsis* plants with elevated levels of inositol we therefore concentrated on plant characteristics which might be linked to such changes in inositol metabolism.

The analysis revealed that there was no significant difference between the wild type and transgenic plants in the characteristics scored (whole plant growth habit, extension growth, germination rate, flowering time, stem thickness, *in vitro* root growth, and germination and survival on high salt or low temperature regimes). Although it is impossible to discount the possibility that the transgenic plants differed from the wild type in some phenotype not scored for, our results indicate that an increase in Ins3P synthase activity leading to a four-fold increase in inositol levels can be absorbed within general inositol metabolism without apparent detriment or advantage to the plant.

With respect to the increase in Ins3P synthase activity achieved in *Arabidopsis*, the relative increases measured in the transgenic plants are comparable to those observed in *S. polyrrhiza* after ABA treatment and which correlate with an easily observable devel-

opmental response [32], and the inositol levels attained (3.4 μmol/g fresh weight) appear similar to those reported in the ice plant (*Mesembryanthemum crystallinum*) [21] and in tomato [31] induced under salt stress. Therefore, it seems likely that the increase in inositol levels achieved in the transgenic plants had the potential to be metabolically significant.

The lack of observable phenotype in plants with elevated inositol levels has important implications for the recently proposed genetic manipulation of inositol metabolism with the aim of engineering resistance to salt stress [7, 8]. Firstly, it has been suggested that inositol might have a role in protecting tissues against internally generated radicals following stress treatments [for review see 7]. The analysis of the transgenic *Arabidopsis* plants reported here suggests either that the induced increase in inositol was too small to have a protective function or that no such function exists. We tend to favour the latter hypothesis in the light of the fact that overproduction of the acyclic polyol mannitol in transgenic tobacco [33] and *Arabidopsis* [34] to very similar levels as we obtained with inositol did lead to some slight alleviation of the effects of salt stress.

Secondly, it has been suggested that D-pinitol (a methylated derivative of inositol) has a role in osmoprotection [21]. Our results, taken in conjunction with those in the literature [38, 39] indicate that if salt resistance can be conferred by elevated levels of D-pinitol, the engineering of increased flux to this metabolite will require the manipulation of a number of intermediary steps. This may include Ins3P synthase and IMP [15] to produce inositol, and inositol *O*-methyl transferase [39] and D-ononitol epimerase to channel the inositol to D-pinitol. Clearly, although a number of tools for the manipulation of D-pinitol levels are now available, the engineering of this metabolic pathway will not be trivial.

Acknowledgements

We would like to thank Dr Reinhard Töpfer for the pRT104 vector and Dr Sönke Holtorf for the pSH4GUS plasmid and advice on *Arabidopsis* transformation. We also thank Prof. N. Amrhein for his support and encouragement, Dr Dieter Rubli for photographic work and Dr Andrew Fleming for critical reading of the manuscript. This work was supported by grant 31-37414.93 from the Schweizerischer Nationalfonds (to C.C.S.).

References

1. Bachmann M: Metabolism of the raffinose family oligosaccharides in leaves of *Ajuga reptans* L. Ph.D. thesis, University of Zürich (1993).
2. Bartels D, Nelson D: Approaches to improve stress tolerance using molecular genetics. Plant Cell Environ 17: 659–667 (1994).
3. Berridge MJ, Irvine RF: Inositol phosphates and cell signalling. Nature 341: 197-205 (1989).
4. Bevan M: Binary *Agrobacterium* vectors for plant transformation. Nucl Acids Res 12: 8711–8721 (1984).
5. Biffen M, Hanke DE: Metabolic fate of *myo*-inositol in soybean callus cells. Plant Sci 75: 203–213 (1991).
6. Blakeney AB, Harris PJ, Henry RJ, Stone BA: A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydrate Res 113: 291–299 (1983).
7. Bohnert HJ, Jensen RG: Strategies for engineering water-stress tolerance in plants. Trends Biotech 14:89–97 (1996).
8. Bohnert HJ, Nelson DE, Jensen RG: Adaptations to environmental stresses. Plant Cell 7: 1099–1111 (1995).
9. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. Anal Biochem 72: 248–254 (1976).
10. Bray EA: Molecular responses to water deficit. Plant Physiol 103: 1035–1040 (1993).
11. Chandler PM, Robertson, M: Gene expression regulated by abscisic acid and its regulation to stress tolerance. Annu Rev Plant Physiol Plant Mol Biol 45: 113–141 (1994).
12. Cosgrove DJ: Inositol Phosphates: Their Chemistry, Biochemistry and Physiology. Elsevier, Amsterdam (1980).
13. De Koning AJ: Determination of *myo*-inositol and phytic acid by gas chromatography using scyllitol as internal standard. Analyst 119: 1319–1323 (1994).
14. Fry SC: The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Wiley, New York (1988).
15. Gillaspy GE, Keddie JS, Oda K, Gruissem W: Plant inositol monophosphatase is a lithium-sensitive enzyme encoded by a multigene family. Plant Cell 7: 2175–2185 (1995).
16. Gilroy S, Read ND, Trewavas AJ: Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. Nature 346: 769–771 (1990).
17. Han JH, Stratowa C, Rutter WJ: Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning. Biochemistry 26: 1617–1625 (1987).
18. Holsters M, De Waele D, Depicker A, Messens E, Van Montagu M, Schell J: Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163: 182–187 (1978).
19. Holtorf S, Apel K, Bohlmann H: Comparison of different constitutive promoters for the overexpression of transgenes in *Arabidopsis thaliana*. Plant Mol Biol 29: 637–646 (1995).
20. Huang H, Ma H: An improved procedure for transforming *Arabidopsis thaliana* (Landsberg *erecta*) root explant. Plant Mol Biol Rep 10: 372–383 (1992).
21. Ishitani M, Majumder AL, Bornhouser A, Michalowski, CB, Jensen RG, Bohnert HJ: Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. Plant J 9: 537–548 (1996).
22. Jacobs DL: An ecological life-history of *Spirodela polyrrhiza* (greater duckweed) with emphasis on the turion phase. Ecol Monogr 17: 437–469 (1947).

23. Johnson MD: The *Arabidopsis thaliana myo*-inositol 1-phosphate synthase (EC 5.5.1.4). Plant Physiol 105: 1023–1024 (1994).
24. Loewus FA: Inositol biosynthesis. In: Morré DJ, Boss WF, Loewus FA (eds) Inositol Metabolism in Plants, pp. 13–19. Wiley-Liss, New York (1990).
25. Loewus FA, Loewus MW: *Myo*-inositol: its biosynthesis and metabolism. Annu Rev Plant Physiol 34: 137–161 (1983).
26. Logemann J, Schell J, Willmitzer L: Improved method for the isolation of RNA from plant tissue. Anal Biochem 163: 16–20 (1987).
27. Longland JM, Fry SC, Trewavas AJ: Developmental control of apiogalacturonan biosynthesis and UDP-apiose production in a duckweed. Plant Physiol 90: 972-976 (1989).
28. Murata N, Ishizaki-Nishizawa O, Higashi S, Hayashi H, Tasaka Y, Nishida I: Genetically engineered alteration in the chilling sensitivity of plants. Nature 356: 710–713 (1992).
29. Rabel FM, Caputo AG, Butts ET: Separation of carbohydrates on a new polar bonded phase material. J Chromatog 126: 731–740 (1976).
30. Rogers SO, Bendich AJ: Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual, pp. A6/1–A6/10. Kluwer Academic Publishers, Dordrecht (1988).
31. Sacher RF, Staples RC: Inositol and sugars in adaptation of tomato to salt. Plant Physiol 77: 206–210 (1985).
32. Smart CC, Fleming AJ: A plant gene with homology to D-*myo*-inositol-3-phosphate synthase is rapidly and spatially up-regulated during an abscisic-acid-induced morphogenic response in *Spirodela polyrrhiza.* Plant J 4: 279–293 (1993).
33. Tarczynski MC, Jensen RG, Bohnert HJ: Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science 259: 508–510 (1993).
34. Thomas JC, Sepahi M, Arendall B, Bohnert HJ: Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. Plant Cell Environ 18: 801–806 (1995).
35. Töpfer R, Matzeit, V, Gronenborn B, Schell J, Steinbiss H-H: A set of plant expression vectors for transcriptional and translational fusions. Nucl Acids Res 15: 5890 (1987).
36. Valvekens D, Van Montagu M, Van Lijsebettens M: *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* root explants by using kanamycin selection. Proc Natl Acad Sci USA 85: 5536–5540 (1988).
37. Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Rosa M: Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220: 245–250 (1990).
38. Vernon DM, Bohnert HJ: A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum.* EMBO J 11: 2077–2085 (1992).
39. Vernon DM, Tarczynski MC, Jensen RG, Bohnert HJ: Cyclitol production in transgenic tobacco. Plant J 4: 199–205 (1993).
40. Wang H, Qi M, Cutler AJ: A simple method of preparing plant samples for PCR. Nucl Acids Res 21: 4153–4154 (1993).