SALT TOLERANCE IN FLORICULTURE SPECIES: CHARACTERIZATION OF SALT TOLERANCE AND THE CLONING OF A NOVEL PETUNIA GENE INVOLVED IN THE TREHALOSE SUGAR BIOSYNTHESIS (TREHALOSE-6-PHOSPHATE SYNTHASE I) AND EVALUATING ITS POTENTIAL ROLE AS A STRESS OSMOLYTE IN MUTANT YEASTS.

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by
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SALT TOLERANCE IN FLORICULTURE SPECIES: CHARACTERIZATION OF SALT TOLERANCE AND THE CLONING OF A NOVEL PETUNIA GENE INVOLVED IN THE TREHALOSE SUGAR BIOSYNTHESIS (TREHALOSE-6-PHOSPHATE SYNTHASE I) AND EVALUATING ITS POTENTIAL ROLE AS A STRESS OSMOLYTE IN MUTANT YEASTS.

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Cornell University 2011.

The use of saline irrigation water in floriculture production may be inevitable in the long run since the freshwater supply is decreasing over time. Many floriculture species are sensitive to salt accumulation in the root zone especially, to sodium chloride. Symptoms caused by high levels of salt include leaf tip burn, reduced height and yield. In some regions of the United States producers of crops are already facing a limited supply of high quality water. Therefore it is necessary to determine the salt tolerance of commonly used greenhouse bedding plants to minimize potential salt damage before use of nonpotable water sources is mandated.

Initially we screened popular bedding plants for salt tolerance during greenhouse production. The least affected species for dry weight was Snapdragon with a 54% reduction and the most affected was Salvia with 98% reduction as NaCl increased from 0 to 80 mM. Pansy and Zinnia exhibited 100% mortality when exposed to 80 mM NaCl. A classification of the fourteen species is created based on plant dry weight to provide guidance as to which

species could be irrigated with more saline water while not compromising plant growth and quality to large degree.

To determine the effect of NaCl on two *Petunia x hybrida* cultivars in a controlled root environment, a hydroponic experiment was conducted. Plants received NaCl concentrations (0, 20, 40, 60 and 80 mM) for a 4 week exposure period. Whereas Petunia ‘Mitchell Diploid’ receiving 80 mM of NaCl had a 75% reduction in leaf surface area, 65% reduction of total dry weight and 28% reduction of plant width compared to 0 mM, Petunia ‘Bravo White’ had a 64% reduction in leaf surface area, 62% reduction of total dry weight and 34% reduction of width under increasing salinity. Our quantitative models indicate that 20 mM NaCl reduces growth parameters and yet mean separation comparisons indicate growth is not significantly different from the control. Growers may be able to use slightly saline water, such as 20 mM NaCl, for the greenhouse production of petunias without detrimental effects on plant marketability.

Increasing resistance of crops to abiotic stresses is one of the first objectives of plant biotechnology. Alteration in cellular environment leads to protein misfolding and inactivation. Trehalose sugar may prevent physical and chemical instability in proteins that occurs upon desiccation and when exposed to high concentration of salt. Trehalose accumulation plays a stress protection role in many bacteria and fungi. In most plants trehalose levels are low. Both tomato and rice have been transformed with a microbial trehalose-6-phosphate synthase 1 gene (*TPS1*) increasing their tolerance to salt. Inactivation of the *Saccharomyces cerevisiae* TPS1 gene (*ScTPS1*) causes a specific growth defect in the presence of glucose in the medium, associated with deregulation of the initial part of glycolysis. In this work we show how we obtained the coding region of the *Petunia x hybrida* TPS1 (*PhTPS1*). We also truncated the first ~80 amino acids to increase its catalytic activity, as has been reported for *Arabidopsis thaliana TPS1*. In our work, the ability of mutant yeast to grow in glucose as the carbon source was successfully restored by the insertion of *PhTPS1* gene, indicating that *PhTPS1* gene is a functional plant gene capable of complementing trehalose biosynthesis function.

BIOGRAPHICAL SKETCH

Gonzalo Villarino greatly enjoyed backpacking in Patagonia several times; riding his bike alone, hitch-hiking alone and with friends. He has done some awesome backpacking so far in the Adirondacks of New York and other regions. Gonzalo joined Cornell University graduate school in August 2008.

This work is dedicated to my parents, who both successfully beat cancer.

“…only now do I realize how many paths there are to knowledge and that the path of the mind is not the only one and perhaps not even the best one…”

Hermann Hesse, Narcissus and Goldmund. 1930.

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LIST OF ABBREVIATIONS

AA	Amino acid
ABA	Abscisic acid
AtTPS1	*Arabidopsis thaliana* trehalose-6-phosphate synthase 1
BP	Base pair
BW	Petunia ‘Bravo White’
cDNA	Complementary DNA
CDS	Coding sequence
DNTAG	Down tag region
DW	Dry weight
EC	Electrical conductivity
ELP	Electrolyte leakage percentage
FW	Fresh weight
FP	Forward primer
Gal	Galactose
gDNA	Genomic DNA
Glu	Glucose
G6P	Glucose-6-phosphate
Ha	Hectare
LSA	Leaf surface area
MCS	Multiple cloning site
Mat a	Mating type a
MD	Petunia ‘Mitchell Diploid’
mM	millimolar
NaCl	Sodium chloride
NCBI	National center for biotechnology information

PCR	Polymerase chain reaction
PhTPS1	*Petunia x hybrida* trehalose-6-phosphate synthase 1
RP	Reverse primer
RT-PCR	Reverse transcriptase-PCR
ScTPS1	*Saccharomyces cerevisiae* trehalose-6-phosphate synthase 1
ScTPS2	*Saccharomyces cerevisiae* trehalose-6-phosphate synthase 2
SE	Standard error
SPAD	Chlorophyll index
T6P	Trehalose-6-phosphate
TPS1	Trehalose-6-phosphate synthase 1
TPS2	Trehalose-6-phosphate synthase 2
TPP	Trehalose-6-phosphate phosphatase
UDP-glucose	Uridine diphosphate glucose
UPTAG	Up tag region

CHAPTER 1

ASSESSING TOLERANCE TO SODIUM CHLORIDE SALINITY IN FOURTEEN FLORICULTURE SPECIES.

Keywords: Salt stress, osmotic stress, bedding plants.

Abstract

The use of saline irrigation water may be inevitable in the long run since the freshwater supply is decreasing over time. In some regions of the United States producers of both ornamental and agronomic crops are already facing a limited supply of high quality water. Given these facts, it is necessary to determine the salt tolerance of commonly used greenhouse bedding plants to minimize potential salt damage before use of nonpotable water sources is mandated. Research screening bedding plants has not taken place for more than two decades. Therefore, we undertook experiments to screen popular bedding plants for salt tolerance during greenhouse production. In this work, we screened fourteen floriculture species and classified them based on their relative tolerance to sodium chloride. Pansy and Zinnia, the most sensitive species examined, exhibited 100% mortality when exposed to 80 mM NaCl. The least affected species for dry weight was Snapdragon with a 54% reduction as NaCl increased from 0 to 80 mM. Only Fuchsia and Snapdragon were unaffected by 20 mM NaCl and in comparing the control to 40 mM NaCl all of the species had a significantly reduced dry weight. Verbena, Petunia, Coleus and Begonia were the only species that did not undergo a significant height reduction in comparing the control to 40 mM of NaCl. A classification of the fourteen species is created here based on plant dry weight to provide

guidance as to which species could be irrigated with more saline water while not compromising plant growth and quality.

Introduction

Water quality and conservation are at the forefront of public environmental concerns; hence greenhouse operations particularly in Europe, Israel and California are reorganizing and investing in their irrigation practices (Morgan and Reed, 1998). As supply of high-quality water decreases overtime growers are being forced to irrigate their crops with poor quality water and/or saline water (Lieth and Burger, 1989). Use of recycled water to irrigate floriculture bedding plants may be inevitable in the long run not merely because freshwater supply is decreasing over time but also because the population continues to grow (Niu and Rodriguez, 2006). In regions lacking outlets for agricultural drainage disposal or where water supply is scarce the recycling of drainage waters for irrigation is increasingly seen as a viable management option (Skaggs et al., 2006).

Treated municipal effluent has been used for irrigation of golf courses and some horticulture crops, particularly citrus orchards, in many areas of the United States (Niu and Rodriguez, 2006). For example, in Florida 92,345 ha of land was irrigated with reclaimed municipal water in 2005; 6,144 ha was agricultural land and the rest was used for irrigating golf courses and landscapes (Morgan et al., 2008).

Besides reclaimed municipal water there are additional sources of salt in irrigation water. Road de-icing salts in northern locations can salinize irrigation water (Lerner, 2006) hampering plant production. Intrusion of sea water into underground aquifers is also a severe problem in some coastal areas (Lerner, 2006). The need to develop new, innovative and more efficient agricultural water management systems is essential to developing sustainable irrigation (Skaggs et al., 2006).

Due to the current and increasing use of saline irrigation water it is imperative for the floriculture industry to determine the salt tolerance of commonly used greenhouse bedding plants to minimize potential salt damage before the use of nonpotable water sources are mandated. Such research has been conducted for five herbaceous perennials grown in the landscape in the arid and semiarid southwestern U.S. (Niu and Rodriguez, 2006). To our knowledge the last significant screening of herbaceous annuals (bedding plants) took place over two decades ago (Tija and Rose, 1987). Clearly, plant materials have changed greatly over the intervening years justifying another look at salt tolerance in bedding plants.

In this work we characterized the response during greenhouse production of fourteen popular floriculture species exposed to different levels of sodium chloride salinity in the irrigation water. We aim to find a distinct pattern so that we may classify species based on how sensitive they are for practical management guidelines.

Materials and Methods

Plant material and treatments

A greenhouse experiment was conducted at Cornell University, Ithaca New York, USA (42.4 °N latitude) from 11 February to 15 April 2010. Fourteen floriculture species were used for the experiment; *Antirrhinum majus* L. ‘Florini Amalia Yellow’, *Begonia hiemalis* Fotsch. ‘Solenia Light Pink’, *Calendula officinalis* Hoen. ‘Bon Bon Yellow’, *Catharanthus roseus* (L.) G.Don

‘Mediterranian Polka Dot’, *Euphorbia chamaesyce* Baill. ‘White Manaus’, *Fuchsia hybrida* Hort. ‘Trailing Dark Eyes’, *Impatiens walleriana* Hook.f. ‘Super Elfin XP White’, *Pelargonium x hortorum* (L.H. Bailey) ‘Classic Scarlet’, *Petunia x hybrida* Hort. ex E.Vilm ‘Ramblin White’, *Salvia splendens* Ker Gawl. ‘Vista Red’, *Solenostemon scutellarioides* (L.) Codd ‘Stained Glassworks Copper’, *Verbena x hybrida* Hort. ex Vil. ‘Lanai Dark Red’, *Viola tricolor* L. ‘Delta Blue Blotch’, *Tagetes patula* L. ‘Crested Bonanza Bolero’, *Zinnia angustifolia* Kunth ‘Star Gold’.

Plug/liner trays were received from a commercial propagator (C. Raker and Sons Inc., Litchfield MI) and were held in trays for 2 weeks in the greenhouse while being watered with municipal tap water along with fertilizer prepared at 150 mg•L-1 N from 20N-2.2P-16.6K (Jack’s Professional LXTM Water Soluble Fertilizer 21-5-20 All-Purpose, J.R. Peter’s Inc. Allentown, PA).

A total of 350 plants (14 species X 5 treatments X 5 replicates) were transplanted into 10 cm pots containing soil-less media (Metromix 280, Sun Gro Horticulture LTD., Vancouver, Canada). Plants were irrigated daily manually turning on a drip-irrigation system until water just began to leach out of the bottom of containers, i.e. leaching fraction ca. 5%. At each irrigation event plants received the same commercial fertilizer prepared as described above.

One week after transplanting the salt treatments were commenced. Five salt treatments 0 (control), 20, 40, 60 and 80 mM of NaCl were prepared in 525 L tanks along with the commercial fertilizer. Treatments were randomly divided so that each bench (14 x 5 ft) contained a reservoir with one salinity treatment. Within each treatment, five plants were randomly sampled each week for electrical conductivity (EC) and pH measurements. These measured using an EC meter (OAKTON Instruments, Vernon Hills, IL) and pH meter (pHTestr 20, OAKTON Instruments, Vernon Hills, IL), using the pour-thru method following the protocol of Cavins et al. (2000).

Six weeks after transplanting (five weeks after salt treatments commenced) final PourThru pH/EC data were recorded from each individual plant. Destructive harvest was used to obtain total shoot fresh weight (FW). Total dry weight (DW) was recorded after plants were oven-dried at 80 °C for 4 d. Plant height (from the soil interface to the tallest part of the plant) and plant width (average of two measurements – the widest part of the plant and width at 90° from this location) were also recorded.

To be able to compare the salinity effects from one species to another we compared the percent reduction in measured parameters as NaCl increased from 0 to 80 mM. For a given species, percent DW reduction was calculated as: (Average DW(0 mM) – Average DW(80 mM)) / Average DW(0 mM).

Experimental design and statistical analysis

The experiment followed a completely randomized design with five NaCl treatments, fourteen plant species and five replicates (1 plant in 1 container) per species by treatment combination. Analysis of variance was used to determine significant NaCl treatment effects on the measured parameters (JMP Version 8, SAS Institute, Cary, NC). Where significant differences were found, Tukey’s Honestly Significant Difference was used to determine mean separations within species across salt treatment.

Results

Whereas the control treatment (0 mM NaCl) EC remained relatively stable throughout the experiment EC increased across the weekly periods for the added salt treatments (Figure 1.1). Conversely, pH remained stable across the treatment period and averaged 6.0 (Figure 1. 2).

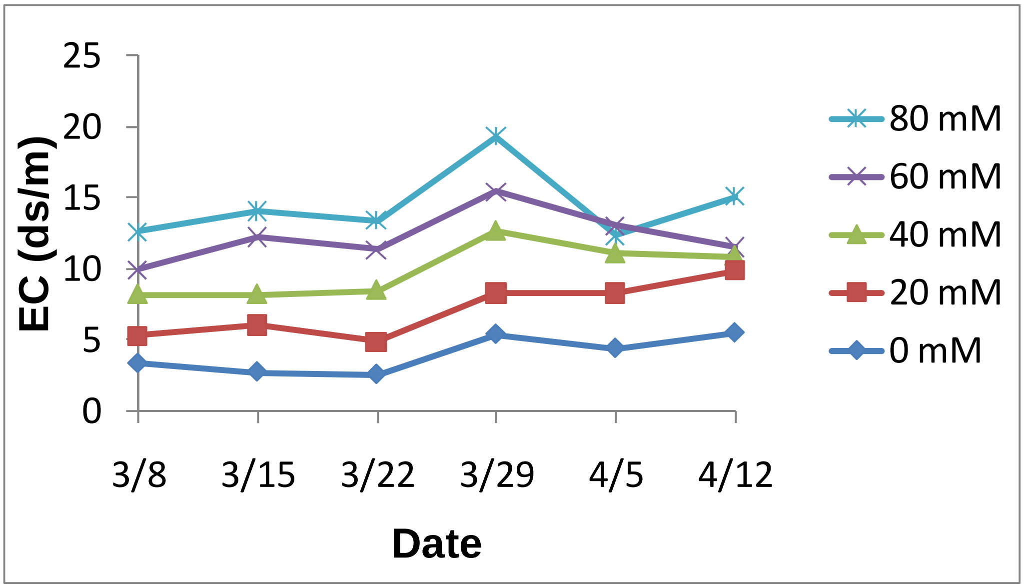

Figure1.1. The effect of increasing NaCl concentration in irrigation water during a five week treatment period on container leachate electrical conductivity (EC). Data are means of five randomly selected plants per NaCl treatment.

Plant fresh weight (FW), dry weight (DW), width and height were significantly reduced with exposure to increasing NaCl for all species. In terms of DW percent decrease the least affected species was Snapdragon with a 54% reduction and the most affected was Salvia with 98% reduction as NaCl

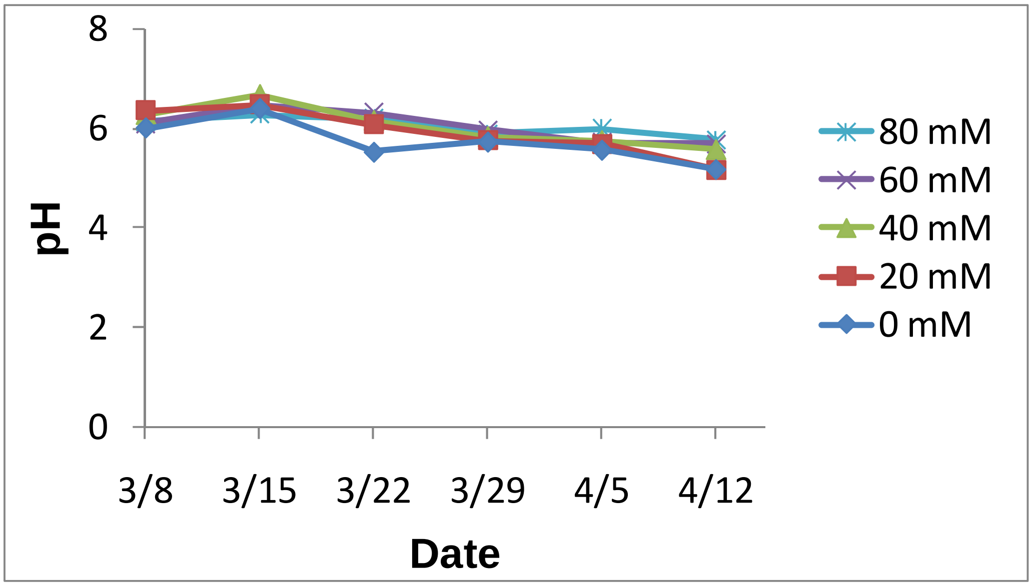

Figure 1.2. The effect of increasing NaCl concentration in irrigation water during a five week treatment period on container leachate pH. Data are means of five randomly selected plants per NaCl treatment.

increased from 0 to 80 mM. Only the DW of Fuchsia and Snapdragon was not significantly reduced as NaCl increased from 0 to 20 mM. While comparing control to 40 mM NaCl all of the species had a significantly reduced DW (Table 1.1). In terms of DW, 60 mM was detrimental to all species examined; interestingly only five species seemed to be further damaged when salt concentration was further increased to 80 mM (Table 1.1), which may be due to the fact that PourThru EC values were pretty similar between these 2 treatments by the end of the experiment, indicating that the 80 mM treatment did not accumulate significantly greater salt than the 60 mM treatment. Zinnia stands out as an example of plants that can be damaged visually with exposure to even 20 mM NaCl as with this treatment all plants exhibited marginal necrosis on lower leaves.

Table 1.1. Dry weight (g) response of fourteen species to increasing NaCl concentration in the irrigation water for a five week treatment period. Data are means ± SE of five plants.

Dry Weight	Salt concentration					
Species	*0 nm NaCL*	*20 nm NaCL*	*40 nm NaCL*	*60 nm NaCL*	*80 nm NaCL*	*P-Value Anova*
Begonia	3.37 ± 0.15 a[z]	1.76 ± 0.15 b	1.67 ± 0.15 b	1.00 ± 0.15 c	0.89 ± 0.15 c	<.0001*
Coleus	5.23 ± 0.24 a	2.79 ± 0.24 b	2.84 ± 0.19 b	2.22 ± 0.19 b	0.88 ± 0.19 c	<.0001*
Euphorbia	7.63 ± 0.21 a	2.02 ± 0.21 b	0.64 ± 0.21 c	0.51 ± 0.21 c	0.41 ± 0.23 c	<.0001*
Fuchsia	7.77 ± 0.44 a	6.73 ± 0.44 a	2.92 ± 0.38 b	2.39 ± 0.34 b	1.69 ± 0.34 b	<.0001*
Geranium	9.64 ± 0.56 a	3.28 ± 0.56 b	4.78 ± 0.56 b	3.33 ± 0.65 b	2.67 ± 0.50 b	<.0001*
Impatiens	7.96 ± 0.14 a	4.90 ± 0.16 b	2.45 ± 0.12 c	1.10 ± 0.12 d	0.55 ± 0.12 e	<.0001*
Marigold	5.76 ± 0.21 a	3.92 ± 0.21 b	3.12 ± 0.23 b	1.88 ± 0.23 c	0.71 ± 0.47 c	<.0001*
Pansy	4.07 ± 0.13 a	2.29 ± 0.16 b	0.50 ± 0.16 c	0.37 ± 0.20 c	.	<.0001*
Petunia	16.03 ± 0.29 a	13.11 ± 0.37 b	10.04 ± 0.29 c	8.93 ± 0.37 c	7.14 ± 0.32 d	<.0001*
Salvia	7.81 ± 0.19 a	4.62 ± 0.24 b	1.77 ± 0.21 c	0.57 ± 0.19 d	0.19 ± 0.30 d	<.0001*
Snapdragon	6.57 ± 0.26 a	6.89 ± 0.23 a	4.03 ± 0.26 b	3.88 ± 0.37 b	2.978 ± 0.23 b	<.0001*
Verbena	12.15 ± 0.40 a	9.35 ± 0.63 b	5.57 ± 0.40 c	6.44 ± 0.40 c	3.48 ± 0.40 d	<.0001*
Vinca	5.17 ± 0.15 a	3.63 ± 0.12 b	2.66 ± 0.12 c	2.18 ± 0.12 c	1.43 ± 0.12 d	<.0001*
Zinnia	3.36 ± 0.34 a	1.66 ± 0.34 b	0.46 ± 0.76 b	0.29 ± 0.76 b	.	0.0007 *

[z] Values within a row (species) followed by the same letter are not significantly different at P=0.05 by Tukey's HSD test.

For FW the least affected species were Petunia and Snapdragon, both exhibited a 52% reduction as NaCl increased from 0 to 80 mM. Pansy and Zinnia were the most sensitive species exhibiting 100% mortality at the highest NaCl treatment. Salvia was also very sensitive, fresh weight was reduced by 96% as NaCl increased from 0 to 80 mM. While comparing control to 40 mM NaCl treatment all of the species exhibited a significantly reduced FW (Table 1.2). Only Coleus, Pansy, Verbena and Zinnia showed further damage when salt concentration increased from 60 to 80 mM (Table 1.2).

Overall, plant width was slightly less affected to increasing NaCl treatment than DW and FW parameters. Begonia, Euphorbia, Petunia, Snapdragon and Verbena did not show a significant reduction of width when salt increased from 0 to 20 mM whereas both FW and DW of these species was significantly reduced when treatment increased from 0 to 20 mM (Table 1.3). The least affected species was Petunia with a reduction of 34% and the most affected was Salvia with 92% as NaCl increased from 0 to 80 mM. For all of the species (expect Begonia) there was a significant reduction of width when comparing control to 40 mM of NaCl. Further damage as NaCl increased from 60 to 80 mM was seen only in Impatiens, Pansy, Salvia, Zinnia and Verbena (Table 1.3).

Table 1.2. Fresh weight (g) response of fourteen species to increasing NaCl concentration in the irrigation water for a five week treatment period. Data are means ± SE of five plants.

Fresh Weight	Salt concentration					
Species	*0 nm NaCL*	*20 nm NaCL*	*40 nm NaCL*	*60 nm NaCL*	*80 nm NaCL*	*P-Value Anova*
Begonia	72.19 ± 4.16 a	54.21 ± 4.16 a	33.75 ± 4.16 b	14.46 ± 4.16 bc	11.73 ± 4.16 c	<.0001*
Coleus	74.43 ± 3.51 a	41.83 ± 3.51 b	43.69 ± 2.71 b	34.41± 2.71 b	14.51 ± 2.71 c	<.0001*
Euphorbia	53.71 ± 1.62 a	18.32 ± 1.62b	6.79 ± 1.62c	4.79 ± 1.62 c	3.31 ± 1.82c	<.0001*
Fuchsia	60.15 ± 3.32 a	40.44 ± 3.32 b	26.62 ± 2.88 c	19.78 ± 2.57 c	14.81 ± 2.57 c	<.0001*
Geranium	103.82 ± 7.05 a	42.91 ± 4.99 b	45.4 ± 4.46 b	16.75 ± 4.99 c	27.44 ± 4.46 bc	<.0001*
Impatiens	162.55 ± 6.37 a	127.77 ± 7.12 b	55.95 ± 6.37 c	36.51 ± 6.37 cd	16.54 ± 6.37 d	<.0001*
Marigold	54.26 ± 2.19 a	38.40 ± 2.19 b	25.41 ± 2.46 c	16.16 ± 2.46 cd	3.70 ± 4.92 d	<.0001*
Pansy	45.04 ± 1.96 a	18.07 ± 1.96 b	6.57 ± 1.96 c	1.67 ± 1.96 c	.	<.0001*
Petunia	48.18 ± 2.65 a	48.68 ± 3.42 b	28.96 ± 2.65 c	26.76 ± 2.96 d	23.13 ± 2.65 d	<.0001*
Salvia	68.43 ± 1.67 a	47.03 ± 1.93 b	13.35± 1.67 c	3.67 ± 1.50 d	3.37 ± 1.93 d	<.0001*
Snapdragon	48.18 ± 2.16 a	48.68 ± 1.93 a	28.96 ± 2.50 b	26.76 ± 3.06 b	23.13 ± 1.90 b	<.0001*
Verbena	58.20 ± 2.83 a	49.63 ± 4.47 a	32.44 ± 2.83 b	33.47 ± 2.83 b	17.60 ± 2.83 c	<.0001*
Vinca	36.46 ± 1.09 a	26.49 ± 1.25 b	19.82 ± 0.97 c	16.19 ± 0.97 cd	12.28 ± 0.97 d	<.0001*
Zinnia	30.61 ± 3.40 a	13.21 ± 3.40 b	1.60 ± 7.60 b	1.76 ± 7.60 b	.	0.0012*

[z] Values within a row (species) followed by the same letter are not significantly different at P=0.05 by Tukey's HSD test.

Table 1.3. Plant width (cm) response of fourteen species to increasing NaCl concentration in the irrigation water for a five week treatment period. Data are means ± SE of five plants.

Plant Width	Salt concentration					
Species	*0 nm NaCL*	*20 nm NaCL*	*40 nm NaCL*	*60 nm NaCL*	*80 nm NaCL*	*P-Value Anova*
Begonia	13.88 ± 0.94 a	11.00 ± 0.94 a	10.00 ± 0.94 ab	6.05 ± 0.94 c	7.55 ± 0.94 bc	0.0007*
Coleus	25.25 ± 0.76 a	18.83 ± 0.87 b	14.60 ± 0.67 c	12.35 ± 0.67 cd	9.10 ± 0.67 d	<.0001*
Euphorbia	36.33 ± 2.03 a	30.40 ± 2.03 a	21.00 ± 2.03 b	18.40 ± 2.03 b	14.00 ± 2.27 b	<.0001*
Fuchsia	44.00 ± 1.52 a	33.63 ± 1.52 b	28.67 ± 1.76 b	20.40 ± 1.36 c	17.80 ± 1.36 c	<.0001*
Geranium	28.13 ± 0.69 a	17.5 ± 0.79 b	16.75 ± 0.69 b	14.00 ± 0.97 c	14.60 ± 0.61 bc	<.0001*
Impatiens	31.30 ± 0.60 a	23.75 ± 0.94 b	18.08 ± 0.77 c	15.30 ± 0.60 c	11.00 ± 0.77 d	<.0001*
Marigold	17.90 ± 0.63 a	15.10 ± 0.63 b	13.25 ± 0.70 bc	10.94 ± 0.70cd	7.50 ± 1.40 d	<.0001*
Pansy	19.98 ± 0.54 a	14.00 ± 0.76 b	7.25 ± 0.54 c	4.75 ± 0.76 c	.	<.0001*
Petunia	43.33 ± 0.69 a	40.83 ± 0.69 ab	44.38 ± 0.59 bc	31.38 ± 0.59 bc	28.45 ± 0.53 c	<.0001*
Salvia	20.08 ± 0.76 a	16.81 ± 0.66 b	9.50 ± 0.59 c	5.55 ± 0.59 d	1.67 ± 0.76 e	<.0001*
Snapdragon	17.90 ± 0.63 a	15.10 ± 0.63 a	13.25 ± 0.81 b	10.94 ± 1.00 b	7.50 ± 0.70 b	<.0001*
Verbena	43.50 ± 1.48 a	43.75 ± 2.10 a	31.90 ± 1.33 bc	36.67 ± 1.71 ab	28.13 ± 1.48 c	<.0001*
Vinca	22.50 ± 0.76 a	17.38 ± 0.76 b	15.55 ± 0.67 bc	14.25 ± 0.67 cd	11.92 ± 0.87 d	<.0001*
Zinnia	25.50 ± 1.15 a	16.58 ± 1.13 b	.	7.75 ± 2.30 c	.	0.0007*

[z] Values within a row (species) followed by the same letter are not significantly different at P=0.05 by Tukey's HSD test.

Begonia was the least affected species for plant height; it was not significantly reduced as NaCl increased from 0 to 80 mM (Table 1.4). The most affected species was Pansy with a 49% reduction as NaCl increased from 0 to 60 mM. Similar to plant width, not all the species showed a significant reduction in height as NaCl increased from 0 to 20 mM. For Begonia, Fuchsia, Marigold, Petunia, Snapdragon, Verbena and Vinca there was not a significant reduction in height (whereas for both FW and DW there was significant reduction when treatment increased from 0 to 20 mM NaCl). Only Begonia, Coleus, Fuchsia, Petunia and Verbena exhibited a significant reduction in height at 40 mM NaCl as compared with 0 mM NaCl; in this regard, height was the least affected parameter. As NaCl increased from 60 to 80 mM only Zinnia, Snapdragon, Petunia, Pansy and Coleus exhibited further damage (Table 1.4).

A classification of the fourteen species would be useful to growers to provide them some guidance as to which species could be irrigated with more saline water while not compromising plant growth and quality to large degree. We develop a classification based on the percent reduction in DW at the 0 versus 80 mM treatments as described in Table 1.5. Although the specific thresholds used were arbitrarily assigned, these correlated to four clusters of species noted in Table 1.5. Photographs at harvest taken from one species representative of each of the four groups are presented in Figure 1.3.

Table 1.4. Plant height (cm) response of fourteen species to increasing NaCl concentration in the irrigation water for a five week treatment period. Data are means ± SE of five plants.

Plant height	Salt concentration					
Species	*0 nm NaCL*	*20 nm NaCL*	*40 nm NaCL*	*60 nm NaCL*	*80 nm NaCL*	*P-Value Anova*
Begonia	17.25 ± 0.98 a	16.75 ± 0.98 a	13.13 ± 0.98 a	15.67 ± 0.98 a	16.88 ± 0.98 a	0.4073
Coleus	24.75 ± 0.78 a	19.33 ± 0.90 bc	22.90 ± 0.69 a	22.40 ± 0.69 ab	17.80 ± 0.69c	<.0001*
Euphorbia	34.60 ± 1.24 a	29.25 ± 1.24 b	24.25 ± 1.24 bc	25.40± 1.24 bc	23.33± 1.38 c	<.0001*
Fuchsia	32.00 ± 2.29 ab	32.00 ±1.99 a	23.25 ±1.99 bc	22.20 ±1.78 c	20.00 ±1.78 c	0.0009
Geranium	20.75 ± 0.98 a	18.75 ± 0.98 b	18.40 ± 0.88 b	17.50 ± 0.88 b	16.90 ± 0.88 b	0.0043*
Impatiens	19.90 ± 0.41 a	16.83 ± 0.53 b	16.25 ± 0.46 b	15.30 ± 0.41 b	15.17 ± 0.53 b	0.4073
Marigold	19.30 ± 0.49 a	18.00 ± 0.49 ab	16.50 ± 0.55 b	16.13 ± 0.55 bc	12.50 ± 1.10c	0.0003
Pansy	20.50 ± 0.37 a	15.33 ± 0.42 b	12.17 ± 0.42 c	10.50 ± 0.52 c	.	<.0001*
Petunia	20.75 ± 2.87 ab	18.75 ± 2.22 b	18.40 ± 2.48 a	17.50 ± 2.48 c	16.90 ± 2.22 d	0.0018
Salvia	19.67 ± 0.39 a	20.75 ± 0.34 a	15.20 ± 0.30 b	11.83 ± 0.39 c	12.33 ± 0.39 c	<.0001*
Snapdragon	19.40 ± 0.59 a	19.00 ± 0.76 ab	16.50 ± 0.66 bc	14.33 ± 0.76 c	18.00 ± 0.76 ab	0.0014
Verbena	12.25 ± 0.21 ab	13.00 ± 0.24 a	13.17 ± 0.24 a	11.88 ± 0.21b	11.50 ± 0.24b	0.0013*
Vinca	18.88 ± 0.59 a	17.50 ± 0.59 ab	15.30 ± 0.53 bc	14.83 ± 0.69 bc	14.60 ± 0.53 c	<.0001*
Zinnia	21.90 ± 0.46 a	15.83 ± 0.59 b	.	13.00 ± 1.03 b	.	0.0014*

^{z}Values within a row (species) followed by the same letter are not significantly different at P=0.05 by Tukey's HSD test.

Table 1.5. Classification of 14 bedding plant species based on tolerance to 80 mM NaCl in the irrigation water during a five week treatment period in the greenhouse.

	Species	**DW reduction with or 80mM NaCl**
Most Tolerant	Snapdragon & Petunia	DW reduced ≥ 50% and < 70%
Somewhat Tolerant	*Begonia, Fuchsia,* Coleus, Marigold, *Vinca, Verbena and Geranium*	DW reduced ≥ 70% and < 90%
Moderately Sensitive	*Impatiens, Euphorbia and Salvia*	DW reduced by ≥ 90% at 80 mM
Extremely Sensitive	*Zinnia* and Pansy	100% mortality at 80 mM NaCl

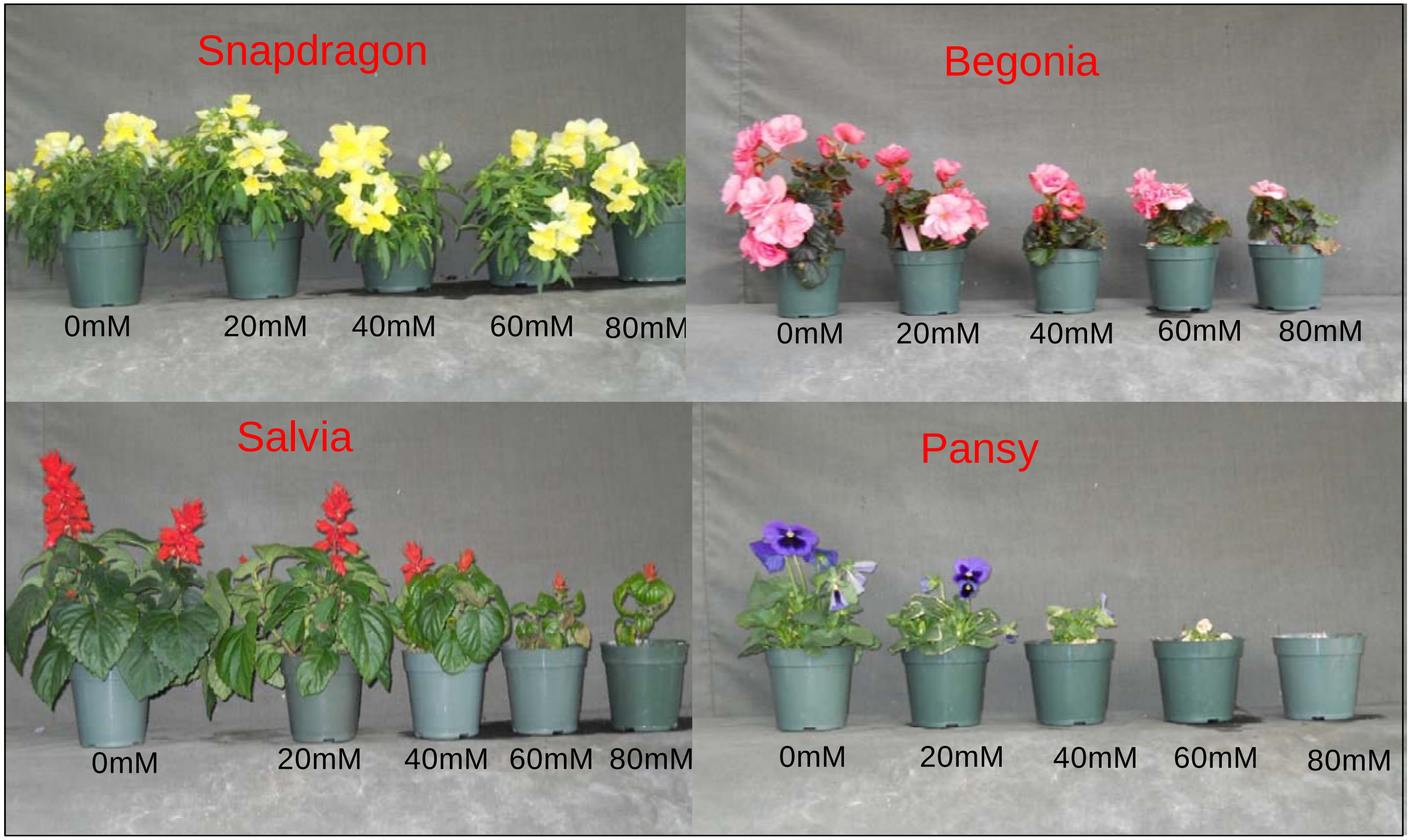

Figure 1.3. Pictures of species representative of the four salt tolerance groups as described in Table 5: Snapdragon (most tolerant), Begonia (somewhat tolerant), Salvia (moderately sensitive) and Pansy (extremely sensitive).

Discussion

Due to the high value of both ornamentals and floriculture species it is imperative to determine the salt tolerance of popular bedding plants during greenhouse production. The results can be used by greenhouse operations to select plants suitable for their water's salinity.

A similar experiment was carried out in containerized plants growing in soil-less substrate in two greenhouses in Texas by Niu and Rodriguez (2006) with five landscape herbaceous perennials: *Achillea millefolium* L. (yarrow), *Agastache cana* (Hook.) Woot. & Standl. (Wild hyssop), *Echinacea purpurea* (L.) Moench (purple cone-flower), *Gaillardia aristata* Foug.(firewheel), and *Salvia coccinea* Juss ex J (scarlet sage). In the summer experiment shoot dry weight of *A. millefolium*, *A. cana* and *G. aristata* were significantly less at 20 or 40 mM NaCl ascompared with the control (0 mM). In the fall experiment shoot dry weight of A*. millefolium and G. aristata* were reduced at 20 and 40 mM NaCl as compared with the control whereas that of *Agastache cana, Echinacea purpurea* and *Salvia coccinea* were similar among the treatments (Niu and Rodriguez, 2006). Therefore, environmental conditions played a role in salt tolerance of *Achillea millefolium, Gaillardia aristata* and *Agastache cana.*

The relative salt tolerance among their tested species was generally consistent. *A. millefolium*, *G*. *aristata and S*. *coccinea* had relatively high salt tolerance*:* at 40 mM NaCl they were about 30% smaller in height than the control. They might be irrigated with saline solution up to 40 mM under both summer and fall condition in the greenhouse with acceptable performance. *A. cana* and *E. purpurea* were sensitive and should not be irrigated with water ≥ 20 mM NaCl (Niu and Rodriguez, 2006).

Within a given genus, species can vary in their salt tolerance. For example, Morgan and Reed (1998) found for New Guinea Impatiens (Impatiens *hawkeri* ‘Barbados’) shoot FW was reduced by 75% as salinity increased from 0 to 14 mol x m^{-3} (14 mM) against their control. In our experiment FW of the related species, *I. walleriana* was reduced by only 21% as salinity increased from 0 to 20 mM.

Sooneveld and Voogt (1983) carried out a greenhouse experiment to determine the effects of NaCl on yield of five cut flower species. *Dianthus caryophyllus* L. and *Chrysanthemum ×morifolium* Ramat. proved to be the least sensitive to NaCl; cut flower yield was reduced by only 6.9% when exposed to 40 mM NaCl. *Gerbera jamesonii* Bolus ex Hook. f. and *Hippeastrum vittatum* (L'Hér.) Herbert were moderately sensitive to salt exhibiting a yield reduction of 12% and 18%, as salinity increased from 0 to 40 mM, respectively. *Anthurium andreanum* was the most sensitive, exhibiting a 22% reduction in yield of cut flowers as salt increased from 0 to 40 mM.

Tjia and Rose (1987) classified 45 horticulture bedding plants in an area on the beach front where they were exposed to salt spray and also irrigated with well water with high salt content. Of those species, 11 overlap with the 14 species we studied. Our findings are relatively similar for the species studied: Pansy did poorly; Marigold, Geranium, Vinca, Verbena and Begonia were moderately tolerant; and Petunia was quite tolerant. Some differences in results were found: Impatiens and Salvia (moderately sensitive in our experiment and fairly resistant in theirs); and Coleus (moderately tolerant in our experiment and sensitive in theirs). The differences between our studies may stem from the fact that we grew plants in highly controlled environment in greenhouse whereas they grew plants in a landscape. Further, the specific cultivars used in the two experiments was different.

Conclusions

Overall our experimental results suggest that some bedding plant species can be grown in moderately saline irrigation water. If a grower has access to only saline water they could use it in crops such as Snapdragon, Petunia, Verbena and Geranium and still have fairly high plant growth whereas other species will demand high quality water in order to have a successful production, especially Zinnia, Pansy, Impatiens, Euphorbia and Salvia. For the rest of the species screened moderately saline water might be applied (perhaps < 20 mM) with a slight reduction in growth characteristics, but still producing marketable bedding plants.

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CHAPTER 2

THE IMPACT OF SODIUM CHLORIDE ON GROWTH, MORPHOLOGY AND PHYSIOLOGY OF PETUNIA *x HYBRIDA* ‘BRAVO WHITE’ AND ‘MITCHELL DIPLOID’.

Keywords: Salt stress, osmotic stress, *Petunia x hybrida,* NaCl.

Abstract

Increasing salinization of irrigation water and soils is one of the major abiotic stresses reducing agricultural productivity. Many floriculture species are also sensitive to salt accumulation in the root zone, especially to sodium chloride, one of the predominant salts that accumulate in soils and substrates. Symptoms caused by high levels of salts include leaf tip burn, osmotic effects such as reduced height or leaf surface area and ultimately reduced plant growth and yield. To determine the effect of sodium chloride on two *Petunia x hybrida* cultivars in a controlled root environment a hydroponic experiment was conducted. Plants were established in 4 L buckets filled with a modified Hoagland’s solution. Subsequently, plants received sodium chloride concentrations (0, 20, 40, 60 and 80 mM) for a 4 week exposure period. Whereas Petunia ‘Mitchell Diploid’ receiving 80 mM of sodium chloride had a 75% reduction in leaf surface area, 65% reduction of total dry weight and 28% reduction of plant width compared to 0 mM, Petunia ‘Bravo White’ had a 64% reduction in leaf surface area, 62% reduction of total dry weight and 34% reduction of width under increasing salinity. Our quantitative models indicate that 20 mM sodium chloride, the lowest salt treatment studied, reduces growth parameters and yet mean separation comparisons indicate growth is not

significantly different from the control. Growers may be able to use slightly saline water, such as 20 mM sodium chloride, for the greenhouse production of petunias without detrimental effects on plant marketability.

Introduction

Soil salinity is a major constraint to the successful use of agricultural lands for crop production, affecting 7% of the world's land area, equivalent to 930 million ha (Mann, 2002). How to effectively use saline soils for agriculture has received worldwide attention (Zhu et al., 2004). Similarly, the impact of saline irrigation water and subsequent salinization of root-zones are of great importance in production of containerized plants due to the ongoing reduction of high quality irrigation water (Lee and van Iersel, 2008). Therefore, reducing the spread of salinization as well as understanding and most importantly increasing the salt tolerance of high yielding crops are important issues across the world (Munns, 2002).

There are several potential ways that irrigation water can become saline. In some regions well water is naturally high in salts due to mineral deposits in contact with the aquifer (Stewart, 2006). Road de-icing salts in northern locations can salinize irrigation water (Lerner, 2006). Saltwater intrusion of well water is also an important problem in coastal areas. Intrusion can be defined as "the movement of saline water into freshwater aquifers and most often is caused by ground water pumping from coastal wells" (Barlow, 2005). This phenomenon occurs not only along coastal parts of the United States but also in other parts of the world (Lee and van Iersel, 2008). Finally, as availability of high quality (low salinity) water declines, reclaimed municipal water is increasingly being proposed for irrigation. For example, in Florida

92,345 ha of land was irrigated with reclaimed municipal water in 2005, primarily golf courses and landscapes were irrigated from this water, but also 6,144 ha of agricultural land (Morgan et al., 2008).

Salt composition of water varies across regions. The dominant cations are typically Na^+, Ca^{2+} and Mg^{2+} while the dominant anions are Cl^-, SO_4^{2-} and HCO_3^- (Grattan and Grieve, 1999). Sodium along with calcium are often the predominant cations exposed to horticultural crops in irrigation water or soil solutions with $Na^+/(Na^+ + Ca_2^+)$ in the range of 0.1 to 0.7 (Carter and Grieve, 2006).

Sodium chloride (NaCl) is a compound in which ions are held together in a lattice structure by ionic bonds. Water readily dissolves many crystalline salts by hydrating their component ions; the NaCl crystal lattice is disrupted as water molecules cluster about the Cl^- and Na^+ ions (Lehninger; Nelson and Cox, 2008) suspending the ions in solution.

The role of Na^+ in plants is not yet fully understood and it is not clear whether or not it is required by all plants. Nonetheless, for C4 and CAM plants Na^+ is considered a micronutrient for regenerating phosphoenolpyruvate, the substrate for the first carboxylation step in C-fixation (Epstein and Bloom, 2005). In greater quantities Na^+ can also replace some of the osmotic functions that potassium serves. The osmotic potential of the leaf at full turgor is not affected by the replacement of K^+ with Na^+ though the extent to which K^+ can be replaced by Na^+ in metabolic processes varies with plant families and species (Subbarao et al., 1999).

Chlorine (Cl) is found primarily as the chloride ion (Cl^-). Trace amounts of Cl^- are required by plants to serve in activation of the photsystem II enzyme in photosynthesis and greater quantities of Cl^- can serve as a cellular osmoticum (Epstein and Bloom, 2005).

Plant drought stress and saline stress effects are quite similar during the onset of stress. The rapid reduction in growth occurring across seconds or minutes is mainly due to changes in cell water relationships and not due to toxic amounts of Na^+ or Cl^- in cells (Munns, 2002). This initial decrease of cellular expansion in both leaves and roots occurs quickly and transiently allowing a rapid recovery thereafter indicating that it must be due to changes in cell water relations (Passioura and Munns, 2002). Inhibition of root growth in maize under salinity is due to reduction in the length of root tip elongation zone (Zidan et al., 1990).

Over the timescale of days exposure to salt stress leads to a decrease in cell division rate first in leaves and eventually also in roots. This is attributed with osmotic stress rather than a salt-specific effect (Munns, 2002). Evidence also suggests that hormonal signals - for both water and salt stress - are controlling growth at this point (scale of days) rather than water deficit or ion toxicity. The evidence for this is that leaf expansion over 24 h of plants in saline soil does not respond to an increase in leaf water status. Abscisic Acid (ABA) is synthesized in roots and mature leaves particularly in response to water stress (Davies, 2010).

Over days to weeks, water and salt stress injury may be visible as chlorosis (yellowing) or death of older leaves (de Lacerda et al., 2003). Salt injury at this stage is due to ion accumulation, mainly, in older transpiring leaves. The rate of ion accumulation in older leaves is greater than the capability of the vacuole to compartmentalize them, hence they buildup in the cytoplasm inhibiting enzyme activity or build up in cells wall dehydrating them

(Munns, 2002). Either case is harmful; cell death may occur due to dehydration or ion toxicity (Munns, 2002).

In terms of ion toxicity due to the excess of ions, once proteins are damaged to the point they cannot be repaired the cell will initiate degradation of the protein or will induce a programmed cell death (apoptosis). Proteases will be activated during apoptosis; proteolytic cleavage and DNA degradation will cause cell death (Vaux and Strasser, 1996).

Plants have developed several mechanisms to cope with salt stress, such as (i) control of ion uptake by roots and transport into leaves, (ii) selective exclusion of ions, (iii) compartmentalization of ions at cellular and whole plant level, (iv) synthesis of compatible solutes, (v) changes in the photosynthetic pathway, (vi) alteration in membrane structure, (vii) induction of antioxidant enzymes to scavenge free oxygen radicals and (viii) induction of plant hormones (Parida and Das, 2005).

If there is ion compartmentalization into the vacuole then organic solutes and ions should also accumulate in the cytoplasm and organelles to balance the osmotic pressure. Osmolytes such as amino acids or their derivates, sugars and polyols are commonly accumulated to balance osmotic pressure (Hasegawa et al., 2000). Trehalose sugar acting as a storage carbohydrate possesses the unique feature of reversible water-absorption capacity to protect biological molecules from desiccation-induced damage; trehalose appears to be superior to other sugars at conferring protection (Penna, 2003).

Floriculture crops are also sensitive to salinity and have been historically irrigated in the past with high quality (low salt, potable) water by growers. However, in some regions high quality water is in high demand due to municipal growth (residential and industrial uses) leaving less room for the usage of this water for agriculture or horticulture production. When using poor quality water, leaching (applying excess water so that some water runs out the bottom of a container carrying some soluble salts with it) is used as a tool to help solve high salt problems (Evans, 2004). Loss of fertilizer and salts through leaching may pose environmental risks by contamination and nutrient loading of ground and surface water. Therefore, capture and reuse has been promoted as an alternative to the high quality water to irrigate floriculture crops and as a means to be more environmentally friendly. Given the wide range of species and cultivars used in floriculture there is a broad range in crop response to saline irrigation water (Carter and Grieve, 2006).

Petunia x *hybrida* (Hook.) Vilm *(n*=7), commonly known as Petunia*,* is a gametophytic self-incompatible plant whose origin is thought to be by hybridization between *Petunia axillaris* (Lam.) Britton et al. and *Petunia integrifolia* (Hook.) Schinz & Tell (Gerats and Strommer, 2009). Petunias are among the most popular bedding plants in the world because of their versatility, variety and flower color range (Kessler, 1998). In the United States petunias have been one of the top five selling bedding plants for over 100 years and there are over 400 cultivars currently available on the market (Kessler, 1998). Petunia is also currently the largest bedding plant species for wholesale value in U.S. with a wholesale value of $126 million (USDA NASS, 2010).

Petunia x hybrida cv.'Mitchell Diploid' is a self fertile plant (unlike commercial Petunias) created to aid in both genetic and biochemical studies. It was obtained from a plant with the genotype *P. axillaris* x *(19. axillaris* × *P. hybrida* "Rose du Ciel") backcross (Mitchell et al., 1980) and was shown to be haploid by root tip chromosome counts and by the number of chloroplasts per guard cell pair. The haploid plant was grown in tissue culture and by doubling the chromosomes a fertile and homozygous diploid line resulted with no variation observed between the two sets of chromosomes (Griesbach and Kamo, 1996). Petunia Mitchell Diploid was used in many of the fundamental experiments in plant transformation (Fraley et al., 1983; Horsch et al., 1985; Deroles and Gardner, 1988 a, b) and consequently a well established transformation protocol is available (Jorgenson et al., 1996). Thus, it quickly became a model plant and valuable organism for analyzing the biochemistry related to development and particularly useful since they allow the expression of recessive alleles (Kamo and Griesbach, 1989).

The objective of this study was to quantify the effect of NaCl in the root-zone on two greenhouse-grown petunia cultivars, one commercially available, ‘Bravo White’ (Syngenta Flowers Inc., Boulder CO) and one used for genetic and biochemical studies, ‘Mitchell Diploid’, to assess the potential of petunias to be grown under increasing irrigation water salinity. We hypothesized that plant growth as well as other morphological responses are negatively affected at high levels of NaCl, such as 40 mM, and that the two cultivars respond differently to increasing NaCl salinity.

Material and Methods

Plant material and treatments

A glasshouse experiment was conducted at Cornell University, Ithaca New York, USA (42.42 °N latitude) in fall 2009. Temperature was maintained using heating set points of 21° C during the day and 16° C during the night. The glasshouse received ambient light with no supplementation. Seeds of

Petunia × *hybrida* Vilm. 'Bravo White' (BW) and Petunia 'Mitchell Diploid' (MD) were germinated in soil-less media (Metromix 280, Sun Gro Horticulture LTD., Vancouver, Canada) for 3 weeks. After seedlings were ca. 8 cm tall and well rooted, 30 seedlings of each cultivar were selected for uniformity, roots were washed to remove substrate and seedlings were individually placed into 4 L containers for establishment in hydroponics. Each container had 3.5 L of municipal tap water along with fertilizer prepared at 150 mg•L^{-1} N from 20N-2.2P-16.6 K (Jack's Professional LXTM Water Soluble Fertilizer 21-5-20 All-Purpose, J.R. Peter's Inc. Allentown, PA). The hydroponic containers were randomly arranged along a greenhouse bench and spaced 0.25 m apart. The solution was kept aerated by continuously bubbling air into the solution to maintain oxygen saturation.

After two weeks of establishment in hydroponics the nutrient solution was replaced with treatment solutions. The base solution consisted of a modified Hoagland's solution (4 mM KNO_3, 1 mM $MgSO_4$, 1 mM NH_4H2PO_4, 4 mM $Ca(NO_3)_2 \cdot 4H_2O$, 18 µM Fe-EDDHA, 2 µM $CuSO_4 \bullet 5H_2O$, 4 µM $ZnSO_4 \bullet 7H_2O$, 0.2 µM $H_2MoO_4 \bullet H_2O$, 28 µM $MnCl_2 \bullet 4H_2O$, 4 µM H_3BO_3), prepared in reverse osmosis filtered water. Treatments of 0, 20, 40, 60 and 80 mM NaCl were obtained by adding 0.00, 4.09, 8.18, 12.27 and 16.36 g of NaCl in the 3.5 L of nutrient solution in each container. Once NaCl treatments commenced the nutrient solution was replaced weekly. Electivity conductivity (EC) and pH were recorded using an EC meter (ECTestr high, OAKTON Instruments, Vernon Hills, IL) and pH meter (pHTestr 20, OAKTON Instruments, Vernon Hills, IL) respectively every week before disposing of the old solution and again following replacement of new solution and NaCl.

After 4 weeks exposure to NaCl several growth parameters were measured: plant height (from the lid of the hydroponic container to the tallest part of the plant), plant width (average of two measurements – the widest part of the plant and width at 90° from this location) and chlorophyll index (average of 3 SPAD meter measurements per plant on most recently expanded leaves). Destructive harvest was used to determine leaf, stem, root, fresh weight and leaf surface area using a leaf area meter (LI-3100, LI-COR Environmental, Lincoln, NE). Leaf, stem and root dry weight was recorded after plants were oven-dried at 80 °C for 4 d.

Prior to destructive harvest, leaf samples from both Petunia Mitchell Diploid (MD) and Petunia Bravo White (BW) were cut into 1 cm round discs (5 discs per plant), rinsed three times with distilled water to remove surface contamination and placed in individual vials containing 10 ml of distilled water and capped. These samples were incubated at room temperature (22 °C) on a shaker (100 rpm) for 24 h. Electrical conductivity of the solution (EC initial) was read after incubation. Samples were then placed into an autoclave at 120°C for 15 min and the second reading (EC final) was determined after cooling the solutions to room temperature. Electrolyte leakage percentage (ELP) was calculated as EC initial/EC final and expressed as percent.

Experimental design and statistical analysis

The experiment followed a completely randomized design with five NaCl treatments, two cultivars and six replicates (1 plant in a hydroponic container) per treatment by cultivar combination. Analysis of variance was carried out to look for cultivar and NaCl treatment effects on the measured parameters (JMP Version 8.0, SAS Institute, Cary, NC). Where significant differences were found, Tukey’s HSD was used to determine mean separations.

Results

EC and pH

Whereas the control treatment (0 mM NaCl) EC remained relatively stable throughout the experiment and weekly replenishment period, EC increased across the weekly periods when additional NaCl was added. This indicates that roots were actively excluding salt; i.e. they absorbed more water in proportion to salt leading to an increase in salt concentration (Figure 2.1). Weekly pH declined slightly for the 60 and 80 mM NaCl treatments, remained relatively stable for 40 mM and increased prior to weekly replenishment for the 0 and 20 mM treatments (Figure 2.2).

Growth Effects

All growth and morphological parameters recorded were significantly reduced with exposure to increasing NaCl (Table 2.1). Leaves, roots, stems and the total dry weight for both MD and BW drastically deceased with higher NaCl treatments (Figure 2.3).

In terms of percent DW reduction as NaCl increased from 0 to 80 mM, stem DW was reduced the most of any organ (75% DW reduction for both cultivars). The total plant DW was reduced by 62% for ‘Bravo White’ and 65% for ‘Mitchell Diploid’. With regards to total DW, exposure to 20 mM NaCl quantitatively reduced growth of both cultivars but according to Tukey’s analysis this was not significantly different than control. Exposure to 40 mM NaCl significantly reduced total DW and exposure to 60 mM further reduced total DW. Further increasing NaCl exposure to 80 mM did not further reduce DW as compared with the 60 mM treatment (Table 2.1 and Table 2.2).

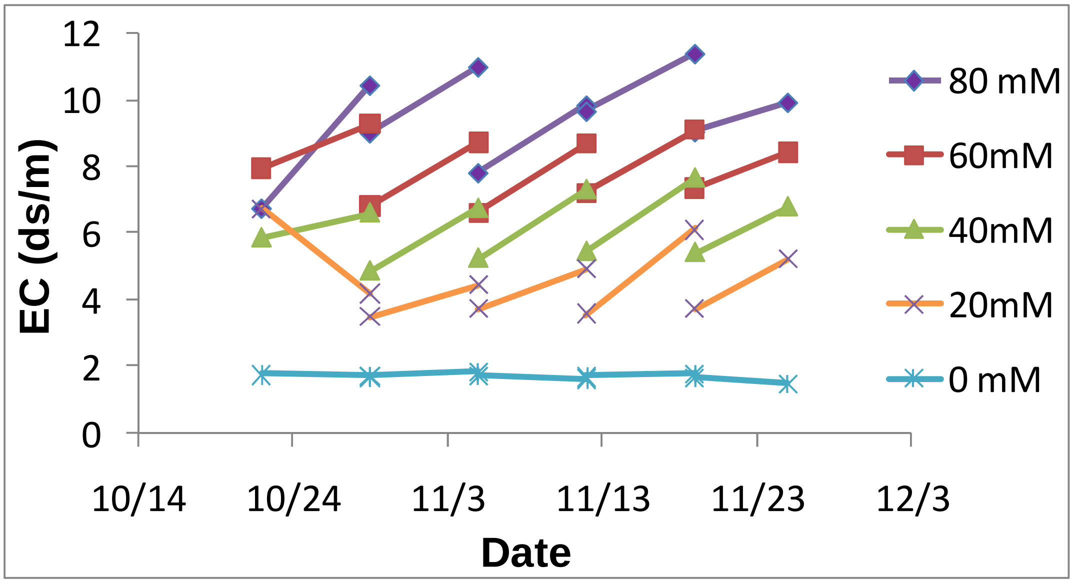


Figure 2.1. Weekly EC measurements taken from the hydroponic solution of *Petunia* plants exposed to 0, 20, 60, or 80 mM NaCl amended to the Hoagland's solution. Disjointed data denotes when weekly replacement of the hydroponic solution took place. EC was recorded just prior to and just after weekly solution replacement. Data points are means of 12 containers.

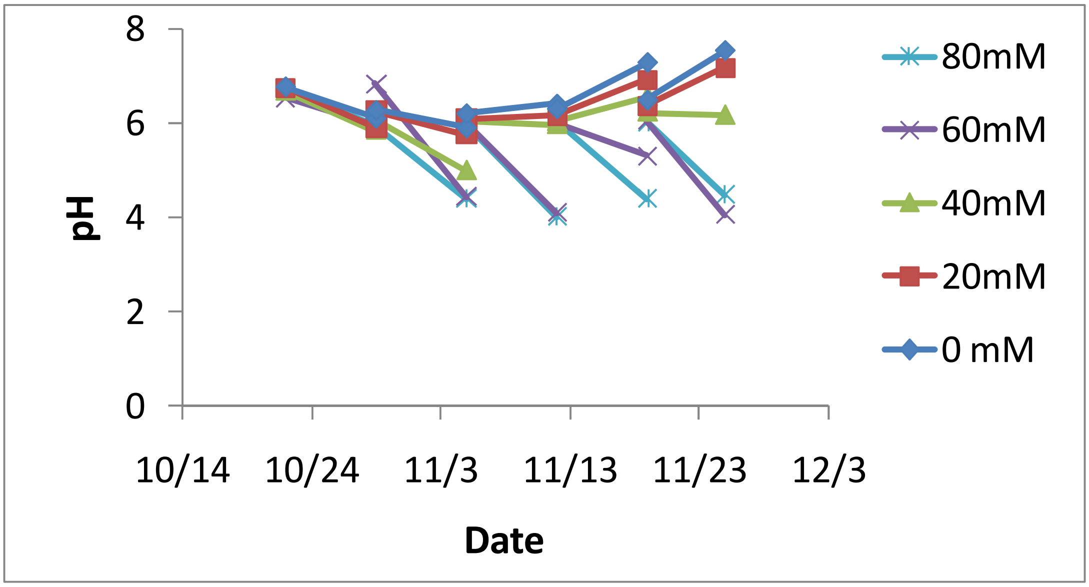
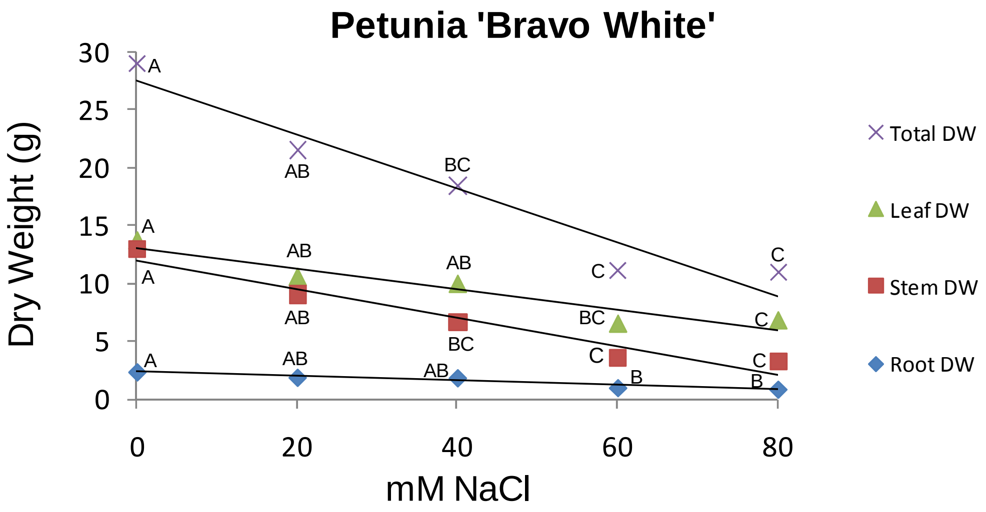


Figure 2.2. Weekly pH measurements taken from the hydroponic solution of *Petunia* plants exposed to 0, 20, 60, or 80 mM NaCl amended to the Hoagland's solution. Disjointed data denotes when weekly replacement of the hydroponic solution took place. pH was recorded just prior to and just after weekly solution replacement. Data points are means of 12 containers.



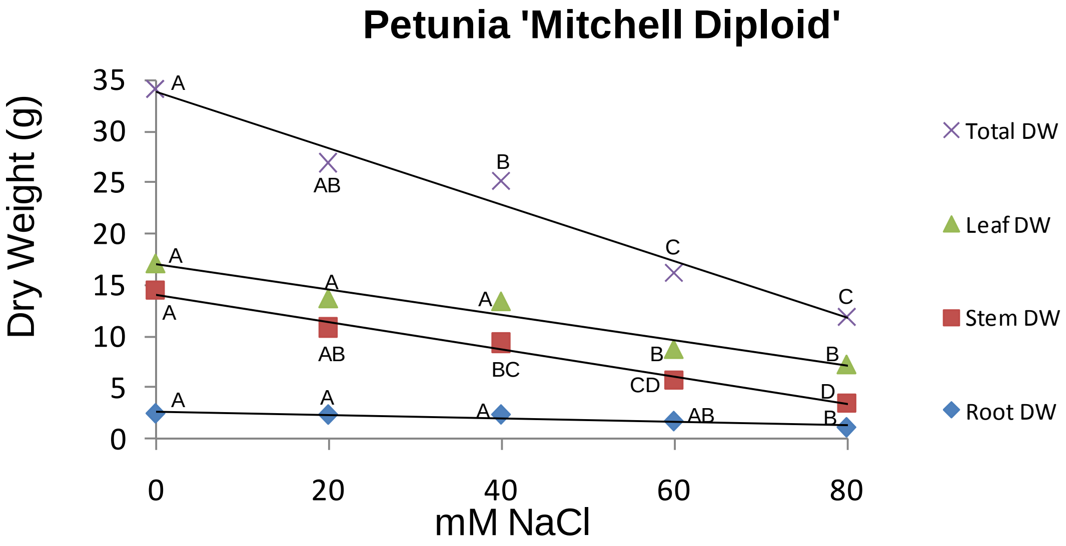


Figure 2.3. The effect of increasing NaCl on total plant leaf, stem and root dry weight (DW) of Petunia 'Bravo White' and 'Mitchell Diploid' in response to NaCl treatment. Plants were grown in solution culture for four weeks in a Hoagland's solution amended with 0 to 80 mM NaCl. Data are means of six plants. Letter's represent mean separation comparison within an organ across NaCL concentrations utilizing Tukey's HSD (P=0.05).

Table 2.1. Analysis of variance (p-values) for the measured parameters in response to NaCl treatment (Trt), cultivar (Cult) and their interaction. Petunia 'Bravo White' and 'Mitchell Diploid' were grown in solution culture with Hoagland's solution amended with 0, 20, 40, 60, and 80 mM NaCl for a four week treatment period.

Source	Stem FW	Leaf FW	Total FW	Root FW	Root DW	Stem DW	Leaf DW	Total DW
Trt	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
Cult	.0301*	.0013*	.0031*	.00434*	.0142*	.0250*	.0002*	.0016*
Trt x Cult	.4165ns	.4236ns	.5266ns	.3872ns	.5896ns	.6854ns	.1374ns	.4107ns

Source	Height	SPAD	Width	LSA	ELP
Trt	<.0001*	<.0001*	<.0001*	<.0001*	<.0001*
Cult	<.0001*	<.0001*	.0047*	.6719ns	.0001*
Trt x Cult	<.0001*	.4386ns	.1131ns	.5400ns	.1848ns

*: Statistically different
NS: Not statistically different

Table 2.2. The effect of increasing root-zone NaCl salinity (0, 20, 40, 60 and 80 mM) in the hydroponic solution during a four week treatment period on the root, stem, leaf, and total fresh weight of Petunia ‘Bravo White’ (BW) and ‘Mitchell Diploid’ (MD). Data are means ± SE of six plants.

BW	Root (g)	Stem (g)	Leaf (g)	Total Plant(g)
0	61.4 ± 8.5 A[z]	221.2 ± 31.3 A	507.2 ± 44.5 A	789.7 ± 80.3 A
20	59.5 ± 9.7 A	145.5 ± 27.3 AB	393.4 ± 55.7 AB	598.4 ± 93.6 AB
40	60.6 ± 10.8 A	112.8 ± 12.5 BC	319.7 ± 38.8 BC	493.1 ± 61.2 BC
60	32.6 ± 3.0 A	60.4 ± 6.2 C	178.9 ± 18.6 C	271.9 ± 26.9 C
80	30.8 ± 6.2 A	61.7 ± 10.1 C	197.0 ± 25.2 C	289.4 ± 40.6 C
MD	Root (g)	Stem (g)	Leaf (g)	Total Plant (g)
0	60.2 ± 6.5 AB	245.6 ± 15.1 A	586.3 ± 28.5 A	892.2 ± 45.0 A
20	72.4 ± 7.3 A	181.1 ± 14.1 B	476.3 ± 31.4 AB	729.7 ± 48.7 AB
40	63.9 ± 7.4 A	148.7 ± 14.9 B	435.6 ± 47.4 B	648.3 ± 68.0 B
60	62.4 ± 7.4 AB	84.0 ± 7.6 C	284.4 ± 26.2 C	430.9 ± 39.4 C
80	36.2± 2.1 B	61.1 ± 7.9 C	199.8 ± 21.4 C	297.1± 30.6 C

[z] Within each column and cultivar values followed by the same letter are not significantly different at P=0.05 by Tukey’s HSD test.

Plant Morphology

Plant height of both cultivars was significantly reduced by increasing NaCl; however, height of MD was more detrimentally affected than BW. As NaCl increased from 0 to 80 mM height of BW decreased from 24.7 to 19.3 cm whereas height of MD decreased from 34.0 to 20.2 cm (Table 2.3). Therefore, height of BW was reduced by 22% whereas MD was reduced by 41%. Height was the only measured parameter where the cultivars responded in a different pattern to increasing NaCl (i.e. there was a significant treatment by cultivar interaction). For MD, 20 and 40 mM NaCl reduced height but not significantly as compared to the control. 60 mM NaCl appears to be a threshold; at that concentration height is quite reduced.

Plant width was reduced by 34% and 28% for BW and MD, respectively, as NaCl increased from 0 to 80 mM (Table 2.3).

For leaf surface area (LSA) there was a strong inverse correlation between NaCl and leaf area which decreased from 7556 cm^2 to 2744 cm^2 for BW and decreased from 7385 cm^2 to 1826 cm^2 for MD (Table 2.3). LSA was the only measured parameter in which the cultivar response was not statistically significant i.e. the actual LSA and the LSA response to NaCl salinity did not differ significantly between the two cultivars (Table 2.1).

Cell membrane integrity

Initial measurements of leaf disk electrolyte leakage (EC Initial) indicate the quantity of solutes that readily disassociated from leaf tissue. For both cultivars this value increased as NaCl increased (Table 2.4). MD showed to be more sensitive to salt for all the treatments than BW leaking more solutes from leaf disks. The EC measurements following autoclaving (EC Final) are an indicator of the total amount of solutes present in leaf disks. As NaCl exposure increased to 80 mM the total amount of solutes increased, particularly in the case of MD where EC Final more than doubled as NaCl treatment increased from 0 to 80 mM.

Table 2.3. The effect of increasing root-zone NaCl salinity (0, 20, 40, 60 and 80 mM) in the hydroponic solution during a four week treatment period on the plant height, width, and leaf surface area of Petunia ‘Bravo White’ (BW) and ‘Mitchell Diploid’ (MD). Data are means ± SE of six plants.

BW	Height (cm)	Width (cm)	LSA (cm^2)
0	24.7 ± 1.7 A[z]	57.3 ± 1.2 A	7555.8 ± 680.4 A
20	22.3 ± 0.6 AB	53.9 ± 2.1 A	5363.5 ± 737.5 AB
40	21.0 ± 0.7 AB	53.6 ± 3.4 A	4241.4 ± 577.3 BC
60	19.0 ± 0.7 B	37.3 ± 2.9 B	2488.5 ± 259.1 C
80	19.3 ± 1.2 B	38.1 ± 1.4 B	2744.2± 222.9 C
MD	Height (cm)	Width (cm)	LSA (cm^2)
0	34.0 ± 1.7 A	60.0 ± 2.9 A	7385.3 ± 648.2 A
20	29.8± 1.0 A	53.5 ± 2.7 AB	5895.2 ± 562.4 AB
40	29.4 ± 1.2 A	57.3 ± 3.7 A	5012.3 ± 495.9 BC
60	20.2 ± 0.7 B	52.6 ± 1.6 AB	2995.8 ± 744.7 CD
80	20.2 ± 0.6 B	43.0 ± 2.7 B	1825.8 ± 128.3 D

[z] Within each column and cultivar values followed by the same letter are not significantly different at P=0.05 by Tukey’s HSD test.

Electrolyte leakage percent (ELP), calculated as EC Init/EC Final (Table 2.4), represents a measure of cell membrane integrity (Zhu, 2004) where a lower percentage indicates that cell membranes are more stable. ELP was fairly small (~20%) for control (0 mM NaCl) treatments and as NaCl increased to 80 mM this reached 60%. The two species were significantly different while calculating ELP; for MD this increased by 38% and for BW by 29% as NaCl increased from 0 to 80 mM.

Chlorophyll Index

Chlorophyll index was reduced in both cultivars by NaCl. For BW chlorophyll decreased from 33.1 to 25.8 SPAD units and for MD 40.7 to 32.6 SPAD units, as NaCl increased from 0 to 80 mM (Table 2.5).

Discussion

In this experiment all the plant parameters measured were significantly affected by NaCl treatment (for all variables $p<0.0001$). Similarly, *Phaseolus vulgaris* experienced a broad spectrum of growth limitations when exposed to different concentrations of NaCl including reductions in biomass and morphological parameters such as plant height, leaf surface area and root length (Gama et al., 2007).

Growth effects

The total plant DW was reduced by 62% for ‘Bravo White’ and 65% for ‘Mitchell Diploid’, which is consistent with the finding of Vasilakakis and Neocleous (2007) where dry weight of Red raspberry (*Rubus idaeus* L. ‘Autumn Bliss’) decreased significantly at salt concentrations of 15, 20 and 30 mM NaCl resulting in 34%, 45% and 48% total plant dry weight reduction, respectively.

Table 2.4. The effect of increasing root-zone NaCl salinity (0, 20, 40, 60 and 80 mM) in the hydroponic solution during a four week treatment period on the cell membrane leakage parameters (EC Initial, EC Final, and Electrolyte Leakage Percentage [ELP]) of Petunia ‘Bravo White’ (BW) and ‘Mitchell Diploid’ (MD). Data are means ± SE of six plants.

		EC Initial	EC Final	ELP
Cultivar	NaCl mM	Mean ± SE	Mean ± SE	Mean ± SE
BW	0	98.1 ± 11.0 F[z]	618.2 ± 43.9 B	16.8 ± 2.2 C
BW	20	123.3 ± 13.9 EF	626.6 ± 34.6 AB	19.9 ± 1.3 BC
BW	40	159.6 ± 7.9 DEF	618.3 ± 22.9 B	26.7 ± 1.3 B
BW	60	187.9 ± 13.7 CDE	656.6 ± 28.9 AB	29.0 ± 2.2 B
BW	80	445.7 ± 96.1 AB	849.7 ± 94.5 A	55.3 ± 5.4 A
PM	0	108.5 ± 13.4 EF	609.2 ± 64.1 B	18.7 ± 1.3 D
PM	20	230.6 ± 26.7 CD	867.3 ± 35.1 A	27.7 ± 3.4 CD
PM	40	309.3 ± 47.3 BC	840.0 ± 14.9 A	37.8 ± 5.2 BC
PM	60	452.5 ± 53.8 AB	1002.4 ± 45.9 A	45.9 ± 3.7 AB
PM	80	672.4 ± 84.3 A	1137.2 ± 80.6 A	60.2 ± 3.8 A

[z] Within each column and cultivar values followed by the same letter are not significantly different at P=0.05 by Tukey’s HSD test.

Table 2.5. The effect of increasing root-zone NaCl salinity (0, 20, 40, 60 and 80 mM) in the hydroponic solution during a four week treatment period on the SPAD chlorophyll index of Petunia ‘Bravo White’ (BW) and ‘Mitchell Diploid’ (MD). Data are means ± SE of six plants.

Petunia Bravo White		Petunia Mitchell Diploid	
mM of NaCl	SPAD Index	mM of NaCl	SPAD Index
0	33.1 ± 1.1 A	0	40.7 ± 1.0 A
20	31.0 ± 1.1 A	20	37.6 ± 1.1 AB
40	32.3 ± 1.0 A	40	34.8 ± 1.2 B
60	29.7 ± 1.2 AB	60	34.4 ± 0.6 BC
80	25.8 ± 1.0 B	80	32.6 ± 0.7 C

[z] Within each column and cultivar values followed by the same letter are not significantly different at P=0.05 by Tukey’s HSD test.

In our experiment, petunia cultivars were not as sensitive as some of the cultivars of *Phaseolus vulgaris* studied by Gama et al. (2007). Of the five *P. vulgaris* cultivars examined there was a huge difference in cultivar sensitivity to 50 mM NaCl; shoot DW of ‘HRS 516’ was reduced by 5% whereas shoot DW of ‘Giza 3’ was reduced by 71% as NaCl increased from 0 to 50 mM (Gama et al., 2007). As shown by Zhu et al. (2004), the dry weight of both shoot and root of cucumbers cultivars (*Cucumis sativus* L.) was significantly reduced by 40 mM NaCl stress, however, this inhibition was significantly alleviated by silicon supplementation.

Morphology

LSA of BW was reduced by 64% whereas MD was reduced by 75%. While Kchaou et al. (2010) reported severe leaf surface area reductions for five Olive (*Olea europea* L) cultivars at 100 and 200 mM leaf thickness increased gradually during the salinity treatment period. This is also in concordance with the increasing concentration of NaCl in hydroponic solutions for bean, cotton and atriplex, where all species responded to salinity by exhibiting increased leaf succulence (thickness) and greater mesophyll thickness (Longstreth and Nobel, 1979).

Physiology

As Zhu et al. (2004) reported, ELP was used to assess membrane permeability (see material and methods) where ELP represents cell membrane injury. Our study shows that as salt concentrations increased cell membranes became more permeable as indicated by an increasing ELP. Similarly in *Cucumis sativus* L., for example, it was shown that salt stress increased ELP by 30% as NaCl increased from 0 to 40 mM (Zhu et al., 2004). Mansour (1998) also used cell membrane permeability to evaluate damage after exposure to salt; treating onion epidermis with 150 mM NaCl for 3 hr significantly increased ELP.

NaCl decreases petunia growth and we believe that reductions in LSA would strongly limit the ability to capture sunlight for photosynthesis, while at the same time large amounts of NaCl would disrupt both cell membranes and cell walls.

Crookes and Grierson (1983) have found changes in the structure of the cell wall beginning with dissolution of the middle lamella and eventual disruption of the primary cell wall. Incubation of mature green tomato pericarp with purified tomato polygalacturonase isoenzymes produced the pattern of wall disruption.

In our experiment, chlorophyll content was also reduced in both cultivars with salt exposure. BW chlorophyll content decreased to 25.8 SPAD units in the 80 mM NaCl treatment as compared with 33.1 SPAD units for the control and in MD chlorophyll index was decreased to 32.6 SPAD units as compared to 40.7 SPAD units for the control treatment. These finding are consistent with those found in *Chrysanthemum;* chlorophyll content was decreased to 29.2 SPAD units in the 9 g L^{-1} (i.e. 154 mM) NaCl treatment as compared with 42.3 SPAD for control plants (Lee and van Iersel, 2008).

Populous deltoides irrigated with 50-200 mM NaCl significantly reduced net photosynthetic rate, chlorophyll and also leaf area (Singh et al., 1999). Excessive amounts of salt in plants may become toxic in the older transpiring leaves causing premature senescence and reducing the photosynthetic leaf area (Munns, 2002). Although we did not measure photosynthesis we hypothesize that photosynthesis was reduced in our experiment in response to increasing NaCl due to declines in LSA, chlorophyll content (as indicated by SPAD index) and cell membrane integrity. Photosynthesis of Chrysanthemum was reduced with NaCl exposure (Lee and van Iersel, 2008).

Conclusions

Petunia growth is reduced with increasing exposure to NaCl in the root-zone. According to our quantitative models even 20 mM NaCl reduces growth, but in comparing treatments this was not significantly different from the control. In terms of mean separation comparisons 40 mM appears to be a threshold for significant decline of many of the measured parameters. Overall, the results suggest that petunias are a good candidate plant for producing acceptable plants with poorer quality (saline) irrigation water. However, it should be noted that we exposed plants past the seedling stage to salts, whereas young seedlings are often more sensitive to salt (Serrato et al., 1992).

Concentrations of NaCl higher than 20mM stunted growth in a manner that decreased marketability (marginal necrosis and lower leaf chlorosis). Plants remained wilted during the end of the production period when irrigated with 40 mM and were severely stunted at even higher concentrations (60 and 80 mM).

Our findings indicate that growers may be able to use slightly saline water at 20 mM of NaCl for the greenhouse production of petunias without detrimental effects on plant growth. The results of this experiment are relevant to the floriculture industry as high-quality irrigation water becomes less available.

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CHAPTER 3

CLONING, SEQUENCING, CHARACTERIZING AND ASSESING FUNCTIONALITY OF THE PETUNIA TREHALOSE-6-PHOSPHATE SYNTHASE (TPS1) GENE THROUGH COMPLENTATION IN YEAST.

Keywords: PCR, cloning, TPS1, yeast complementation, yeast homologous recombination, trehalose, disaccharide sugar.

Abstract

Increasing resistance of crops to abiotic stresses is one of the primary objectives of plant biotechnology. Alteration in cellular environment leads to protein misfolding and inactivation. Trehalose sugar may prevent physical and chemical instability in proteins that occurs upon desiccation and when exposed to high concentration of salt. Trehalose is a non-reducing disaccharide sugar composed of two glucose units linked in an α,α,1,1 bond. Trehalose accumulation plays a stress protection role in many bacteria and fungi. Whereas in most plants trehalose levels are low the concentration of this sugar is high in "resurrection plants" (*Selaginella lepidophylla)*. Both tomato and rice have been genetically modified with microbial *TPS1* increasing their tolerance to salt. There is no petunia trehalose-6-phosphate synthase 1 sequence available (*PhTPS1*); however, its ortholog is available for tomato (*SLTPS1*) and arabidopsis (*AtTPS1*). With an aim toward overexpressing *PhTPS1* to improve salt tolerance in the model floriculture species petunia, we designed primers based on the tomato and arabidopsis orthologs to search for this gene. In this work we show how we obtained the coding region of *PhTPS1*and sequence it. Inactivation of the *Saccharomyces*

cerevisiae TPS1 gene (*ScTPS1*) causes a specific growth defect in the presence of glucose in the medium associated with deregulation of the initial part of glycolysis. We also truncated the first ~80 AA towards the 5' end to increase its catalytic activity as has been reported before for *AtTPS1*. In our work, the ability of mutant yeast to grow in glucose as a carbon source was successfully restored by the insertion of *PhTPS1* gene, indicating that *PhTPS1* gene is a functional plant gene capable of complementing trehalose biosynthesis function.

Introduction

Increasing the resistance of crops to osmotic stresses is one of the primary objectives of plant metabolic engineering (LeRudulier et al*.,* 1984) and continues to the present day as a major goal (Sakamoto and Murata, 2001).

Drought and salt sensitivity impose major limitations on crop productivity and quality in the United States and globally (Rontein et al., 2002). Adaptations to withstand drought and salt stress have been widely reported in several organisms; yeast cells, fungal spores, invertebrate species and also some plants (Zentella et al., 1999).

One mechanism employed by plants to withstand osmotic stress is by synthesizing and accumulating osmoprotectant compounds and/or compatible solutes. Usually, compatible solutes are small uncharged molecules that are nontoxic in cells at low concentrations. The levels can build up in cells to help to stabilize proteins and membranes by minimizing the exposure of hydrophobic moieties to salt and/or other harmful solutes (Rontein et al., 2002).

Osmoprotectants are typically hydrophilic compounds capable of replacing water at the surface of proteins, protein complexes or membranes (Hasegawa et al., 2000). These osmolytes are believed to facilitate osmotic adjustment lowering the internal osmotic potential and thus contributing to tolerance (Hasegawa et al., 2000).

Frequently observed metabolites with an osmolyte function are simple sugars (mainly sucrose and fructose) and complex sugars (trehalose, raffinose, fructans and mannitol), alcohols (glycerol, methylated inositols), as well as ions or charged metabolites [glycine betaine (GlyBet), dimethyl sulfonium propionate (DMSP), proline and ectoine (1,4,5,6-tetrahydro-2-methyl-4-carboxyl pyrimidine)] (Hasegawa et al., 2000). Of the aforementioned osmoprotectants, proline, GlyBet and mannitol occur commonly in plants while DMSP and trehalose occur rarely and ectoine is found only in bacteria (Rontein et al., 2002).

Almeida et al. (2005) showed that tobacco (*Nicotiana tabacum*) genetically modified via *Agrobacterium* mediated increased levels of trehalose using a trehalose-6-phosphate synthase gene of *Arabidopsis thaliana* (*AtTPS1*) validating the hypothesis that trehalose accumulation improves abiotic tolerance to plants when exposed to NaCl.

Increasing trehalose accumulation can improve drought and salinity tolerance (Penna, 2003) as was reported in transgenic rice, offering novel strategies for improving abiotic stress tolerance in crop plants. In transgenic rice trehalose appears to efficiently stabilize dehydrated enzymes, proteins, and lipid membranes, as well as protect biological structures from damage during desiccation (Garg et al., 2002).

Trehalose may also prevent physical and chemical instability that occurs upon desiccation. Physical instability refers to the alteration of the three-dimensional structure of the protein. Lack of water implies fewer hydrogen bonds with hydrophilic residues on the protein surface, thus, destabilizing its structure. Trehalose forms fewer internal hydrogen bonds (when compared to other nonreducing disaccharides) and hence is available for a stronger interaction with hydrophilic residues in the polypeptide (Kumar and Roy, 2010). This, in turn, helps to maintain the proper protein conformation.

Chemical instability refers to any change in the primary structure of proteins due to modifications of any amino acids through deamidation, hydrolysis, oxidation, reduction and β-elimination. The end result is a change in conformation and shape of the protein which loses its native conformation and interacts non-selectively with other molecules to form supramolecular structures with either altered or diminished functionality (Kumar and Roy, 2010).

Trehalose sugar (chemical nomenclature α-Dglucopyranosyl-1,1-α-Dglucopiranoside) is a non-reducing disacharide sugar with two glucose molecules linked by α,α-1,1 glycosidic linkage. It is widely present in bacteria, yeast, spores, some invertebrates (*Drosophila melanogaster* and *Apis mellifera*) and some plants (Avonce et al., 2004). Its accumulation occurs in many bacteria and fungi and in a few extremely desiccation-tolerant plants, the “resurrection plants” (such as *Selaginella lepidophylla)*, all of which can rehydrate and reactivate vital functions upon contact with water (Rontein et al., 2002).

The building blocks of the trehalose sugar are uridine diphosphate glucose (UDP-glucose) and glucose-6-phosphate (G6P). UDP-glucose is a nucleotide sugar or an activated form of monosaccharide, a compound in which the anomeric carbon is activated by attachment to a nucleotide through a phosphate ester linkage. UDP-glucose consists of the pyrophosphate group, the pentose sugar ribose, glucose, and the nucleobase uracil. UDP-glucose acts as a glycosyl donors in a glycosylation reaction catalyzed by enzyme family of the glycosyltransferases in the metabolism of nucleotide sugars (Vogel, 1998).

G6P is a glucose molecule phosphorylated on carbon six. This compound is very common in cells as the vast majority of glucose entering a cell will become phosphorylated in this way. G6P has been shown to be linked to other metabolic pathways such as glycolysis and pentose phosphate (Gibson et al., 2004).

In the first step of the trehalose sugar biosynthesis pathway one molecule of glucose is transferred from UDP-glucose (donor sugar) to glucose-6-phosphate. The OH group of glucose-6-phosphate makes a nucleophillic attack on the anomeric carbon of UDP-glucose forming a new glycosidic bond and thus creating an intermediate product; α,α-1,1 trehalose-6-phosphate (T6P) (personal communication; G. Feigenson, Cornell University). This reaction is catalyzed by trehalose-6-phosphate synthase 1 enzyme (TPS1) a glycosyltransferase that transfers a monosaccharide unit from an activated nucleotide sugar (UDP-glucose) to a glycosyl acceptor molecule (G6P) (Gibson et al., 2004).

Secondly, the T6P intermediate is dephosphorylated by the enzyme trehalose-6-phosphate phosphatase (TPP) to yield trehalose (Almeida et al., 2005). Nonspecific phosphatases can also carry out this reaction. The whole equation is UDP-glucose + D-glucose-6-phosphate + H_2O = trehalose + UDP + Pi (Almeida et al., 2005).

Although the underlying processes in trehalose formation are well understood there is no clear agreement as to how trehalose actually confers stress resistance to salt stress. Both substrates for TPS are readily available in the cytosol, however, attempts to constitutively express the *E. coli* TPS gene (*otsA*) or the yeast gene (*ScTPS1*) in tobacco showed that plants accumulated only low amounts of trehalose (0.5 *m*mol g $^{-1}$ fresh weight) and unexpected phenotypes such as stunted growth were observed (Goddijn and Smeekens, 1998).

Müller et al. (1995) reported that trehalose is toxic to legumes such as soybean *(Glycine* max L. Merr.) and cowpea *(Viyna unguiculata* L.) hypothesizing that plant trehalase has evolved to function as a detoxifying enzyme. Particularly high activity of trehalase were found in nitrogen fixing root nodules of soybean and other legumes in which trehalose is produced by the bacterial symbionts.

When the specific trehalase inhibitor validamycin A (an anti-fungal aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* var. *limoneus,* Liao 2009) was added to culture media transgenic tobacco accumulated several fold more trehalose than wild type plants and transgenic potato tubers accumulated significant amounts (up to 12 *m*mol g^{-1} fresh weight) for the first time (Goddijn et al., 1997), suggesting that trehalase is common in plants and that trehalose sugar is depleted faster than it is produced in cells (Vogel et al., 1998).

Trehalose itself might not be a stress protectant, except in certain specialized resurrection species that accumulate the compound quantitatively. Instead the intermediate product in the trehalose biosynthesis, T6P, might be responsible due to its role in the regulation of sugar metabolism. It was found that T6P inhibits hexokinase activity *in vitro* in *Saccharomyces cerevisiae* (Eastmond et al., 2003). Further work is necessary to confirm this hypothesis *in vivo* and to determine the underlying mechanism by which T6P down regulates hexokinase (Leymann et al., 2001).

T6P is essential for carbon utilization in *Arabidopsis thaliana* and its control may affect similar steps in glycolysis (Schluepmann et al., 2003). Eastmond et al. (2002) previously observed that an *Arabidopsis thaliana tps1* mutant is embryo lethal, which is most likely due to reduced T6P levels and the apparent inability to utilize sugars. Moreover, T6P levels have been shown to influence photosynthetic capacity per unit leaf area (Pellny et al., 2004).

In *Saccharomyces cerevisiae* it is thought that the TPS1 gene exerts an essential control on the influx of glucose into glycolysis. The deletion of *ScTPS1* causes an inability to grow on glucose because of a hyperaccumulation of sugar phosphates and depletion of ATP and phosphate (Ernandes et al., 1998). However, the control of glucose influx into glycolysis was restored in yeast *tps1* mutant when the *E. coli* homologous gene was expressed using a PGK strong promoter (Bonini et al., 2000).

Plant TPS genes contain specific N- and C- terminal extensions not found in bacterial and fungal TPS genes (Leymann, 2001). Two conserved residues in the N-terminus region have been identified that act as an inhibitory domain when present in the AtTPS1 gene. Truncation of the *AtTPS1* specific

N-terminal extension restored growth defect of *tps1* yeast (Van Dijck et al., 2002).

In this present work we demonstrate how we cloned and sequenced the TPS1 gene from *Petunia x hybrida* cv. 'Mitchell Diploid' and proved that it is a functional gene in terms of rescuing yeast growing in glucose as a carbon source.

3. **Petunia trehalose-6-phosphate synthase 1 gene**

3.1 Identification of the TPS1 gene in petunia

The presence of the petunia trehalose-6-phosphate synthase 1 gene (*PhTPS1*) was confirmed through polymerase chain reaction (PCR) analysis carried out on a thermocycler (Bio-Rad, Hercules, CA) using *Taq* 2X Master Mix (New England BioLabs, USA). Genomic DNA (gDNA) from mature leaves of *Petunia x hybrida* cv. Bravo White (Syngenta, USA) grown for 8 weeks in soil-less media (Metromix 280, Sun Gro Horticulture LTD., Vancouver, Canada) in a greenhouse located at Cornell University was extracted as follows: one piece of young leaf tissue was harvested and put into in a microfuge tube. 560 μl of extraction buffer (see below) was added and the tissue was gently grinded with a pestle. 40 µl of 20% SDS was added and mixed by gentle inversion and incubated at 65°C for 30 minutes with occasional inversion. 3 μl of RNase A (10 mg/ml) solution was added to the cell lysate and tubes were incubated at 37° C for 15 minutes. 200 μl of 5M KOAc was added and vortex for 20 sec. Tubes were spun at 13,000 rpm for 10 min. The supernatant was decanted into a clean microfuge tube and 600 μl; isopropanol was added and mixed well. Pellet DNA was obtained by centrifugation at 13,000 rpm for 2 min. The supernatant was decanted and

600 μl of 70% ethanol was added, spun for 2 minutes and the pellet was dried under vacuum in a speed-vac. The DNA pellet was redissolved in 100 μl of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) and incubated at 65°C water bath for 1 hour while gently inverting it every 20 min. Extraction buffer was prepared as follows: 100 mM TrisHCl, 50 mM EDTA, pH8.0, 500 mM NaCl, 10mM β-mercaptoethanol.

Three sets of primers #1FP & #1RP, #2FP & #2RP and #3FP & #3RP (Table 3.1) were designed based on the alignment (DNAstar-Lasergene software) of the coding region (CDS) of conserved domains of exons from *Solanum lycopersicum TPS1* (*SlTPS1*) and *Arabidopsis thaliana TPS1* (*AtTPS1*). The *AtTPS1* gDNA sequence has 17 exons and 18 introns. These primers should amplify bands ranging between nearly 1 and 1.7kb from gDNA. The PCR products observed were different sizes than predicted most likely due to different sizes of introns in the petunia genomic sequence (Figure 3.1). An elongation factor gene coding for the alpha subunit 1 (EF-1 alpha) was also amplified from petunia gDNA as a positive control using primers #4FP & #4RP (Table 3.1).

PCR parameters used were: 94°C for 2 min, followed by 30 cycles of: 94°C for 20 sec, 55°C for 20 sec and 68°C for 30 sec. A final extension of 5 min at 68°C was used. PCR products were then separated by a 1% agarose (0.5 g of agarose in 50 ml of 0.5 TBE buffer; 218 g Tris base, 100 g Boric acid, 9.3g EDTA) gel electrophoresis (Figure 3.1).

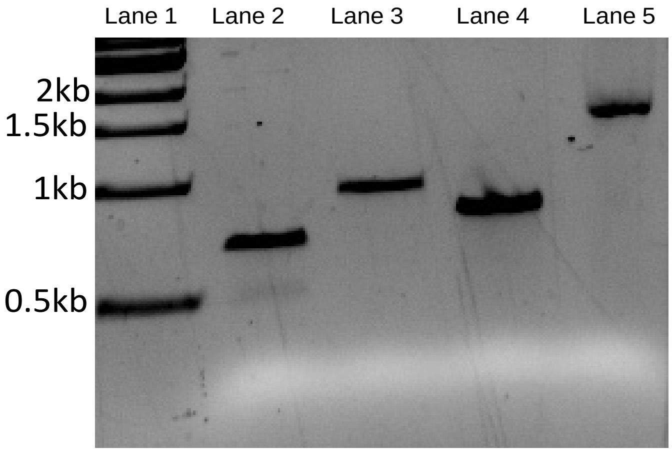


Figure 3.1. Amplifying *TPS1* from genomic DNA of Petunia *x hybrida* cv. 'Bravo White'. Positive PCR results show that the TPS1 gene is also present in Petunia. Lane 1: 1kb DNA Invitrogen ladder. Lane 2: primers #1FP & #1RP yielding a 700-bp PCR product. Lane 3: primers #2FP & #2RP yielding 1000-bp PCR product. Lane 4: primers #3FP & #3RP yielding 800-bp PCR product. Lane 5: elongation factor used as a positive control (#4FP & #4RP, Table 3.1) yielding 1700-bp PCR product.

3.2 Cloning the PhTPS1gene

3.2.1 Petunia cDNA preparation

Petunia x hybrida cv. Mitchell seedlings were grown for 4 weeks in substrate (Metromix 280, Sun Gro Horticulture LTD., Vancouver, Canada). Total RNA (5 μg) was extracted using Zymo *research,* Quick-RNA™ MicroPrep. Complementary DNA (cDNA) was synthesized using RT-PCR (reverse transcriptase-PCR) (SuperScript III, Invitrogen, USA).

3.2.2 DNA manipulation and constructs

In order to clone the whole CDS of the *PhTPS1*, primers from the 5' and 3' UTR regions flanking the start and stop codon of the tomato ortholog gene were designed. The tomato gene was used because there is neither petunia genome nor CDS sequence available for this gene. PCR parameters and reagents were the same as mentioned above (section 3.1). This PCR gave no product (data not shown). Next, a comparison of the *Solanum lycopersicum* and *Arabidopsis thaliana* CDS was used to design a degenerate forward primer #5FP (Table 3.1). This degenerate primer included an ATG triplet and *K* = G or T; *Y* = C or T; *S* = G or C; and *R* = G or A. The reverse primer used was #1RP described above (section 3.1) and the approximate relative positions on the *PhTPS1* cDNA are shown in Figure 3.2. This PCR reaction gave a 2kb band (Figure 3.3).

Table 3.1. All primers (P) cited in this paper; forward (F) and reverse (R). For #24FP, #24RP, #25FP and #25R green indicates filler nucleotides, red indicates NotI restriction site and blue indicates pDB20 yeast vector sequence (only for #25FP and #25R). For the same four primers, bold case indicates the start codon (#24FP and #25FP) and italic case indicates the stop codon TAA.

#	Primer direction	Nucleotide sequence (5'→3')	Primer used	Section
		Used only in Petunia *x hybrida*		
1	F	CCCATTGGTATAGATTCAGAAC	on *PhTPS1* genomic DNA	3.1
1	R	ACCACTTTATCACGCCAG	on *PhTPS1* genomic DNA	3.1
2	F	GATCAGAGCTGCTCCGAGCAG	on *PhTPS1* genomic DNA	3.1
2	R	TGCCTTGCATAGTCATAGG	on *PhTPS1* genomic DNA	3.1
3	F	GGGAGCAGCAATAGATCGTATAC	on *PhTPS1* genomic DNA	3.1
3	R	CCAGCACATTCCATGACACA	on *PhTPS1* genomic DNA	3.1
4	F	ATGGGTAAAGAGAAGATTCACATC	on *PhTPS1* genomic DNA (E. factor)	3.1
4	R	TCACTTCCCCTTCTTCTGGGCAGC	on *PhTPS1* genomic DNA (E. factor)	3.1
5	F	KKYKGTYTTGKTSTRRRCRKATG	on *PhTPS1* cDNA (degenerate)	3.2.2
6	F	CAGGAAACAGCTATGAC	to sequence 2kb PCR - TOPO	3.2.2
7	F	CCAATAACCGTCTACTAATACT	on *PhTPS1* cDNA - ends of the 2kb	3.2.2
8	R	GAATTCGCCCTTTTCATGA	on *PhTPS1* cDNA 3' (+stop codon)	3.2.2
9	F	CGGGGAACAAGTATAACGG	to sequence *PhTPS1* into TOPO	3.2.2
10	F	ATGACTATGCGAGGCATTTTGTTA	to sequence *PhTPS1* into TOPO	3.2.2
11	F	GGAATATCACAGAGGTTGCTGCGT	to sequence *PhTPS1* into TOPO	3.2.2
12	F	AGCTAGAGACATGCTGCAGCAT	to sequence *PhTPS1* into TOPO	3.2.2
13	F	ATGCCGGGGAACAAGTATAAC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
13	R	GACTACATCGCCTTCTTCATAATG	on BULK PCR (*PhTPS1* cDNA)	3.2.3
14	F	ACTACCAGAAGTTTCCAGTCTCAA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
14	R	AAGTTCCGAATCGGCCATTTATGC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
15	F	AAATTGCTGTACCAACAAGAAC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
15	R	TTGCGGTCAATGGTTCTTTCA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
16	F	GACAGAATCAGTGGATACCCCTGG	on BULK PCR (*PhTPS1* cDNA)	3.2.3
16	R	GACTTTTGGGGCCGCTCGCCTTTC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
17	F	GAAGGATGAGGATGTATAT	on BULK PCR (*PhTPS1* cDNA)	3.2.3
17	R	ACTAGTCCTGCAGGTTTA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
18	R	GCTCCCATCCCTCATTAATT	on BULK PCR (*PhTPS1* cDNA)	3.2.3
19	F	TTCATATGTCGAACAGTACTTGG	on BULK PCR (*PhTPS1* cDNA)	3.2.3
19	R	TCTGAAGATGGAAAGGGTGTG	on BULK PCR (*PhTPS1* cDNA)	3.2.3

Table 3.1 (Continued)

#	Primer direction	Nucleotide Sequence (5'→3')	Primer used	Section
20	F	AGAGGTGTATCCCAGTATTCCT	on BULK PCR (*PhTPS1* cDNA)	3.2.3
20	R	TGCCTCGCATAGTCATAGGTAT	on BULK PCR (*PhTPS1* cDNA)	3.2.3
21	F	TAGGAGTTGATCGCCTTGATA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
21	R	CAAATTCACTGAGAATAAGAACC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
22	F	GTTATTGAAGCTCAACAGAGGATA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
22	R	CAAATTCAACATCTGCATACTTG	on BULK PCR (*PhTPS1* cDNA)	3.2.3
23	F	CAAAGACAACAGTCGTCGTCC	on BULK PCR (*PhTPS1* cDNA)	3.2.3
23	R	CAGGCACTTTCAGTGCATCA	on BULK PCR (*PhTPS1* cDNA)	3.2.3
24	F	TTAAAGCGGCCGC**ATG**CAACGACTCTTAGTTGT	to trim *PhTPS1*, introduce NotI & ATG codon	3.3.1
24	R	ATTTGCGGCCGC*TTA*AGAAGAGGCTTCAGCTAGT	to trim *PhTPS1*, introduce NotI & stop codon	3.3.1
25	F	GCTATACCAAGCATACAATCAACTTAAAGCGGCCGC**ATG**CAACGACTCTTAGTTGT	to trim *PhTPS*1, introduce NotI, ATG & pDB20 sequence	3.3.2
25	R	TGGCGAAGAAGTCCAAAGCTTATTTGCGGCCGC*TTA*AGAAGAGGCTTCAGCTAGT	to trim *PhTPS*1, introduce NotI stop codon & pDB20 sequence	3.3.2
		Used only in *Saccharomyces cerevisiae*		
26	F	AATATTTCAAGCTATACCAAGCAT	to PCR 5 yeast colonies	3.3.5
27	F	TATAAGGGGATTGACGAGG	to amplify *ScTPS1*	4.2
27	R	ACTTGAGGCGTAGACCTGCT	to amplify Sc*TPS1* (DNTAG)	4.2
28	R	ATATTCTATGGTCTTCTCCGTC	to amplify *ScTPS1*	4.2
29	F	GGTGCCATACAGGGAAATC	to amplify *ScTPS2*	4.3
29	R	GAGATGCCGTTCTTCTCCTCAG	to amplify *ScTPS2*	4.3

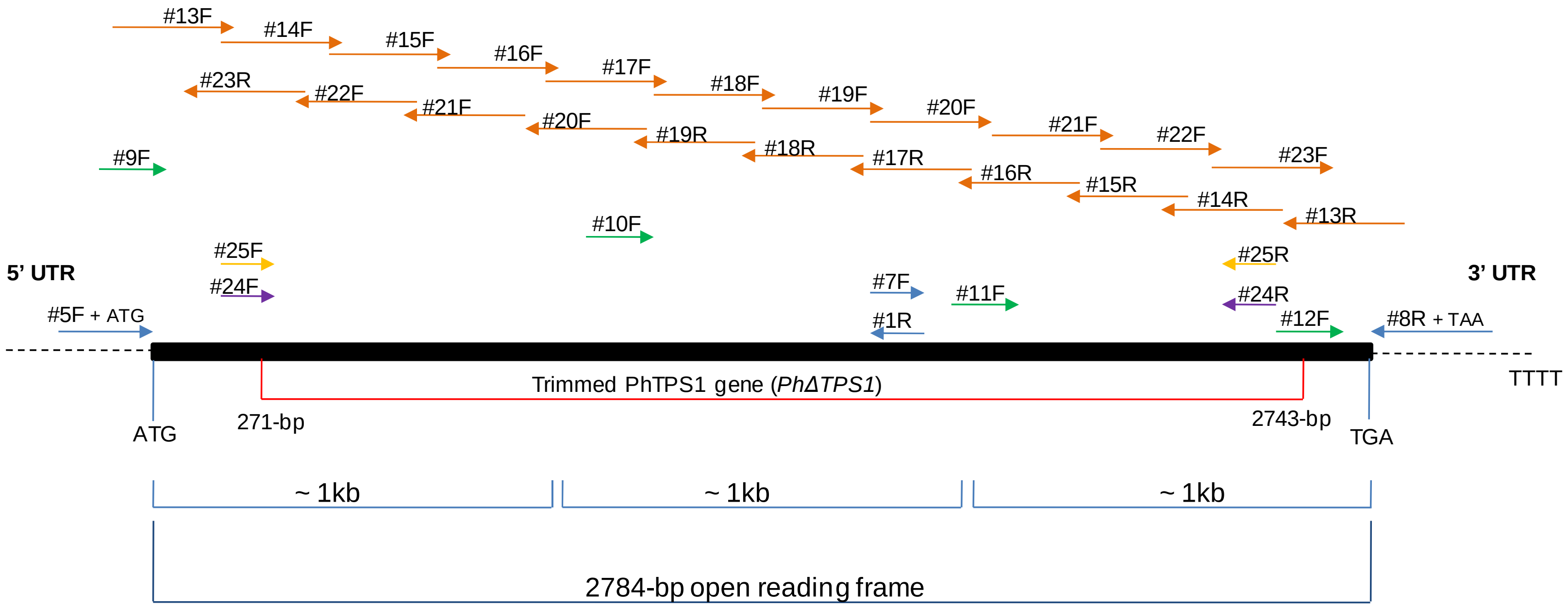


Figure 3.2. Forward (F) and reverse (R) primers used and their approximate relative positions on the *PhTPS1* cDNA. The green (#9F - #12F) primers used to sequence *PhTPS1* in TOPO cloning vector, green and orange (#13 - #23) used in BULK PCR, purple (#24F and #24R) primers used to trim (between 271-bp and 2743-bp) the petunia TPS1 gene while introducing a NotI restriction site and yellow primers (#25F and #26R) were used to trim *PhTPS1* while introducing the pDB20 yeast vector sequence and a NotI restriction site. All primer sequences are found in Table 3.1.

The 2kb PCR product (Figure 3.3) was cloned into the TOPO PCR cloning vector (pCR® *4 TOPO® Vector*, Invitrogen, USA). During PCR an ‘A’ is added by the *Taq* 2X Master Mix enzyme to the 3’ of the PCR product so that it can base-pair with the ‘T’ overhanging in the multiple cloning site (MCS) of the TOPO vector, MCS which has an *EcoRI* site upstream and downstream of the site where the PCR products gets cloned. The ligation was used to transform competent *E. coli* cells (Lucigen, *E. coli* 10G CLASSIC Electrocompetent Cells). The transformed cells were plated on rich LB media (5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g of agar and 0.5 L of dH_20) with the addition of 500 μl of ampicillin (100 mg/mL) after the autoclaved agar was cooled to ~50°C.

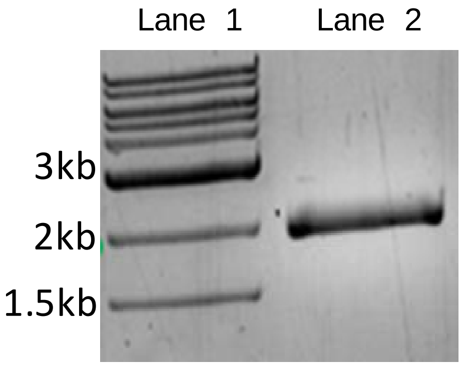


Figure 3.3. Amplification of cDNA with a forward degenerate primer including an ATG triplet (#5FP) and a reverse primer used in section 3.1 (#1RP). This PCR reaction gave an expected 2kb band (Lane 2). Lane 1: 1kb DNA Invitrogen ladder.

Plasmid DNA was isolated from *E. coli* (Zyppy™ Plasmid Miniprep Kit, Zymo research, USA) and digested with EcoRI (New England BioLabs, USA).

The three bands seen in Figure 3.4 were the expected sizes; 4kb TOPO, 1.3kb and 0.7kb = 2kb insert, suggesting that an EcoRI restriction site is present in the 2kb insert. Only the ends of the 2kb PCR product was sequenced (Life Sciences Core Laboratory Center, Biotechnology Bldg, Cornell University, 14853) using primer #6FP (Table 3.1) that align in TOPO MCS.

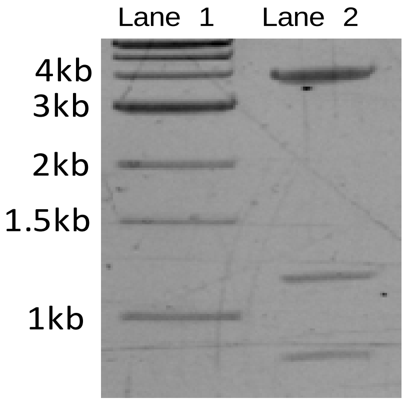


Figure 3.4. Plasmid DNA isolated from *E. coli* and digested with EcoRI. The three bands seen match the expected sizes; 4kb TOPO, 1.3kb and 0.7kb = 2kb insert; an EcoRI restriction site is present in the 2kb insert (Lane 2). Lane 1: 1kb DNA Invitrogen ladder.

In order to obtain the remaining 3' region of the *PhTPS1* (see Figure 3.2) a new forward primer #7FP (Table 3.1) was designed based on the sequence obtained from the core laboratory center at Cornell University. An oligo dT reverse primer (SuperScript III, Invitrogen, USA) was used to amplify the still unknown 3' UTR and 3' CDS of the *PhTPS1*. PCR reactions as described in section 3.1. This PCR reaction yielded an ~ 1.3kb band (Figure 3.5) which was also cloned into the TOPO PCR cloning vector (pCR® 4

TOPO® Vector, Invitrogen, USA). Competent *E .coli* cells were transformed (Lucigen, *E. coli* 10G CLASSIC Electrocompetent Cells) and plated on rich LB media with the addition of ampicillin as mentioned above. A restriction digestion with EcoRI confirmed the success of the latest cloning (Figure 3.6); 4kb band, the expected size of TOPO and a ~ 1.3kb band the remaining *PhTPS1* gene. This clone was also sequenced (Life Sciences Core Laboratory Center, Biotechnology Bldg, Cornell University, 14853) using #FP6 (Table 3.1), as mentioned above.

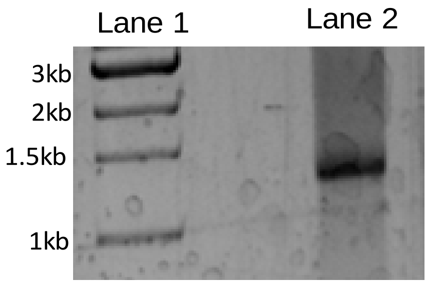


Figure 3.5. Amplification of petunia cDNA. Expected size ~ 1.3kb band (Lane 2) while amplifying with a forward primer at the end of the newly 2kb sequence obtained (#7FP) and an oligo dT provided with the SuperScript III kit. Lane 1: 1kb DNA Invitrogen ladder.

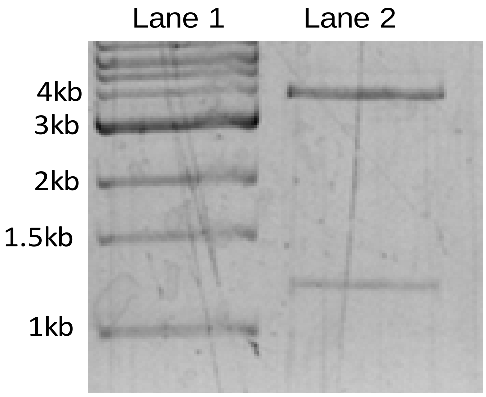


Figure 3.6. A restriction digestion with EcoRI was on TOPO cloning vector to confirm the success of the latest cloning (Figure 3.5); 4kb is the expected size for the vector and ~1.3kb for the remaining *PhTPS1* gene (Lane 2). Lane 1: 1kb DNA Invitrogen ladder.

Based on the sequence of the ends of the *PhTPS1* insert previously obtained from the core laboratory center a new reverse primer was designed to amplify across the whole CDS. This primer #8RP (Table 3.1) included the TAA stop codon. The previously described degenerate forward primer #FP5 (Table 3.1) was used (Figure3.2). This PCR yielded a faint 3kb band that matched the expected size of the CDS of *SlTPS1* ortholog (2781-bp) and *AtTPS1* ortholog (2829-bp) (data not shown). The 3kb band was gel-purified (Zymoclean™ Gel DNA Recovery Kit, USA) and re-amplified through PCR using the same primers and PCR reaction conditions described above in section 3.1 (Figure 3.7). The 3 kb band was then cloned into the TOPO PCR cloning vector (pCR® 4 TOPO® Vector, Invitrogen, USA) and was used to transform competent *E. coli* cells (Lucigen, *E. coli* 10G CLASSIC Electrocompetent Cells). The transformed cells were plated onto rich LB media plus ampicillin (see description above). A single colony was picked, grown overnight in the same liquid media and miniprepped (Zyppy™ Plasmid Miniprep Kit, Zymo research, USA). Samples sequenced included the plasmid plus the 3kb insert and forward primers #9, #10, #11 and #12 (Table 3.1). This generated overlapping products of 700-bp. The sequences obtained from core laboratory center at Cornell University were aligned using the DNAstar-Lasergene software. The final assembled contig has 2783-bp and closely aligns with *SlTPS1*, having 849 amino acids in common out of a total of 927. This petunia reference sequence has fourteen restriction sites for nine different enzymes; Bsp106, BstXI, SacI, BSp, SpeI, DraII, EcoRI, HindIII, PstI & XbaI.

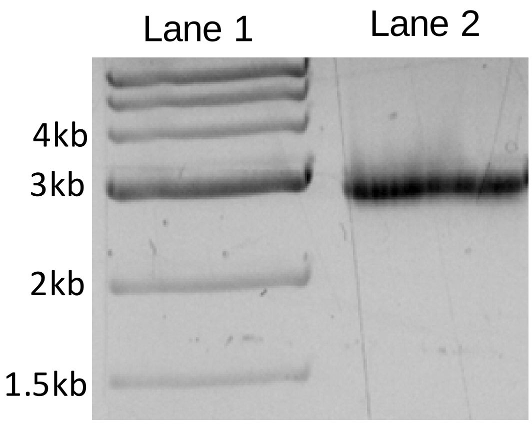


Figure 3.7. Amplification of cDNA with a forward degenerate primer including an ATG triplet (#5FP) and a new reverse primer (#8RP) designed to amplify across the whole CDS This PCR reaction gave an expected 3kb band (Lane 2) which matches the size of the tomato and arabidopsis ortholog TPS1 genes Lane 1: 1kb DNA Invitrogen ladder.

3.2.3 Bulk PCR

Multiple sequences were obtained and aligned from the plasmid plus insert described in section 3.2.2. To confirm that the sequence (2783-bp) described above was correct and no PCR mistakes were introduced while amplifying the *PhTPS1* a bulk PCR was carried out in a thermocycler (Bio-Rad, Hercules, CA) using a high-fidelity Taq polymerase (Herculase II Fusion DNA Poly, VWR, USA) to double check the fidelity of the 2783-bp. Primers #9F - #12F and eleven new ones #13F– #23 F (Table 3.1) were designed to amplify directly from cDNA (section 3.2.1). The relative positions of these primers are found in Figure 3.2. Thirty five short (400-bp) and overlapping PCR products were generated through this bulk PCR. The sequenced products were aligned and compared to the reference sequence obtained earlier. The PCR parameters used were: 94°C for 2 min, followed by 30 cycles of 94 °C for 20 sec, 52 °C for 20 sec and 68 °C for 1 min and a final extension of 5 min at 68 °C.

Six differences/mutations were found when comparing the reference sequence with the alignment of all the short PCR products generated. Of those mutations four were silent and two changed the predicted amino acids. A single base change (T→C, bp#143, AA#48) in the second base of Leucine (CTC) in the reference sequence became a Proline (CCC) in the new aligned sequence. Another single base change (T→A, bp#2573, AA #858) in the second base of Valine (GTG) of the reference sequence changed to Glutamic Acid (GAG) in the new sequence. Both of these PCR artifact/errors introduced by polymerase were in non-conserved regions, as indicated by the red triangle in Figure 3.8. This corrected final sequence generates a 2784-bp open reading frame that predicts a 58kDA protein of 927 amino acids. This final and doubled checked sequence was submitted to both SOL (Sol Genomics Network) and NCBI (National Center for Biotechnology Information) database and the accession number of HQ259080 was granted by the later database.

3.2.4 DNA alignments

Alignments of amino acid sequences of *Selaginella lepidophylla, Populus trichocarpa, Solanum lycopersicum, Arabidopsis thaliana, Vitis vinifera* and *Petunia x hybrida* show that the N-terminal and C-terminal ends of the TPS1 enzyme are diverse whereas the middle of the sequences remains highly conserved across different species (Figure 3.8). Also, the predicted amino acid sequence from the petunia cDNA confirms that the PhTPS1gene is most closely related to the Solanaceae family, as shown in the phylogenetic tree (Figure 3.9).

3.3 Expression of the PhTPS1 gene

3.3.1 Truncating *PhTPS1* (*ΔPhTPS1*)

Analysis of the TPS1 N-terminus from *Arabidopsis thaliana* and *Selaginella lepidophylla* led to the identification of two conserved residues which decrease enzyme activity when present. Therefore, to verify *ΔPhTPS1* activity in yeast we undertook to truncate the same region from*PhTPS1*. To truncate *PhTPS1* a PCR was conducted on a thermocycler (Bio-Rad, Hercules, CA) using a high-fidelity Taq polymerase (Herculase II Fusion DNA Poly, VWR, USA) on previously made cDNA (section 3.2.1). The following parameters were used: 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 sec, 52 °C for 20 sec and 68 °C for 1 min. A final extension time of 5 min at 68 °C was used.

Two newly designed sets of primers #24FP & #24RP (Table 3.1) were used. The 5’ end of the forward primer #24FP has 5-bp of filler sequence followed by the 8-bp NotI site, a start codon and 17 bases complementary to the *PhTPS1* sequence. This primer was designed at the 5’ region of the gene (first base pair of primer starts at 271-bp of the double checked sequence in bulk PCR) and by designing it there we were trimming the first 100 AA.

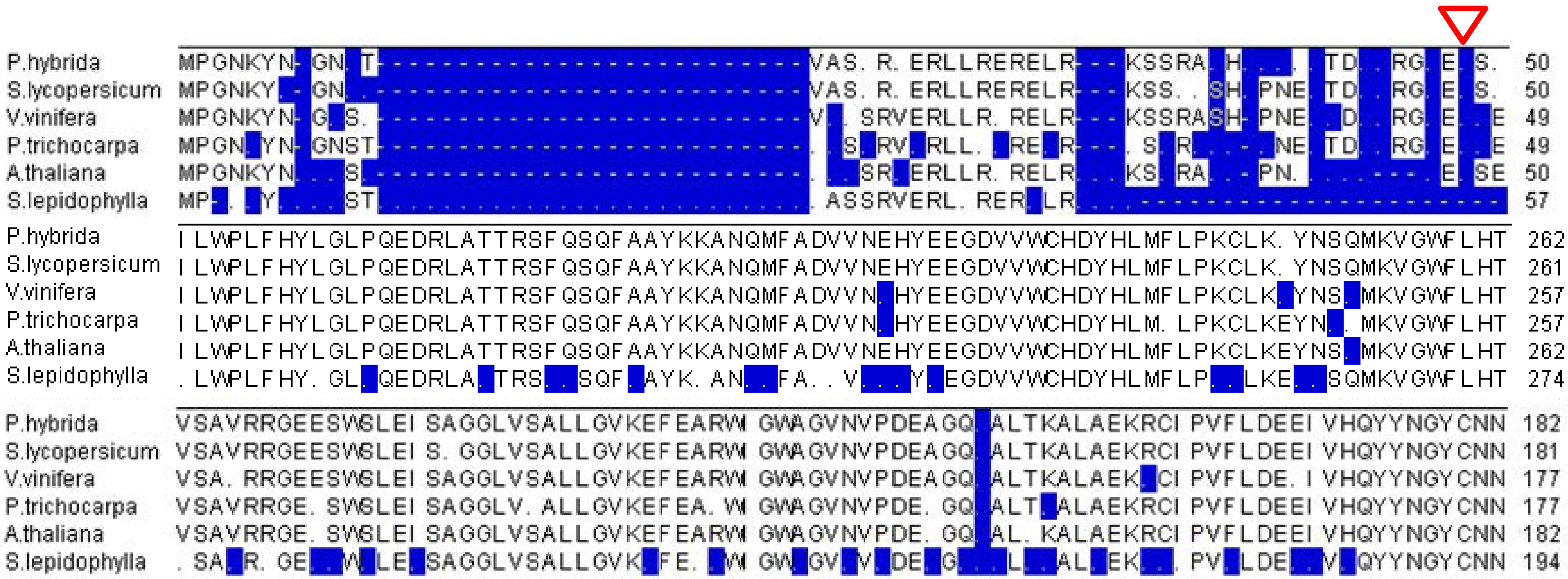


Figure 3.8. Alignment of the predicted TPS1 amino acids sequences for *Petunia x hybrida* (on top), *Solanum lycopersicum*, *Vitis vinifera Populus trichocarpa, Arabidopsis thaliana* and *Selaginella lepidophylla.* The N-terminal and C-terminal ends of the TPS1 enzyme are diverse whereas the middle of the sequences remains highly conserved across different species. The two red triangles point out errors introduced during PCR amplification of *PhTPS1*. Both of these PCR artifacts/differences are in non-conserved regions (blue).

Figure 3.8 (Continued)

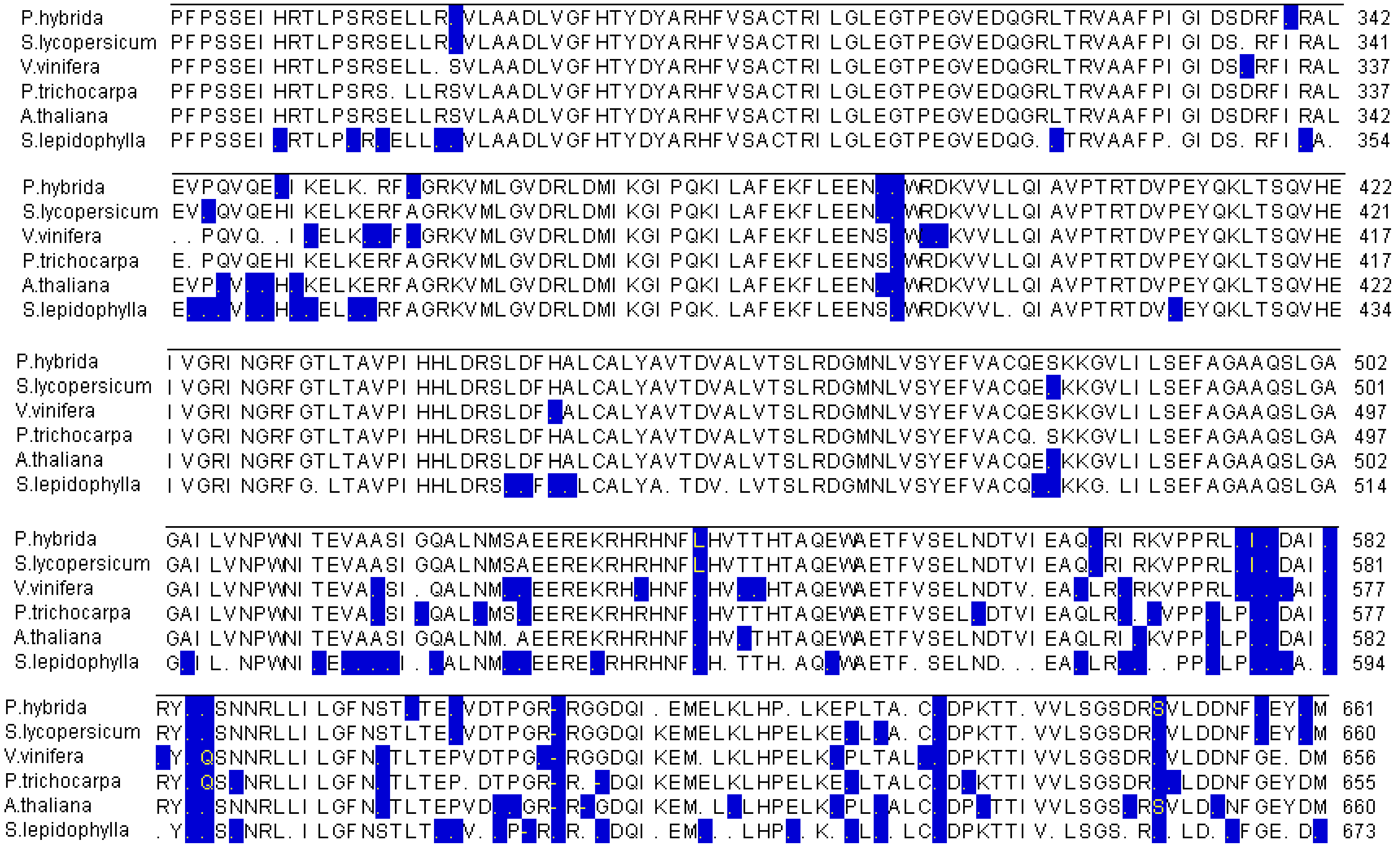


Figure 3.8 (Continued)

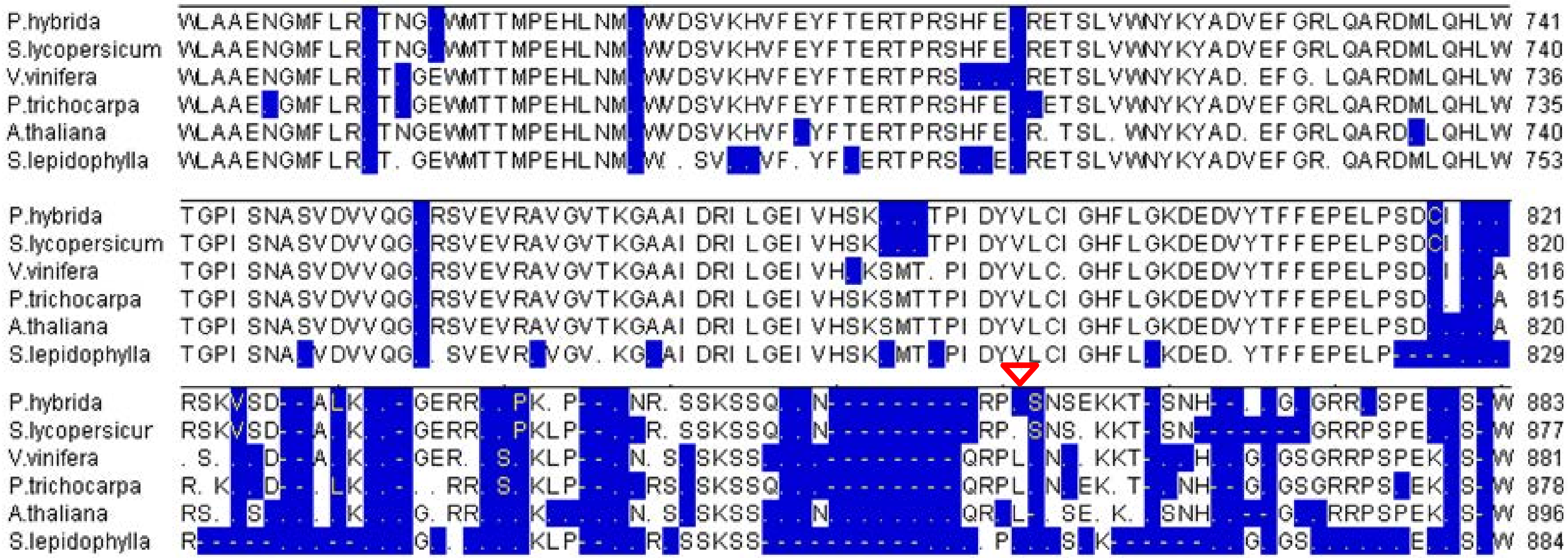
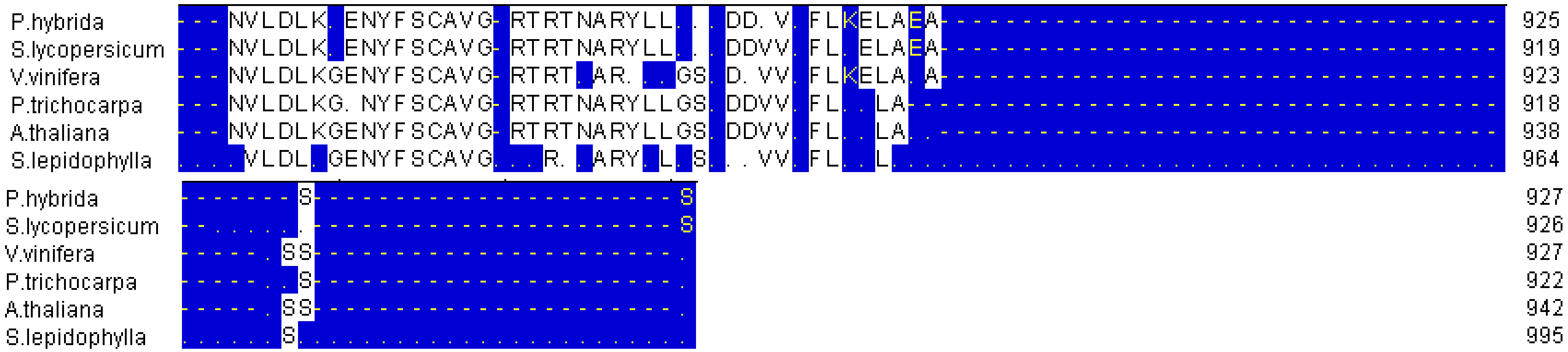


Figure 3.8 (Continued)



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P.hybrida        ---NVLDLK.ENYFSCAVG-RTRTNARYLL...DD.V.FLKELAEA----------------------------------   925
S.lycopersicum   ---NVLDLK.ENYFSCAVG-RTRTNARYLL...DDVV.FL.ELAEA----------------------------------   919
V.vinifera       ---NVLDLKGENYFSCAVG-RTRT.AR...GS.D.VV.FLKELA.A----------------------------------   923
P.trichocarpa    ---NVLDLKG.NYFSCAVG-RTRTNARYLLGS.DDVV.FL..LA-----------------------------------   918
A.thaliana       ---NVLDLKGENYFSCAVG-RTRTNARYLLGS.DDVV.FL..LA..---------------------------------   938
S.lepidophylla   ....VLDL.GENYFSCAVG...R..ARY.L.S...VV.FL..L.....................................   964

P.hybrida        -------S----------------------S   927
S.lycopersicum   --......----------------------S   926
V.vinifera       -----.SS----------------------.   927
P.trichocarpa    -----..S----------------------.   922
A.thaliana       -----.SS----------------------.   942
S.lepidophylla   ......S........................   995
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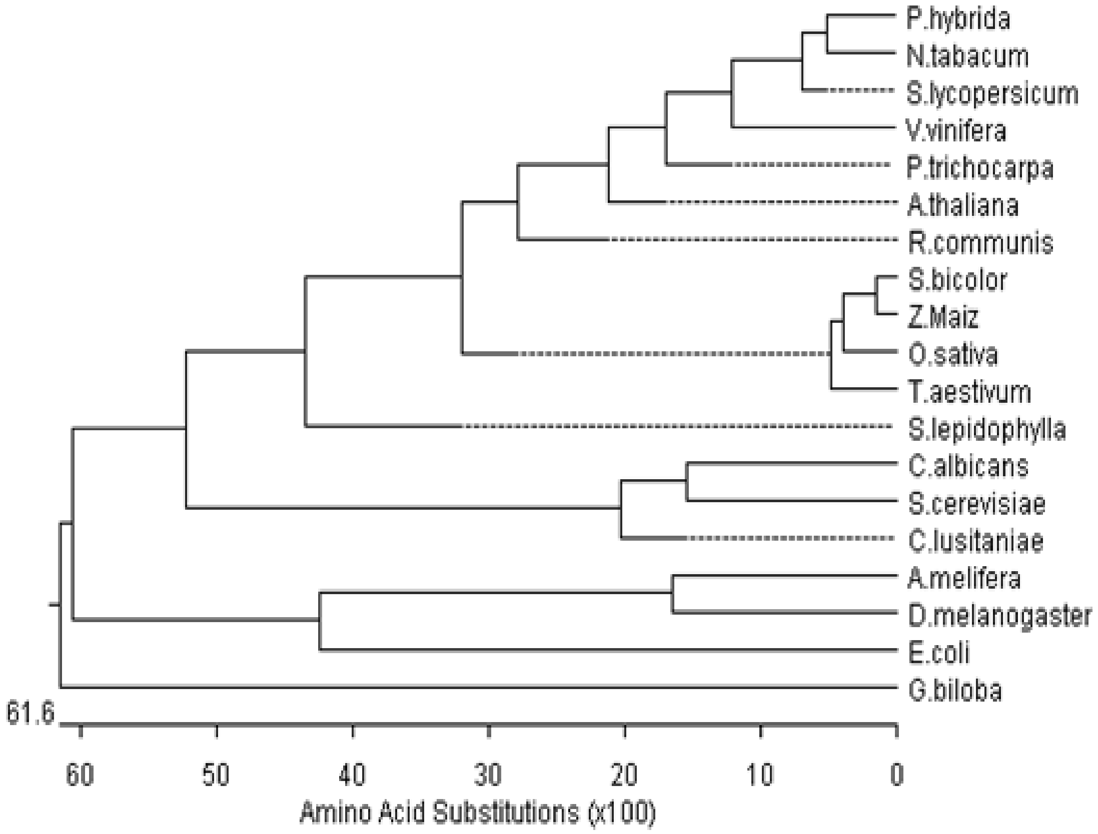

Figure 3.9. Phylogenic tree (including non-plant organisms) showing position of cloned *Petunia x Hybrida* cv. 'Mitchell Diploid' TPS1(on top) relative to TPS1 protein sequences from other species: *Nicotiana tabacum*, *Solanum lycopersicum, Vitis vinifera, Populus trichocarpa, Arabidopsis thaliana, Ricinus communis, Sorghum bicolor, Zea mays, Oryza sativa, Triticum aestivum, Selaginella lepidophylla, Candida albicans, Saccharomyces cerevisiae, Clavispora lusitaniae, Apis mellifera, Drosophila melanogaster, Escherichia coli* and *Ginkgo biloba.* The predicted amino acid sequence from the Petunia cDNA confirms that the PhTPS1 gene is most closely related to the Solanaceae family, as shown in the phylogenetic tree.

The reverse primer, beginning at the 5’ end has 4-bp of filler sequence followed by 8-bp of a NotI site, a stop codon and 19 bases complementary to the *PhTPS1* sequence. This primer was designed at the 3’ end of the gene (first base pair starts at 2743-bp of the sequence that was double checked in bulk PCR. The PCR product (0.23μg or 231.4ng/μl) was purified using QIAquick PCR Purification Kit, USA and separated in a 1% agarose gel (Figure 3.10). The NotI restriction site was selected and incorporated into the PCR product because it is not present in the *PhTPS1* sequence and it is present in the polylinker of the pDB20 yeast vector.

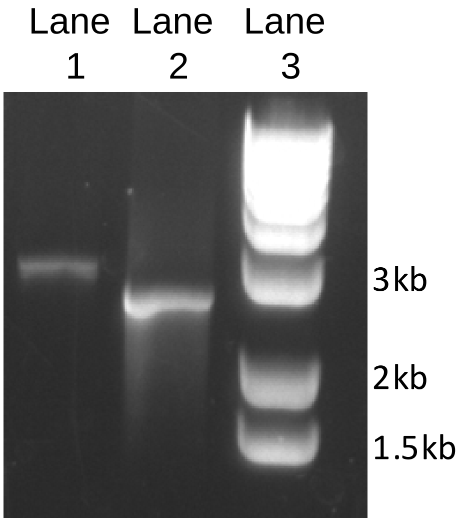

Figure 3.10. Trimming *PhTPS1* with a forward primer (#24FP) and reverse primer (#24FP). Primer descriptions are found in section 3.3.1 and sequences in Table 3.1. Lane 1: Untrimmed *PhTPS1*. Lane 2: Trimmed *PhTPS1*. Lane 3: 1kb Plus DNA Invitrogen ladder.

3.3.2 Truncating *PhTPS1* (*ΔPhTPS1*) and incorporating pDB20 yeast promoter sequences

The PCR product obtained in section 3.3.1 was diluted 1/45 and used as a template to set up a new PCR reaction. The same high-fidelity Taq polymerase and PCR conditions described previously were used. Two different primers were designed to truncate the 3kb *PhTPS1* while at the same time introducing sequence homologous to the promoter of the pDB20 vector, used in later cloning steps. A new forward primer #25FP (Table 3.1) was designed. The 5' end of this primer has 23-bp of the promoter sequence of pDB20 vector followed by 5 bases of filler sequence, a NotI restriction site, a start codon and 17 bases complementary to the *PhTPS1* sequence. This primer was designed starting at nucleotide 271 of the sequence. A reverse primer #25RP (Table 3.1) was also designed. The 5' end this primer has 19-bp corresponding to 3' end of the gene, a stop codon, a NotI site, five base pairs of filler sequence and 21-bp of the promoter of the pDB20 vector. This trimmed Petunia TPS1 gene (*ΔPhTPS1*) which now included a yeast promoter sequence was clone into the pDB20 yeast vector (Section 3.3.4).

3.3.3. The pDB20 yeast vector

The backbone of the pDB20 yeast expression vector comes from the pUC18 vector. pDB20 carries the ADCI (ADHJ) promoter which drives high levels of gene expression. In the polylinker region this vector has 6 cloning sites; HIND3-NOTI-BSTXI-BSTXI-NOTI-HIND3. The vector has URA3 as a yeast selectable marker and amp^R as an *E coli* selectable marker. The vector also has HpaI restriction site outside the polylinker region.

NotI restriction enzyme (Invitrogen, USA) was used to digest pDB20 (Figure 3.11). To confirm that the NotI digested the vector some of the DNA was double digested with HpaI and NotI (Invitrogen, USA). The three bands below show that the NotI and HpaI digestion worked (Figure 3.10).

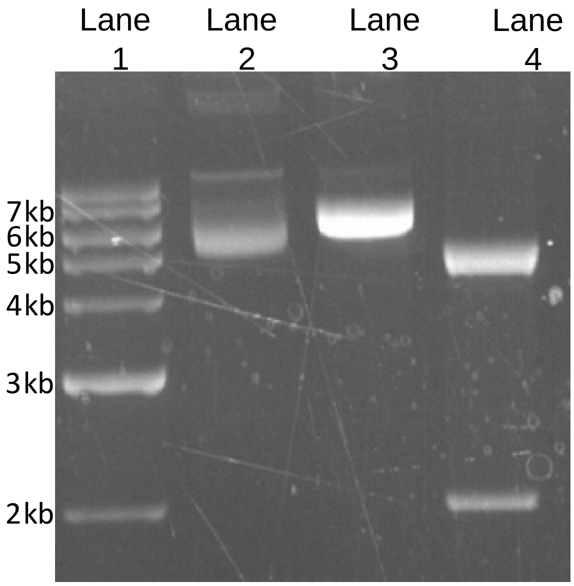

Figure 3.11. pDB20 yeast expression digested with NotI and HpaI restriction enzymes (Invitrogen). Lane 1: ladder (1kb Invitrogen, USA). Lane 2: undigested vector. Lane 3: pDB20 digested with NotI. Lane 4: pDB20 double digested with NotI and HpaI.

3.3.4 Cloning ∆*PhTPS1* into the pDB20 yeast vector

0.25μg (250 ng/μl) of pDB20 digested with NotI (Invitrogen, USA) was treated with phosphatase following the protocol provided by the New England BioLabs kit (Antarctic Phosphatase, New England BioLabs, USA). The trimmed *∆PhTPS1* (0.23μg), as described in section 3.3.1, was digested with the same NotI enzyme. Ligation was carried out with T4 DNA ligase enzyme

(Lucigen, USA) and the ligation mix was used to transform competent *E. coli* cells (Lucigen, *E. cloni* 10G CLASSIC Electrocompetent Cells). The transformed cells were plated on rich LB media (5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g of agar and 0.5 L of dH$_2$0) with the addition of 500 μl of ampicillin (100 mg/mL) after the autoclaved agar was cooled to ~50°C. Plasmid DNA was isolated from transformed *E. coli* (Zyppy™ Plasmid Miniprep Kit, Zymo research, USA) and sequenced at Cornell's core lab. Sequences revealed the lack of the ∆*PhTPS1* insert. Since this standard 'cut and paste' procedure between the vector and trimmed petunia gene was unsuccessful a new approach was undertaken (3.3.5).

3.3.5 Cloning Δ*PhTPS1* using yeast homologous recombination

Yeast strain DFS188 (MATa; ura3-52; leu2-3,112; lys2; his3-deltaHinDIII; arg8::hisG; D273-10B background) was grown overnight in complete liquid medium containing glucose (YPD: 10 g peptone, 20 g yeast extract, 20 g dextrose and 1000 ml dH$_2$0). These cells were transformed (Frozen-EZ Yeast Transformation II Kit™, Zymo research, USA) with 0.25 μg (250 ng/μl) of the NotI digested pDB20 vector plus 0.23 μg (231.4 ng/μl) of *∆PhTPS1* DNA. Another aliquot of the competent cells were transformed separately with NotI digested pDB20 vector. The transformed yeast was plated on complete supplement mixture–Ura (CSM–Ura; Yeast nitrogen base without amino acids, glucose, agar and Complete Supplement Mixture minus uracil; Sunrise Science Products, USA) media to select for Ura$^+$ cells.

The trimmed gene was cloned based on a DNA gap repair system which relies on the ability of yeast to repair gapped DNA sequences *in vivo* by means of homologous recombination (Kostrub et al., 1998). The incorporation of pDB20 sequence into *ΔPhTPS1* (section 3.3.2) allow that homologous recombination takes place at a high frequency between *ΔPhTPS1* and the linearized vector while co-cultivated with competent DFS188 cells, resulting in a circularized plasmid and not Ura3^+ inserted into the yeast chromosome (Figure 3.12, picture 3).

The NotI digested pDB20 vector be inserted into yeast genome via homologous recombination at the Ura^3 locus making cells Ura$^+$, in the same co-cultivation mentioned above. This is, however, a rare event and only a handful of colonies should be found, which matched our observed results (Figure 3.12 picture 2)

To corroborate that cloning was successful PCR was carried out in a thermocycler (Bio-Rad, Hercules, CA) using *Taq* 2X Master Mix (New England BioLabs, USA) using gDNA from five yeast colonies using a forward primer (#26FP) complementary to the ADCI (ADHJ) promoter sequence upstream of the MCS of pDB20 and the previously used reverse primer #22RP (Table3.1). PCR conditions were as follow: 94°C for 10 min, followed by 30 cycles of 94°C for 20 sec, 55°C for 20 sec and 68°C for 30 sec, final extension of 5 min at 68°C. PCR products were separated in a 1% agarose gel (Figure 3.13). The expected PCR product is a bit larger than 1kb as was expected based on the set of primers used.

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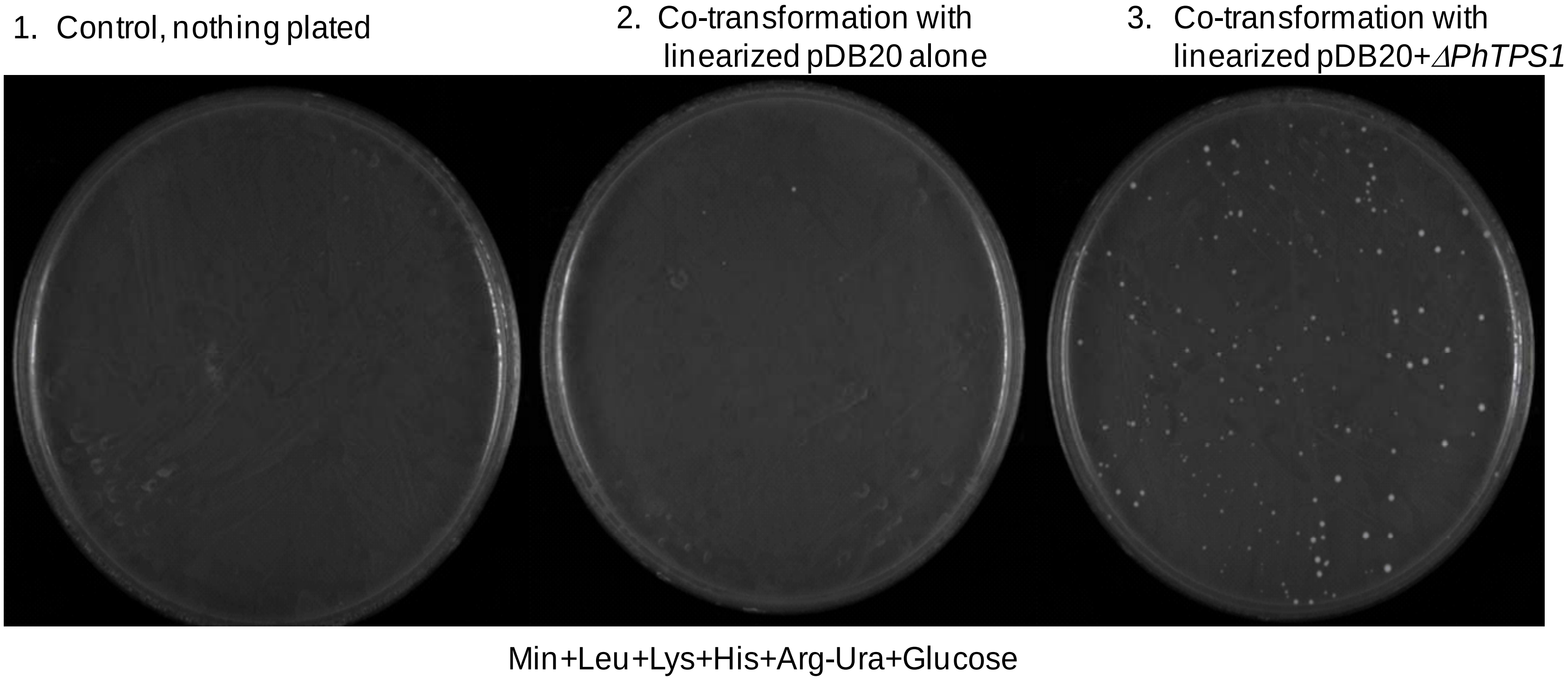
Figure 3.12. Cloning *ΔPhTPS1* into pDB20 yeast vector using DSF188 yeast strain to repair gapped DNA sequences *in vivo* by means of homologous recombination. 1. Control, nothing plated. 2. Co-transformation of competent DS188 cells with linearized pDB20 vector containing the selectable marker (Ura^3) along with *ΔPhTPS1*. Homologous recombination did not take place between the linearized vector and *ΔPhTPS1*; instead, pDB20 was inserted into yeast genome at the Ura^3 locus making cells Ura$^+$. This is a rare event; only a handful of colonies were observed. 3. Co-transformation of competent DS188 cells with linearized pDB20 vector containing the selectable marker (Ura^3) along with *ΔPhTPS1*. Homologous recombination took place at a high frequency between the linearized vector and the trimmed *PhTPS1 in vivo,* resulting in a circularized plasmid containing the *ΔPhTPS1*. Media for all three plates is as follows: Min+Leu+Lys+His+Arg-Ura+Glucose.



1. Control, nothing plated

2. Co-transformation with linearized pDB20 alone

3. Co-transformation with linearized pDB20+*∆PhTPS1*

Min+Leu+Lys+His+Arg-Ura+Glucose

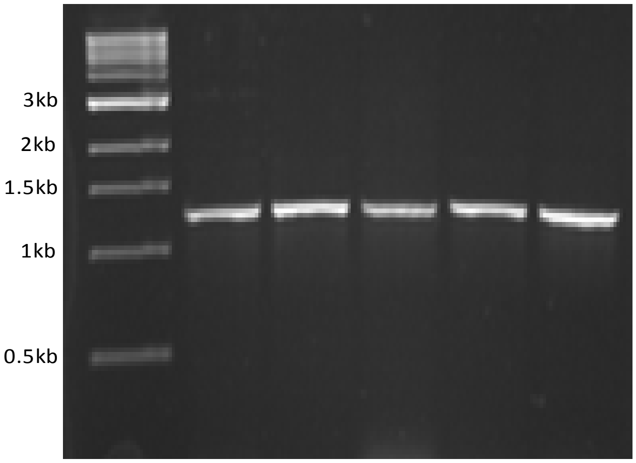


Figure 3.13. PCR using gDNA from five yeast colonies transformed with the pDB20 yeast vector plus the trimmed petunia TPS1 gene. Expected size is 1.3kb using forward primer (#26FP) and a reverse primer annealing to the middle of the petunia trimmed gene (#22RP) (Table 3.1).

Ten colonies of the successful transformation (Figure 3.12, 3) were grown overnight in liquid CSM–Ura media and miniprepped (Zymoprep™ Yeast Plasmid Miniprep II, USA) from these yeast cells. *E. coli* strain 10G supreme Duos (Lucigen, E. cloni 10G CLASSIC Electrocompetent Cells) was then transformed with the plasmid DNA purified from yeast, selecting colonies for ampicillin resistance in the rich LB media (for media preparation see sections 3.2.2).

3.3.6 Sequencing pDB20+Δ*PhTPS1*

To make sure that no new mutations/PCR artifacts were introduced during PCR or as a result of the yeast homologous recombination, the pDB20+Δ*PhTPS1* was sequenced and compared to the previous sequence obtained (section 3.3.2). The primers used for sequencing were #26FP,

#14FP, #15FP, #16FP, #17FP, #22FP, #23FP (Table 3.1). Only one difference was found; a silent mutation in the third position of Glycine (GGA→ GGT) at nucleotide 2283.

3.3.7 Yeast strains BY4742

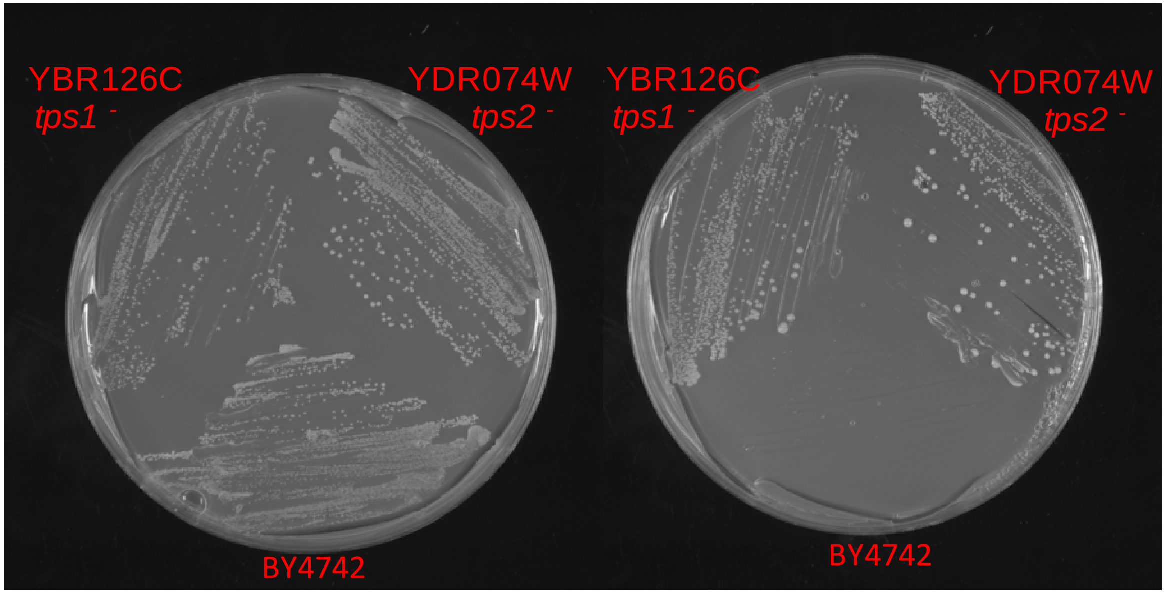
Three different yeast strains were ordered (Thermo Scientific, Open Biosystem, USA). All of them are of the background BY4742. Wild type parental BY4742 (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), YBR126C *tps1*$^-$ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0), and YDR074W *tps2*$^-$ (MATalpha his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0). To confirm the presence of the antibiotic G-418 (kanamycin-like) resistance in the *cassette* causing the deletion of the selected genes (either *tps1*$^-$ or *tps2*$^-$) these strains were grown in min+his+leu+lys+ura+gal+G-418 (Sigma-Aldrich, USA) and the same media without G-418. Media was prepared as follows: 6.7 g of yeast nitrogen base without amino acids (Sigma-Aldrich, USA) 20 g of D-(+)-Galactose (Sigma-Aldrich, USA), 20 g agar (BD Bacto™ Agar, Becton Dickinson and Company, USA) and 1 L of dH_2O were mixed in a 2 L flask. The media was autoclaved for 20 min. The amino acids solution was prepared in a 0.5 L flask by adding 0.2 g His, 0.2 g Leu, 0.2 g Lys, 0.2 g Ura (Sigma-Aldrich, USA) plus 100 ml of dH_20. This solution was filtered through a Stericup-VP Filter Unit (Millipore, USA). 5 ml of the previously purified G-418 was added to half of the plates. The G-418 was prepared as follows; 0.44 g of G-418 plus 10 ml of dH_2O and filtered through a Stericup-VP Filter Unit (Millipore, USA).

As shown in Figure 3.14, YBR126C *tps1*$^-$ and YDR074W *tps2*$^-$ were able to grow in the presence of the antibiotic whereas the parental strain could not. In the antibiotic-free plate all the strains grew.

A handful of kanamycin resistant (kan^R) colonies of YBR126C *tps1*$^-$ and YDR074W *tps2*$^-$ were selected from the Min+Lys+His+Leu+Ura+Gal+Kan plate and grown in liquid CSM+Gal overnight at 30° C. The parental BY4742 was selected from Min+Lys+His+Leu+Ura+Gal-Kan plate and grown overnight at 30°C in the same media (Figure 3.14).

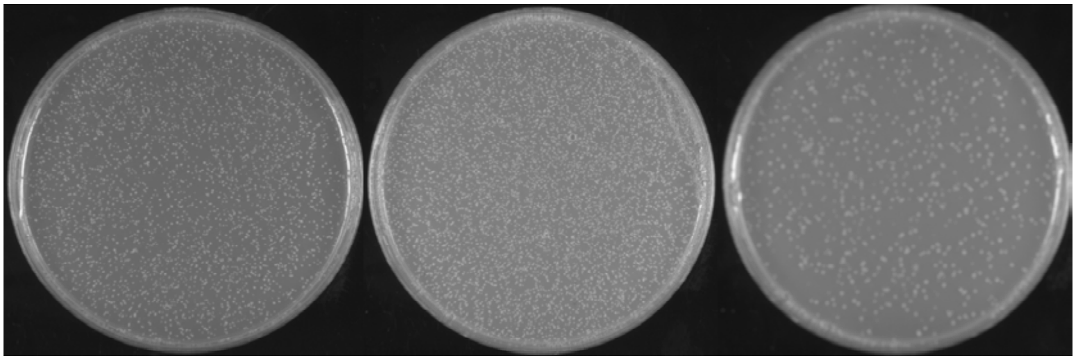
Six transformations (for procedure see section 3.3.5) were carried out: pDB20+*ΔPhTPS1* for YBR126C *tps1*$^-$, YDR074W *tps2*$^-$ and BY4742 cells and empty vector (pDB20) for YBR126C *tps1*$^-$, YDR074W *tps2*$^-$ and BY4742 cells. Transformants were selected by plating on CSM+Gal-Ura media to select for those that became prototrophic (+) for the ability to synthesize Ura (Figure 3.15).

The six transformations described above were plated on CSM-Ura+galactose and CSM-Ura+glucose [6.7 g of yeast nitrogen base without amino acids (Sigma-Aldrich, USA), 20 g of D-(+)-Glucose (L D Carlson company, Kent OH), 20 g agar (BD Bacto™ Agar, Becton, Dickinson and Company, USA) and 1 L of dH_2O, autoclaved for 20 min]. The amino acid solution was prepared by adding 0.2 g His, 0.2 g Leu, 0.2 g Lys (Sigma-Aldrich, USA) plus 100 ml of dH_20 and the solution was filtered through a Stericup-VP Filter Unit (Millipore, USA).

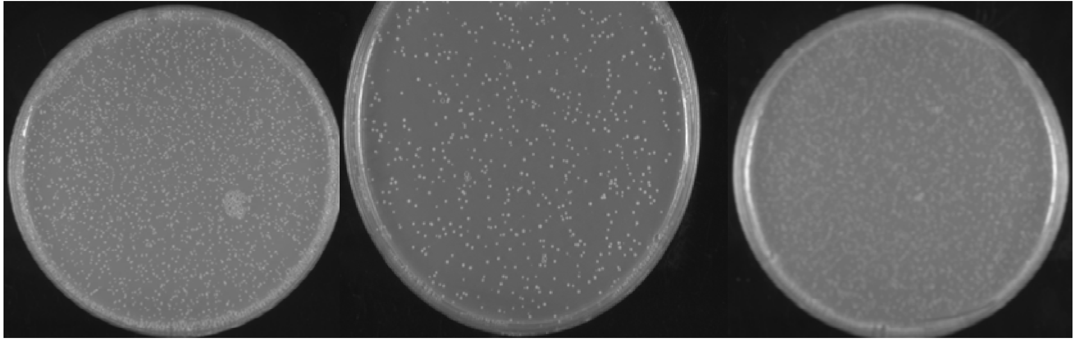


Min+Lys+His+Leu+Ura+Gal-Kan Min+Lys+His+Leu+Ura+Gal+Kan

Figure 3.14. Checking the presence of the kan^R antibiotic G-418 (kanamycin-like) in the *cassette* that causes the deletion of the selected genes (*tps1*$^-$ and *tps2*$^-$). YBR 126C tps1$^-$, YDR074W tps2$^-$ and BY4272 (wt) strains were grown Min+Lys+His+Leu+Ura+Gal-Kan (left) and Min+Lys+His+Leu+Ura+Gal+Kan (right). Without kanamycin the parental strain BY4742 is able to grow and when kanamycin is applied to the media they fail to grow. The only growth observed on the kan media is the knockout stains *tps1*$^-$ and *tps2*$^-$.



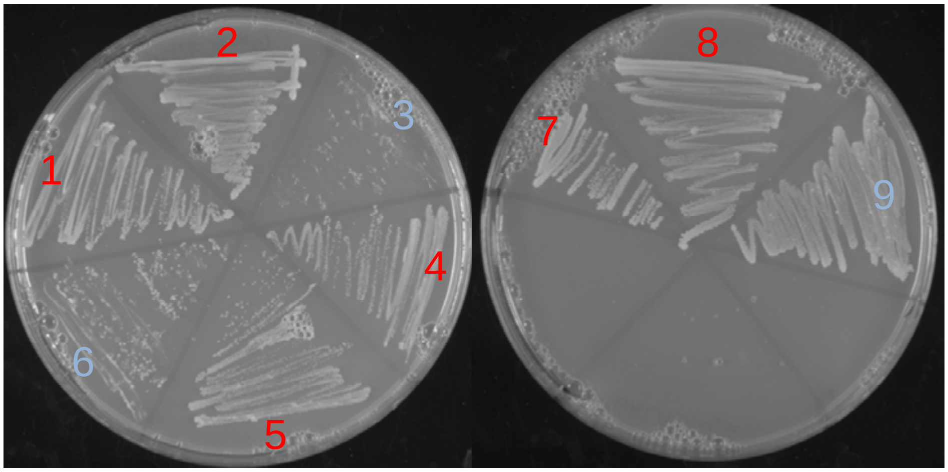
BY4742 - EV YBR126C *tps1*$^-$ - EV YDR074W *tps2*$^-$ - EV



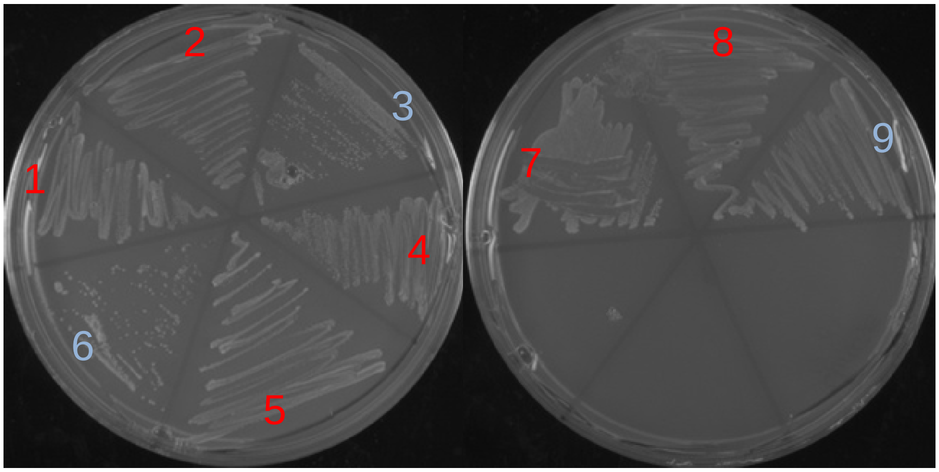
BY4742 - V+G YBR126C *tps1*$^-$ - V+G YDR074W *tps2*$^-$ - V+G

Figure 3.15. Yeast transformations with strains YBR126C *tps1*$^-$, YDR074W *tps2*$^-$ and BY4742 (parental) for both empty vector (EV) and vector plus ∆PhTPS1 gene (V+G).

The expected result is that all the strains can grow when galactose is used as the sole carbon source. Only YBR 126 C *tps1*$^-$ vector plus gene should be able to grow when glucose is the sole carbon source as the *∆PhTPS1* will restore the function of the *ScTPS1* in the knockout strain. Unexpectedly, everything grew in plates with glucose (Figure 3.16).



Min+Lys+His+Leu+Ura+Gal Min+Lys+His+Leu+Ura+Gal



Min+Lys+His+Leu+Ura+Glu Min+Lys+His+Leu+Ura+Glu

Figure 3.16. Growth of yeast strains streaked onto glucose and galactose. The six transformations described above (numbers in red); YBR 126 C *tps1*$^-$ V+G, YBR 126 C *tps1*$^-$ EV, YDR074W *tps2*$^-$ V+G, YDR074W *tps2*$^-$ EV, BY4742 V+G, BY4742 EV and the original untransformed strains from stock center (numbers in blue) were also plated. 1) YBR 126 C *tps1*$^-$ V+G, 2) YBR 126 C *tps1*$^-$ EV, 3) YBR 126 C *tps1*$^-$*,* 4) YDR074W *tps2*$^-$ V+G, 5) YDR074W *tps2*$^-$ EV, 6) YDR074W *tps2*$^-$, 7) BY4742 V+G, 8) BY4742 EV, 9) BY4742.

4 Troubleshooting

In order to examine the unexpected growth patterns on glucose more closely the following experiments were carried out. We first verified the yeast knockouts as described below.

4.1 PCR of gDNA of yeast strains

kan^R YBR126C *tps1*$^-$, YBR126C *tps1*$^-$, kan^R YDR074W *tps2*$^-$, YDR074W *tps2*$^-$, BY4742 (see section 3.3.7) cells were grown in YPD media (see 3.3.5) liquid media at 30°C. gDNA of these five yeast cultures was extracted following the chloroform protocol I (Zymoresearch, YeaStar™ Genomic DNA, USA) as follow: 1.5 ml of cells were centrifuged at 500 g for 2 min and the supernatant was completely removed. Next, 120 μl of YD Digestion Buffer and 5 μl of R-Zymolyase (RNase A + Zymolyase) were added. The pellet was resuspend by vortexing and incubated at 37°C for 60 min. 120 μl of YD lysis buffer was added and mixed well by gentle vortexing. 250 μl of chloroform was added to the pellet and thoroughly mixed for 1 min, then centrifuged at 10,000 rpm for 2 min. The supernatant was loaded onto a Zymo-spin III column and centrifuged at 10,000 rpm for 1 min. 300 μl of DNA wash buffer was placed onto the column and centrifuged for 1 min at 10,000 rpm. The latter step was repeated. Contents of the Zymo-spin III column was transferred to a new 1.5 ml centrifuge tube and 60 μl of water added directly to the membrane. After waiting for one minute the tube was centrifuged for 10 sec at max speed. An aliquot of the gDNA of the DSF188 strains was used as a positive control (section 4.2).

4.2.1 Amplifying *ScTPS1* from yeast gDNA

ScTPS1 is about 1.5kb based on the genome sequence available for *Saccharomyces cerevisiae,* roughly the same size as the *cassette* (1.6kb) used to knockout the ScTPS1 gene. Due to the similar sizes of the *cassette* and *ScTPS1* two different reverse primers #27R & #28R (Table 3.1) were designed in different region of the 3' sequence and used with a forward primer upstream the ATG codon (Figure 3.17).

A reverse primer #27RP (Table3.1) was designed in the down tag region (DNTAG region) of the *cassette* and the forward primer #27FP (Table 3.1) was designed 366-bp upstream to the ATG codon to check for the presence of the *cassette* in the following strains: kan^{R}YBR126C *tps1*$^-$ and YBR126C *tps1*$^-$. Another reverse primer (#28RP) was designed 609-bp downstream of the stop codon to amplify the wild type *ScTPS1* with the same forward primer (#27FP) from strains kan^R YDR074W *tps2*$^-$, YDR074W *tps2*$^-$ and BY4742 (Figure 3.17). Strain DSF188 was used as positive control.

PCR was carried out with the aforementioned primers and gDNA extracted from kan^R YBR126C *tps1*$^-$, YBR126C *tps1*$^-$*,* kan^{R}YDR074W *tps2*$^-$, YDR074W *tps2*$^-$ and BY4742*.* An aliquot of gDNA of DSF188 strain was used as positive control. PCR conditions were: 94°C for 2 min, followed by 30 cycles of 94°C for 20 sec, 50°C for 20 sec and 68°C for 30 sec, final extension of 5 min at 68°C) in a thermocycler (Bio-Rad, Hercules, CA) using EconoTaq® plus 2X Master Mix (Lucigen, USA).

Assuming that the strains kan^{R}YBR126C *tps1*$^-$ and YBR126C *tps1*$^-$ had the knockout where the *ScTPS1* gene was replaced with the kan^R

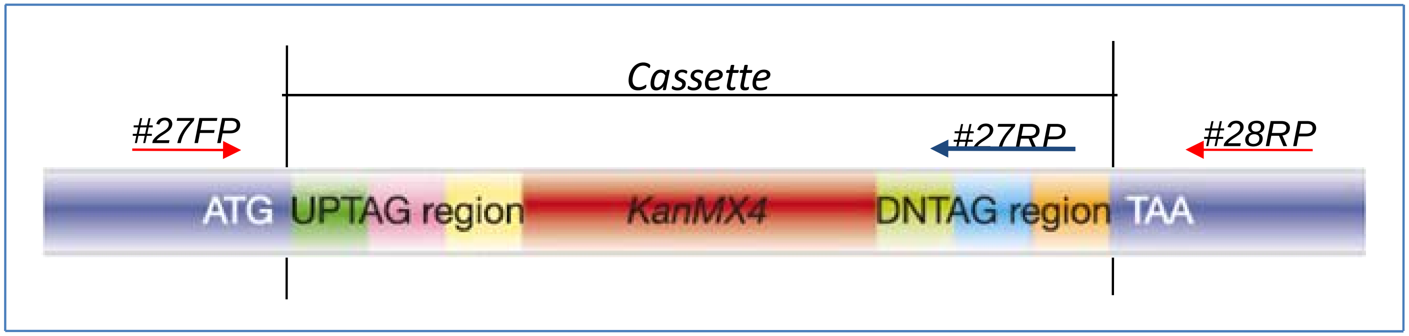


Figure 3.17. Map of the *cassette* used to knock-out *ScTPS1* and positions of primers. *ScTPS1* is replaced with a *cassette* harboring the kan^{R} gene, a 5’ molecular barcode (UPTAG region) and a 3’ molecular barcode (DNTAG region). A forward primer (#27FP) was designed 366-bp upstream ATG and reverse primer (#27RP) in the DNTAG region to amplify strains that contain the *cassette* [kan^{R} YBR126C *tps1*$^{-}$ and YBR126C *tps1*$^{-}$, kan^{R}]. The same forward primer was used (#27FP) with a new reverse primer (#28RP) designed 609-bp downstream of the stop codon to amplify the wild type ScTPS1 gene from the rest of the strains: kan^{R} YDR074W *tps2*$^{-}$, YDR074W *tps2*$^{-}$, BY4742 and DSF 188. Sequences for primers are found in Table 3.1.

cassette, the expected sizes of the PCR product should be ~2kb. The length of the cassette is ~1.6kb and the #27FP begins ~400-bp upstream of the ATG codon. Figure 3.18 show that the observed results matched the expected results.

To amplify wild type *ScTPS1* from kan^{R}YDR074W *tps2*$^{-}$*,* YDR074W *tps2*$^{-}$*,* BY4742 and DSF188 (positive control) the same forward primer (#27FP) was used with a new reverse primer designed (#28RP) as shown in Figure 3.17. This PCR should yield a 2.5kb band – the length of the gene is 1.5kb plus the forward primer #27 anneals 366 bases upstream of the ATG codon and the reverse primer #28 anneals 609 bases downstream of the stop codon.

Figure 3.18 shows that the observed results matched the expected results, thus verifying the knockouts.

4.3.1 Amplifying *ScTPS2* from yeast gDNA

ScTPS2 is about 2.6kb based on the genome sequence available for *Saccharomyces cerevisiae*. Since the size of *ScTPS2* is clearly distinct from the size of the *cassette* (1.6kb) only two new primers were designed flanking the ATG and stop codons. The forward primer #29FP (Table 3.1) was designed 364-bp upstream of the ATG codon and the reverse primer #29RP (Table 3.1) was designed 392-bp downstream of the stop codon to amplify the *cassette* from kan^{R}YDR074W *tps2*$^{-}$ and YDR074W *tps2*$^{-}$ (Figure 3.19).

The same primers were used to amplify the wild type *ScTPS2* from kan^{R}YBR126C *tps1*$^{-}$, YBR126C *tps1*$^{-}$*,* BY4742 and DSF188 (positive control) (Figure 3.19). PCR conditions and reagents were the same as those described above (section 4.2).

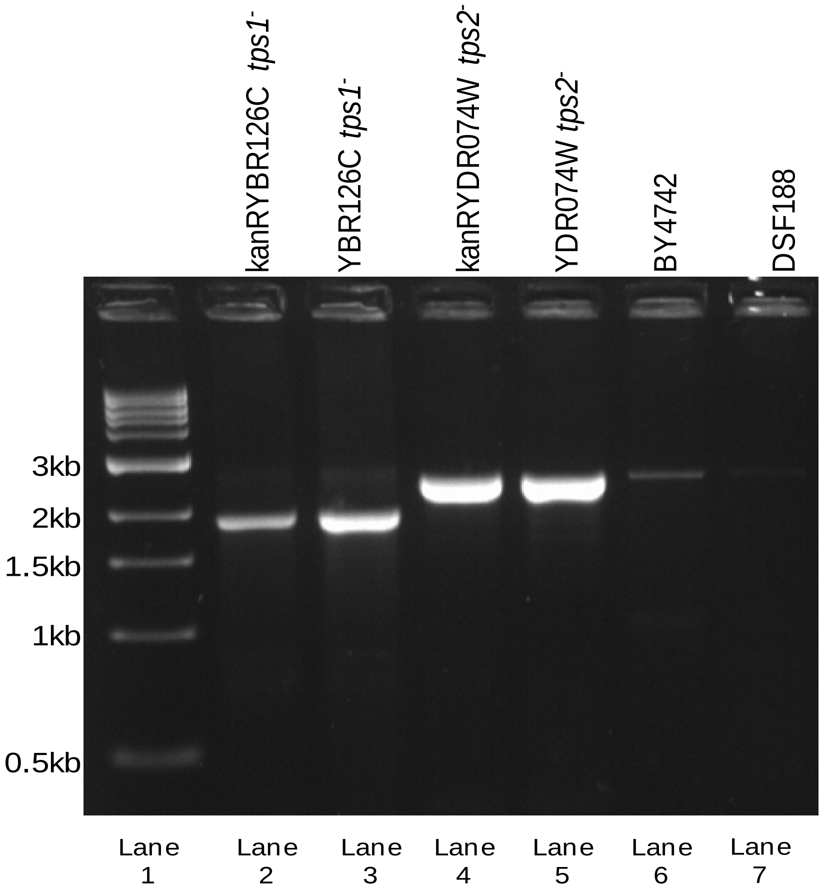


Figure 3.18. Checking the presence the *cassette* in strains kan^R YBR126C *tps1*$^-$ and YBR126C *tps1*$^-$ and the presence of the wild type *ScTPS1* in strains kan^{R}YDR074W *tps2*$^-$, YDR074W *tps2*$^-$ and BY4742. Lane 1: 1kb DNA ladder (Invitrogen). Lane 2: kan^R YBR126C *tps1*$^-$. Lane 3: YBR126C *tps1*$^-$*.* Lane 4: kan^R YDR074W *tps2*$^-$. Lane 5: YDR074W *tps2*$^-$*.* Lane 6: BY4742. Lane 7: DSF188. Lanes 2 and 3 used #27FP and #27RP, lanes 4-7 used #27FP and #28RP. Primers sequences are found in Table 3.1.

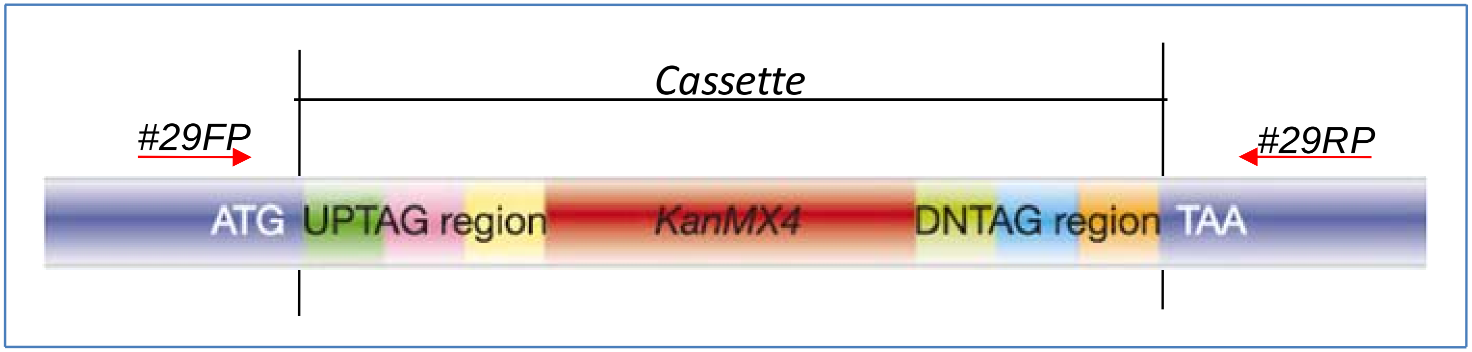


Figure 3.19. Map of the *cassette* used to knock-out *ScTPS2* and positions of primers. *ScTPS2* is replaced with a cassette harboring the kan^R gene, a 5' molecular barcode (UPTAG region) and a 3' molecular barcode (DNTAG region). A new forward primer (#29FP) was designed 364-bp upstream of the ATG codon and a reverse primer (#29RP) 392-bp downstream of the stop codon to amplify the cassette from kan^R YDR074W *tps2*$^-$ and YDR074W *tps2*$^-$ and to amplify the wild type *ScTPS2* in kan^{R}YBR126C *tps1*$^-$, YBR126C *tps1*$^-$, BY4742 and DSF188 ScTPS2.

The knockout allele of the ScTPS2 gene should give a ~2.3kb band – the *cassette* is 1.6kb, the forward primer #27 anneals 365 bases upstream of the ATG and the reverse primer #29 anneals 392 downstream of the stop codon.

The amplification of the wild type *ScTPS2* in kan^{R}YBR126C *tps1*$^{-}$, YBR126C *tps1*$^{-}$, BY4742 and DSF188 (positive control) should yield a ~3.5kb band – the length of *ScTPS2* is 2.6kb, the forward primer #27 anneals 365 bases upstream of the ATG codon and the reverse primer #29 anneals 365 bases downstream of the stop codon.

Figure 3.20 shows that the observed results matched the expected results, thus verifying the *ScTPS2* knockout in YDR074W *tps2*$^{-}$.

Thus, the strains provided had the correct knockouts of the ScTPS1 and ScTPS2 genes. Unfortunately the strains did not show the expected phenotype. One theory as to why we observed the unexpected phenotype associated with these knockouts is due to other genes in the genetic background of these strains.

The experiment was repeated with a new set of strains from a W303-1A background (section 4.4).

4.4 W303-1A yeast strains

A new set of four yeast strains were received from 'VIB laboratory of Molecular Cell Biology (K.U. Leuven)' Flanders, Belgium. Wild type 'W303-1A' (Mat a leu2-3, 112ura3-1, trp1-1, his3-11, 15 ade2-1, can1-100, GAL, SUC2), *tps1Δ* 'YSH290' (W303-1A, ggs1/*tps1Δ*), *tps2Δ* 'YSH450' (W303-1A, *tps2Δ*::LEU2,RP1) and a double knockout *tps1Δtps2Δ* 'YSH652' (W303-1A, ggs1/*tps1Δ::*TRP1, *tps2Δ*::LEU2).

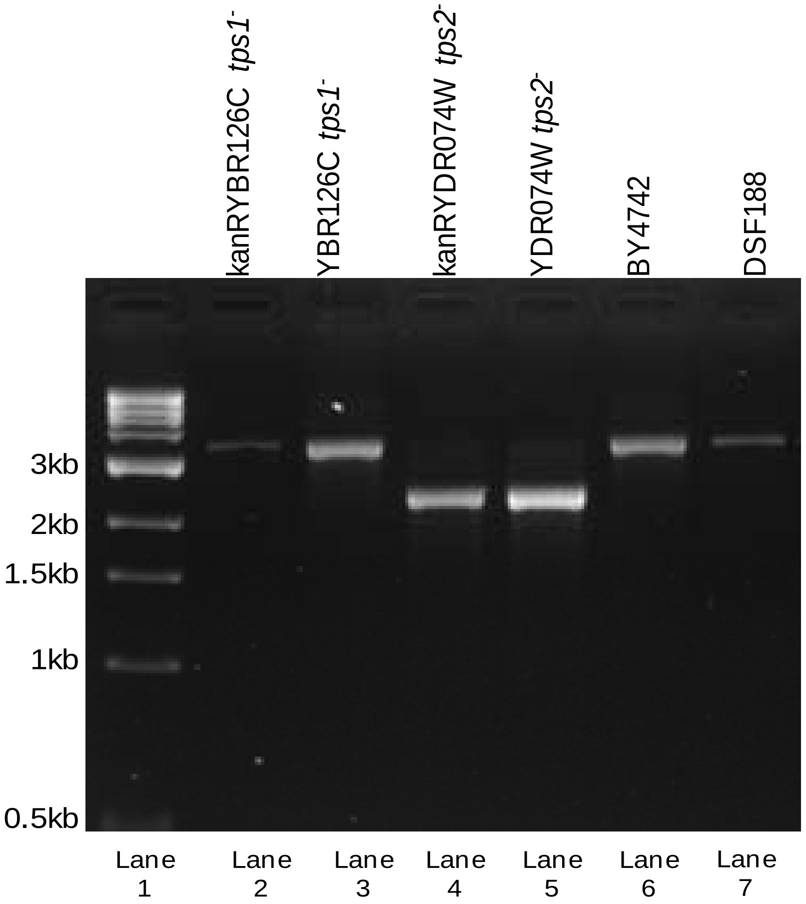


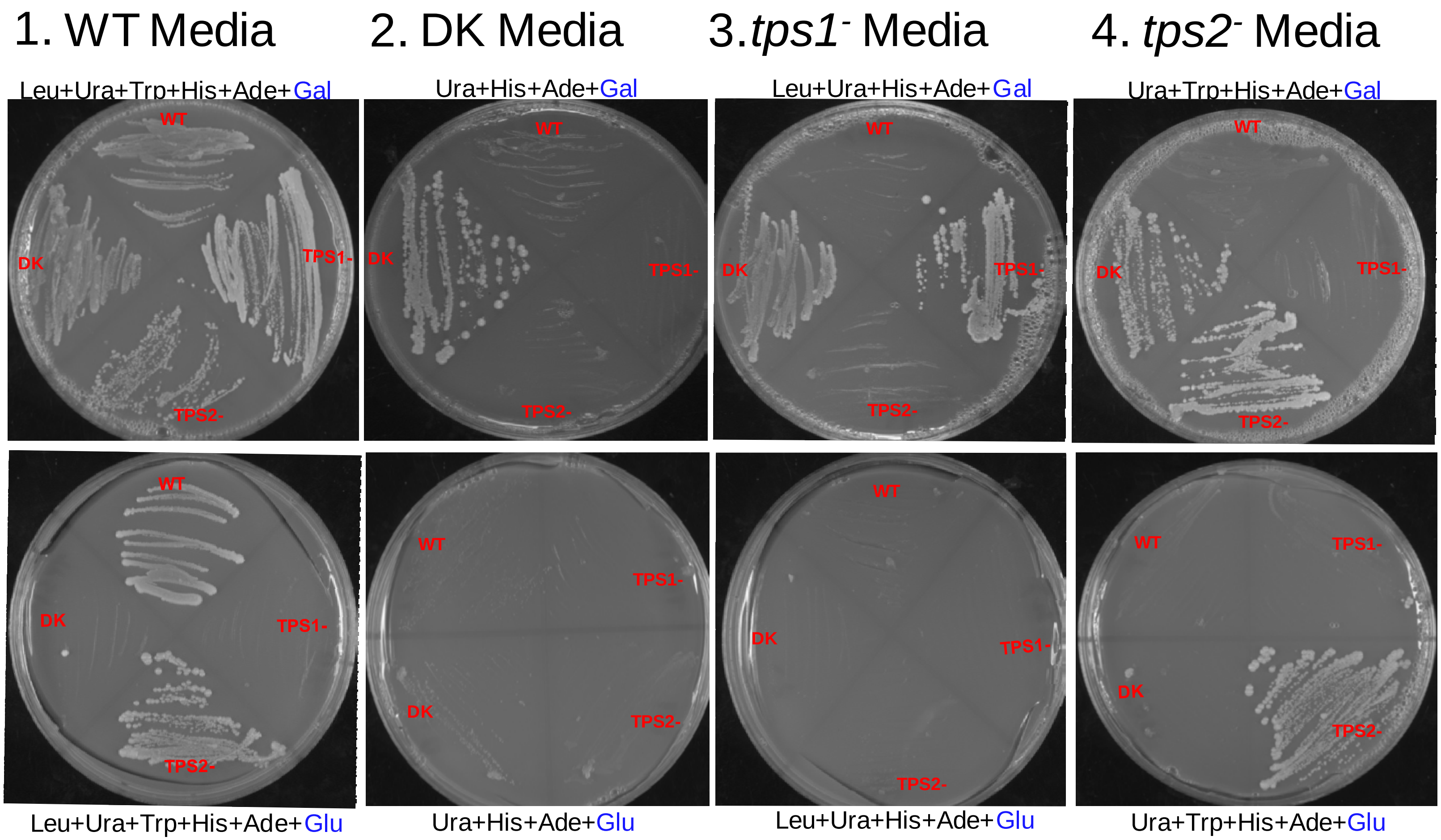
Figure 3.20. Checking the presence of the *cassette* in strains kan^{R}YDR074W *tps2*$^-$ and YDR074W *tps2*$^-$.and the presence of the wild type *ScTPS2* in strains kan^{R}YBR126C *tps1*$^-$, YBR126C *tps1*$^-$, BY4742 and DSF 188. Lane 1: 1kb DNA ladder (Invitrogen). Lane 2: kan^R YBR126C *tps1*$^-$. Lane 3: YBR126C *tps1*$^-$. Lane 4: kan^R YDR074W *tps2*$^-$. Lane 5: YDR074W *tps2*$^-$. Lane 6: BY4742. Lane 7: DSF188. Primer sequences are found in Table 3.1.

Upon their arrival all the strains were screened for their amino acid auxotrophies and for their growth in different carbon source (glucose and galactose). Four different media were prepared and the following amino acids were used: 1) for W303-1A WT Leu+Ura+Trp+His+Ade, 2) for YSH652 double knockout (DK, *tps1*Δ*tps2*Δ) Ura+His+Ade, 3) for YSH290 (*tps1*Δ) Leu+Ura+His+Ade, and 4) for YSH450 (*tps2*Δ) Ura+Trp+His+Ade (procedure and reagents described in 3.3.7).

Each plate was subdivided to four quarters onto which each one of the four different strains was streaked and growth was observed (Figure 3.21). In order for cells to grow the media had to have the correct amino acids and carbon source. WT and *tps2*Δ grew on both galactose and glucose when the correct combinations of amino acids were present; double knockout (*tps1*Δ*tps2*Δ) and *tps1*Δ grew only in galactose with the correct combination of amino acids (Figure 3.21).

Cells from plates with galactose (Figure 3.21) were grown overnight in YPD as described in section 3.3.5 and eight transformations were carried out: pDB20+*ΔPhTPS1* for W303-1A (WT), YSH290 (*tps1*Δ), YSH450 (*tps2*Δ), YSH652 (*tps1*Δ*tps2*Δ) (upper half of Figure 3.22) and empty vector (pDB20) for W303-1A (WT), YSH290 (*tps1*Δ), YSH450 (*tps2*Δ), YSH652 (*tps1*Δ*tps2*Δ) (lower half of Figure 3.22).

Figure 3.21. Screening the yeast W303-1A strains for amino acid auxotrophies and for growth on different carbon source. Each of the four strains has different amino acid requirements. The following combinations of amino acids were used: 1) for W303-1A WT Leu+Ura+Trp+His+Ade. 2) for YSH652 double knockout (DK, *tps1*Δ*tps2*Δ) Ura+His+Ade. 3) for YSH290 (*tps1*Δ) Leu+Ura+His+Ade. 4) for YSH450 (*tps2*Δ) Ura+Trp+His+Ade. Each plate containing galactose (upper) and glucose (lower) was subdivided in four quarters onto which each of the four different strains was streaked. Growth was observed on both galactose and glucose for WT and *tps2*Δ and growth was observed only on galactose for double knockout (*tps1*Δ*tps2*Δ) and *tps1*Δ.


1. WT Media
2. DK Media
3.$\mathit{tps1}^{-}$ Media
4. $\mathit{tps2}^{-}$ Media
Leu+Ura+Trp+His+Ade+Gal
Ura+His+Ade+Gal
Leu+Ura+His+Ade+Gal
Ura+Trp+His+Ade+Gal
WT
DK
TPS1-
TPS2-
Leu+Ura+Trp+His+Ade+Glu
Ura+His+Ade+Glu
Leu+Ura+His+Ade+Glu
Ura+Trp+His+Ade+Glu

Transformations done as described in section 3.3.5 and transformants were selected by plating on Min+ their respective amino acids (described above) minus Ura media to select for those that became prototrophic (+) for the ability to synthesize Ura. As expected, all of transformants, except for YSH652 (*tps1*∆*tps2*∆) empty vector, appear to have taken up the circularized plasmid (Figure 3.22).

Since the transformations were successful a handful of cells from W303-1A (WT), YSH290 (*tps1*∆), YSH450 (*tps2*∆) and YSH652 (*tps1*∆*tps2*∆)[1] with pDB20+*∆PhTPS1* (upper Figure 3.22) and empty pDB20 vector alone (lower Figure 3.22) were streaked onto Min+Leu+His+Ade-Ura+Gal and onto Min+Leu+His+Ade-Ura+Glu to see if the *∆PhTPS1* would restore the function of mutant yeast while growing on glucose.

Figure 3.23 shows that all strains grew on galactose, as expected. On glucose, when looking at the TPS1 allele, only YSH290 (*tps1*∆) and YSH652 double knockout (*tps1*∆*tps2*∆) with the petunia TPS1 gene plus the pDB20 vector were able to grow, showing that *PhTPS*1 is a functional gene capable of restoring TPS1 function in mutant yeast. YSH290 (*tps1∆*) empty vector and YSH652 double knockout (*tps1*∆*tps2*∆) empty vector were not able to grow in glucose.

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[1] A new transformation with YSH652 double knockout (*tps1*∆*tps2*∆) empty vector was successfully carried out (not shown) and was streaked onto the media described above.

Figure 3.22. Transformation of W303-1A (WT), YSH290 (*tps1*Δ), YSH450 (*tps2*Δ), YSH652 (*tps1*Δ*tps2*Δ) with pDB20+*ΔPhTPS*1 (upper) and transformation of W303-1A (WT), YSH290 (*tps1*Δ), YSH450 (*tps2*Δ), YSH652 (*tps1*Δ*tps2*Δ) with empty pDB20 vector (lower). Cells growing in galactose as a carbon source and Min+His+Ade (double knockout, DK), Min+Trp+His+Ade (*tps2Δ*), Min+Leu+His+Ade (*tps1Δ*), Min+Leu+Trp+His+Ade (WT) show that the plasmid (pDB20+*ΔPhTPS*1 and pDB20 alone) was taken up by cells and that they became prototrophic (+) for Ura, except for YSH652 (*tps1*Δ*tps2*Δ) empty vector (not shown).

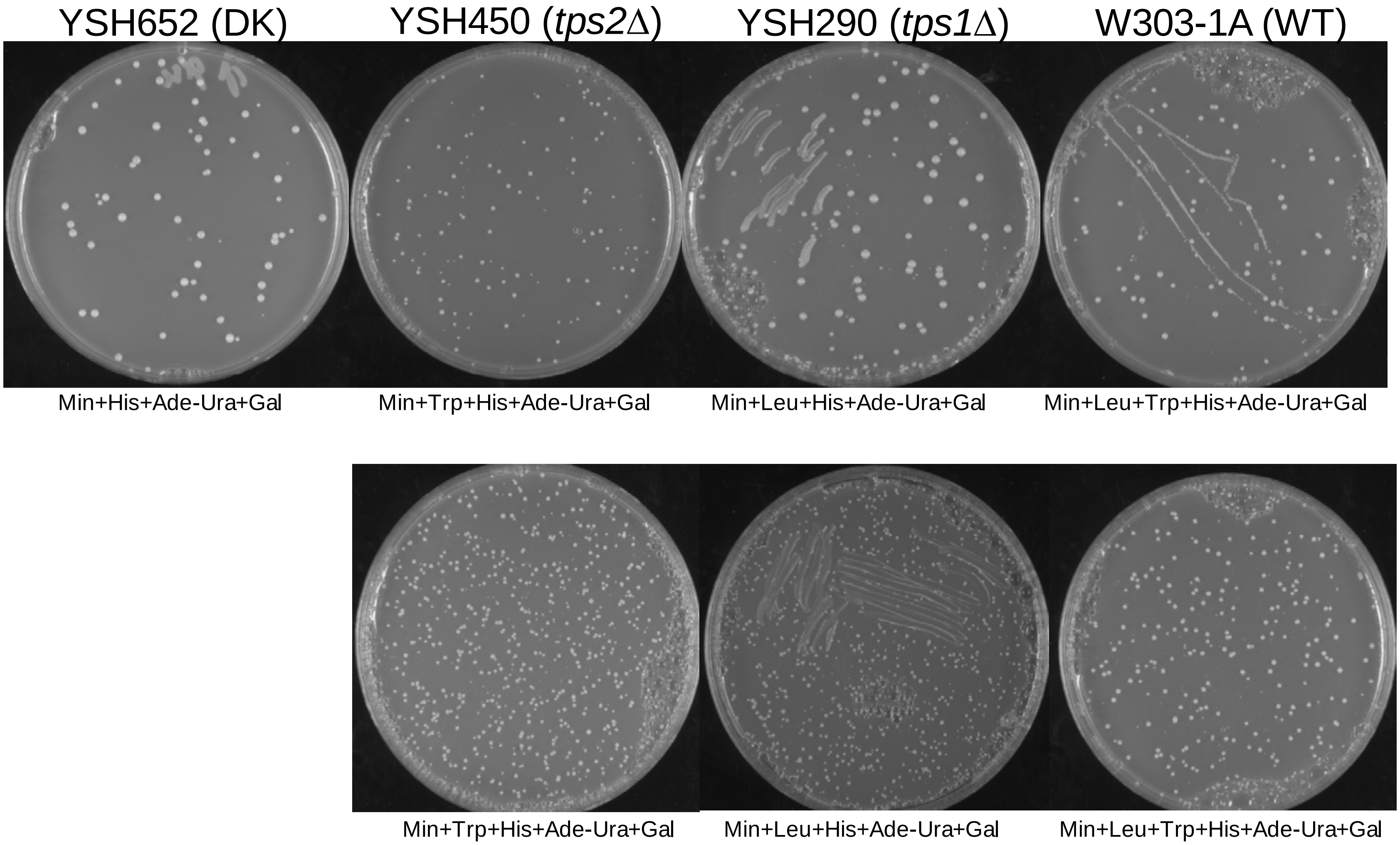
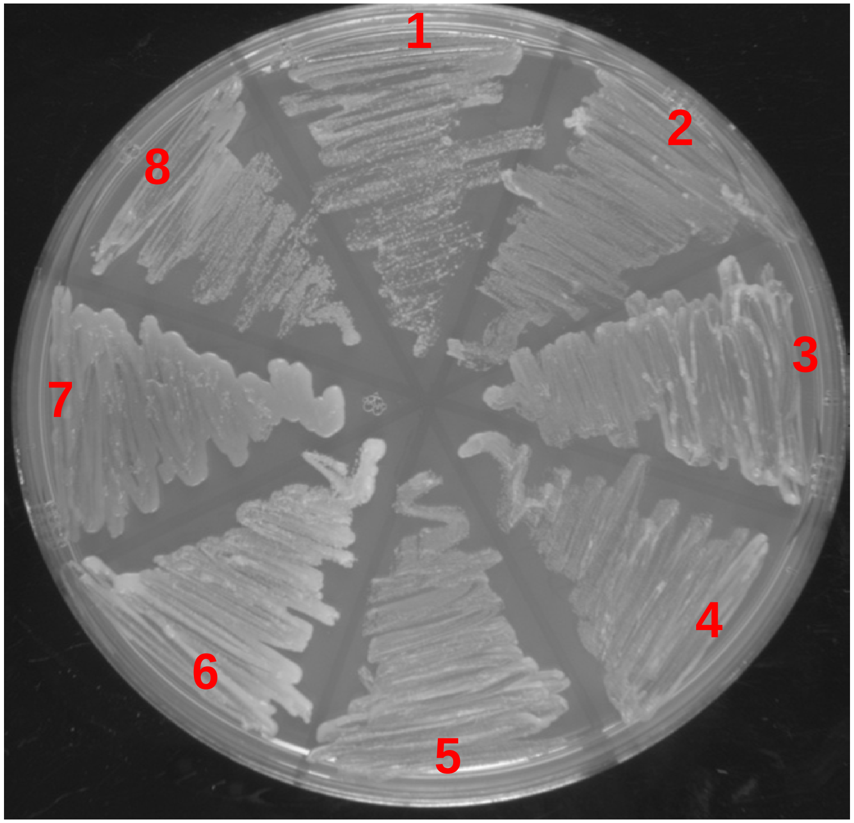
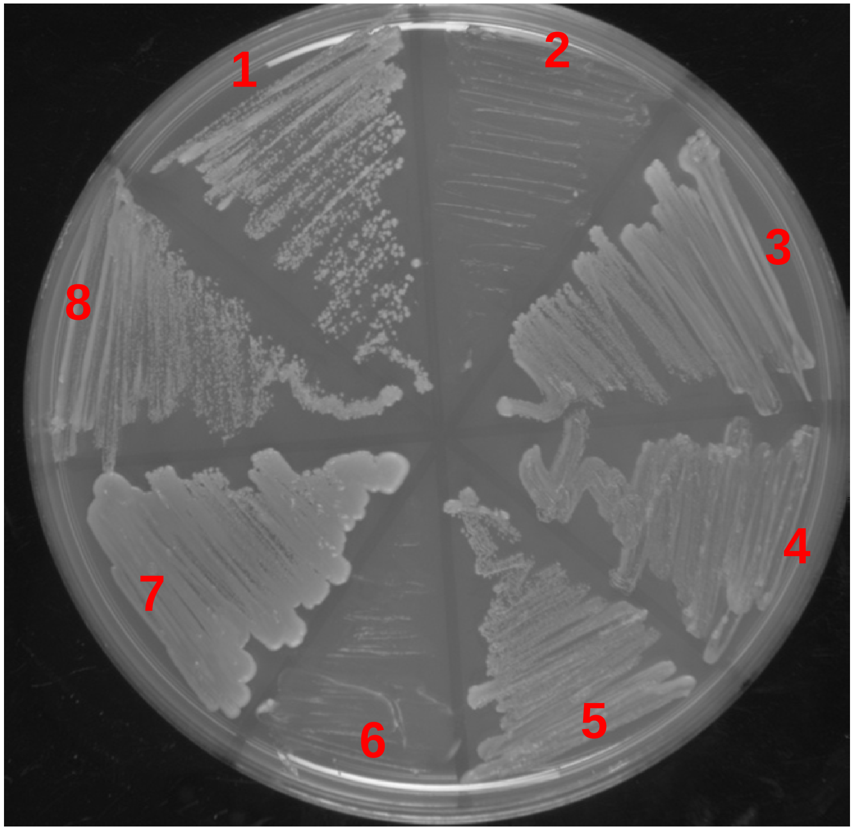

YSH652 (DK)
YSH450 (*tps2*Δ)
YSH290 (*tps1*Δ)
W303-1A (WT)
Min+His+Ade-Ura+Gal
Min+Trp+His+Ade-Ura+Gal
Min+Leu+His+Ade-Ura+Gal
Min+Leu+Trp+His+Ade-Ura+Gal
Min+Trp+His+Ade-Ura+Gal
Min+Leu+His+Ade-Ura+Gal
Min+Leu+Trp+His+Ade-Ura+Gal

Figure 3.23. A handful of colonies of the eight transformations (described in Figure 3.22) were streaked onto Min+Leu+Trp+His+Ade-Ura+Gal and Min+Leu+Trp+His+Ade-Ura+Glu to check if the *ΔPhTPS*1 would restore the ability of mutant yeast to grow on glucose. Note that V+G is vector plus gene and EV is empty vector. 1. YSH290 (*tps1*Δ) V+G. 2. YSH290 (*tps1*Δ) EV. 3. YSH450 (*tps2*Δ) V+G. 4 YSH450 (*tps2*Δ) EV. 5. YSH652 double knockout (*tps1*Δ*tps2*Δ) V+G. 6. YSH652 double knockout (*tps1*Δ*tps2*Δ) EV. 7. Wild type W303-1A V+G. 8. Wild type W303-1A EV. Only YSH290 (*tps1*Δ) V+G and YSH652 double knockout (*tps1*Δ*tps2*Δ) V+G were able to grow in glucose when looking at the TPS1 allele showing that *PhTPS1* it is capable of restoring TPS1 function in a mutant yeast. Empty vector alone failed to rescue function and YSH290 (*tps1*Δ) EV and YSH652 double knockout (*tps1*Δ*tps2*Δ) EV cells were not able to grow in glucose.



Min+Leu+Trp+His+Ade-Ura+Gal



Min+Leu+Trp+His+Ade-Ura+Glu

Conclusions

The TPS1 gene, cloned from *Petunia x hybrida* cv. Mitchell Diploid, generates a 2784-bp open reading frame that predicts a 58kDA protein of 927 amino acids. The sequence is available in the NCBI (Accession HQ259080) and SOL databases.

The cloned *Petunia x hybrida* TPS1 gene was shown to be a functional gene by restoring function in mutant yeast. *tps1*Δ vector plus gene and double knockout (*tps1*Δ*tps2*Δ) vector plus gene were able to grow in glucose when used as a sole carbon source showing that *PhTPS1* it is capable of restoring the influx of glucose into glycolysis. Empty vector alone failed to rescue function and these cells were not able to grow in glucose.

The rate limiting step in the trehalose biosynthesis appears to be the TPS1 gene, since the double knockout vector plus gene was able to grow in glucose suggesting that the T6P intermediate can be dephosphorylated by nonspecific phosphatases.

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