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SOMATIC CELL SELECTION TO GENETICALLY IMPROVE PLANT WATER-USE EFFICIENCY AND TOLERANCE TO STRESSES

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ABSTRACT

Water conservation may depend on development of improved crop plant water-use efficiency and tolerances to drought and heat stresses. This project tested the hypothesis that increased production of uncommon polyamines may confer greater crop water-use efficiency and/or tolerances to drought and heat stresses, using alfalfa (*Medicago sativa* L.) and cotton (*Gossypium hirsutum* L.) as model systems. The genetic strategy involved selection for cellular tolerance to known inhibitors of three biosynthetic enzymes in the polyamine pathway, which should lead to overproduction of the targeted polyamines; and evaluation of selected and nonselected cell lines for cellular tolerance to induced water deficit (alfalfa) or heat (cotton) stresses. Cotton and alfalfa genotypes were chosen that were known to be tolerant or susceptible to heat or drought stress conditions, respectively. Under certain growth conditions, cell cultures expressed stress tolerance traits that correlated with whole plant stress tolerance. Selected cell lines of cotton were recovered that exhibited stable adaptations to the inhibitors or high temperature growth conditions. Unfortunately, all alfalfa cell lines were lost in a devastating laboratory fire. Many of the selected cell lines of cotton with adaptations to the inhibitors exhibited enhancements in the activity of one or more, in several cases all three, of the targeted enzymes. The activities of all three enzymes increased in response to cotton cell adaptation to high temperature alone, suggesting a relationship between adaptation to high temperature and uncommon polyamine biosynthesis. Attempts to regenerate plants from selected cell lines for heritability studies were not successful.

Keywords: plant stress, drought, water conservation, cotton, alfalfa, crops, genetics, plant tissues

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION AND LITERATURE REVIEW

Rationale and Objectives

Our region and nation are facing growing water demands from the urban and industrial sectors, compounded by increasingly limited supplies of high-quality water. Irrigated agriculture accounts for 80-90 percent of the consumptive water use in our state and region, with alfalfa being the greatest consumptive water user (Phillips 1987). Losses in crop production due to water shortage may be greater than those caused by all other environmental conditions (Kramer 1980). High temperature and water deficiency stresses frequently occur together and their effects on crops are confounded (McWilliam 1980). Efforts to conserve water resources therefore are dependent on developing a general strategy to improve crop tolerances to drought and heat stresses in such a manner that will lead to improved crop water-use efficiency.

Previous research in alfalfa led to the suggestion of specific biochemical criteria to use in genetic selection for improved water-use efficiency and/or drought tolerance (Phillips 1987); namely, the polyamines. Drought tolerant/water-use efficient alfalfas accumulate higher levels of polyamines under water deficit stress, while drought susceptible/water-use inefficient alfalfas exhibit a net reduction of polyamines under stress (Kuehn et al. 1990b). The uncommon polyamines, norspermine (caldine) and norspermidine (thermine), specifically are associated with alfalfa water-use efficiency/drought tolerance (Rodriguez-Garay et al. 1989). Related research identified the same pathways, and the additional production of the uncommon caldopentamine, in heat tolerant cotton (Kuehn et al. 1990b). These uncommon polyamines previously had been identified in thermophilic bacteria, where they are implicated in conferring thermotolerance.

Polyamines appear to stabilize cellular membranes and macromolecular (RNA and DNA) function under extreme environmental conditions.

The overall goal of this project was to develop drought and heat tolerant crop plants that conserve water. The approach involves advanced genetic research using alfalfa (*Medicago sativa* L.) and cotton (*Gossypium hirsutum* L.) as model systems. This project tested the specific hypothesis that the increased production of certain polyamines would confer greater crop water-use efficiency and/or plant tolerances to drought and heat stresses. The specific objectives of this project involved:

1) Selection for cellular tolerance to known inhibitors of the three critical biosynthetic enzymes in this pathway. This approach should lead to overproduction of the targeted polyamines.
2) Evaluation of selected and control (unselected) cell lines for cellular tolerance to induced water deficit (alfalfa) or heat (cotton) stresses. Overproduction of the targeted polyamines should translate to water-use efficiency and/or tolerances to water deficit and heat stresses in the plants regenerated from these selected cells, as well.
3) Regeneration of plants from selected cells, and evaluation of plants for tolerance to drought and heat stresses and for water-use efficiency.

Hypothesis confirmation should result in plants suitable for developing new cultivars with genetically improved water-use efficiency and/or drought and heat tolerances. In the short term, such plants could be used in the breeding of alfalfa and cotton, two major crops in our state and region, that would require less water to be productive, leading to significant water savings. Further study of the genetic basis of any improved performance in such plants would facilitate the

implementation of this approach with other crop plants, leading to a general genetic strategy for developing water conserving crops to meet regional and national needs.

Polyamines and Plant Stress Responses

Specific mechanisms permitting higher plants to tolerate environmental stresses are not well understood. It is imperative that we gain an understanding of stress tolerance mechanisms in plants if we are to engineer new crops that conserve water and tolerate periods of drought and high temperature. Drought or heat tolerance is the ability of plants to survive the respective stress (Kramer 1980; McWilliam 1980), but they do not necessarily need to be productive to survive. Crop water-use efficiency is measured as the amount of yield or biomass produced per unit of water consumed (Quisenberry et al. 1985; Salter et al. 1984). The ideal crop plant of the future, in our region, is one that is productive in spite of drought or heat, and that makes efficient use of water when it is available (Currier et al. 1987).

Breeding at the whole-plant level for drought tolerance (Quisenberry et al. 1985; Salter et al. 1984) or heat tolerance (Rodriguez-Garay and Barrow 1988) has advanced at a slow pace. Cell selection approaches promise to be more precise and effective as means to crop genetic improvement. Cell selection for tolerance to osmotic stress has been attempted with the use of polyethylene glycol to induce water deficit conditions, with successful isolation of variant cell lines (Bressan et al. 1981). These variant cells exhibited osmoregulation, but to date no plants have been regenerated from selected cells to assess stress tolerance at the whole-plant level (Handa et al. 1986). This approach therefore remains unvalidated. Moreover, cellular adaptation to osmotic stress in this case may be due to physiological rather than genetic changes (Hasegawa et al. 1984). Cell selection for heat tolerance has been reported only once, in a study involving cotton cells (Trolinder and Goodin 1991). Cotton plants regenerated from selected cells retained

the heat tolerance trait but were male sterile, and heritability studies have not yet been reported. Cell selections for tolerance to low temperatures have been attempted, but the selected cellular trait is not expressed at the whole-plant level and it is not heritable (Tal 1983). In general, plant cell selections have proven effective at the genetic level only when the criteria were based on the manipulation of specific, limiting enzymatic steps in targeted biochemical pathways (Flick 1983).

The accumulation of organic compounds such as proline (Stewart and Lee 1974; Greenway and Munns 1980; Handa et al. 1986), betaine (Grumet et al. 1985), and polyamines (Flores and Galston 1984a) have been observed in plant drought stress response. Proline and betaine are implicated in osmoregulation, whereas polyamines may promote cellular structural integrity under stress conditions. One of the initial observed responses of plants to abiotic stresses is the generation of ammonium ion as proteins are degraded. One mechanism of stress tolerance may be initiated with incorporation of ammonium ion into carbamylphosphate (Figure 1). Carbamylphosphate is the ultimate source of pyrimidines required for nucleotide biosynthesis, as well as of proline which is produced from ornithine generated via the urea cycle (Figure 1). Polyamines also are generated fom the urea cycle, either from arginine or ornithine (Figure 1). Thus, the accumulation of proline and of polyamines are compatible in plant responses to stress.

Heat adaptation in plants can be expressed through leaf pubescence conferring greater reflectance and lowering leaf temperature (Ehleringer 1980), or by enhanced thermostability of membranes and proteins (Sullivan and Kinbacher 1967; Bull and Breese 1973; Raison et al. 1980; Teeri 1980). The specific cellular components leading to membrane thermostability are unclear, however, certain heat shock proteins have been implicated (Huess-LaRosa et al. 1987).

Polyamines accumulate in plants under induction by a wide array of stress conditions (Flores et al. 1989; Smith 1985a; Galston 1983; Bachrach et al. 1983; Tabor and Tabor 1984),

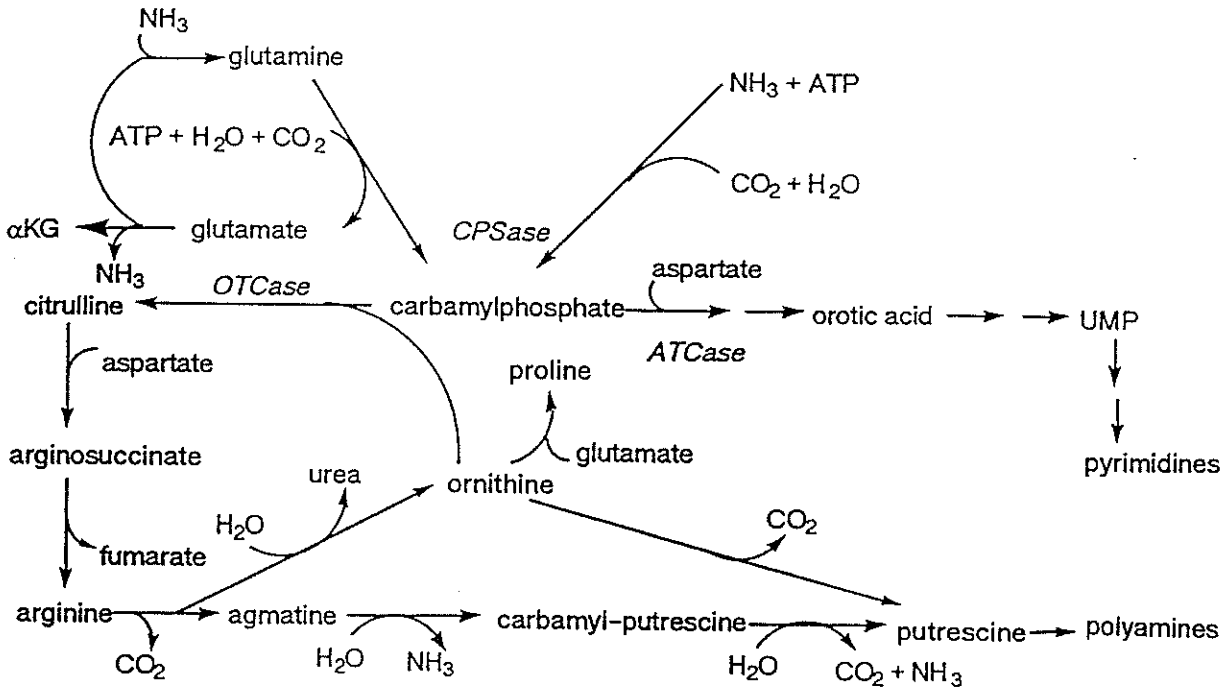

FIGURE 1. Pathways in plants for incorporation of free ammonia or ammonium ion into carbamylphosphate, formation of pyrimidines from carbamylphosphate, and formation of proline and polyamines from carbamylphosphate via the urea cycle

and specifically under osmotic shock or drought conditions (Flores and Galston 1982, 1984a; Erdei et al. 1990; Kubis and Krzywanski 1989). Polyamines also accumulate in plants upon exposure to low temperature chilling stress (Guye et al. 1986; Nadeau et al. 1987; Kramer and Wang 1989). Topical application of polyamines to crop plants protect them from adverse effects of extreme moisture and temperature conditions (Okii et al. 1980). Evidence suggests a protective role for polyamines at the membrane level in plants (Altman et al. 1977; Naik et al. 1980; Popovic et al. 1979; Altman and Bachrach 1981; Apelbaum et al. 1981; Speranza et al. 1984).

The evidence regarding a role for polyamines in adaptation to extreme environments is highly suggestive in microbial systems. Polyamines modulate membrane fusion, exhibit differential preferences for alignment of membrane phospholipids, and show preferential attachment or expulsion of membrane surface proteins (Meers et al. 1986). These observations have important implications for bi-layer structural integrity and function of membranes during osmotic shock in prokaryotic and eukaryotic organisms (Marton and Morris 1987). Polyamines stabilize DNA, RNA and proteins under extreme environments (Kneifel et al. 1986; Basu and Marton 1987), and induce specific DNA conformations (Thomas and Messner 1986).

Uncommon and long-chain polyamines, such as norspermine (thermine), are found in thermophilic and halophilic bacteria (Oshima 1983; Hamana et al. 1985). Long-chain polyamines confer thermoprotection during in vitro protein synthesis (Oshima 1983). Uncommon polyamines may serve specific roles in adaptation to high temperatures in thermophiles (Oshima and Senshu 1985; Paulin et al. 1983). Norspermidine (caldine), norspermine (thermine) and related uncommon polyamines had not been reported previously in higher plants (Hamana and Matsuzaki 1985), until our recent report (Rodriguez-Garay et al. 1989).

Our research has focused on defining the appropriate criteria for performing cell selection aimed at water deficit stress tolerance, using alfalfa as a model system (Phillips 1987). This research was designed to correlate the expression of differential water-use efficiency and/or drought tolerance between the cellular and whole-plant levels, and to correlate the stress physiology of water-use efficiency with differential genetic composition. Initial results supported the concept that simulated water deficit stress alone may not be adequate as cell selection criteria; but preliminary data suggested that certain polyamines may be correlated with stress tolerance.

Correlation of uncommon and long-chain polyamines with the most water-use efficient populations of alfalfa and with heat tolerance in cotton was confirmed in our laboratory, for both whole plants and corresponding cell cultures grown under stress conditions (Kuehn et al. 1990b). Water deficit stressed alfalfa plants contain increasing total polyamine pools that correlate precisely with their preselected levels of increasing tolerance; but the alfalfa lines can not be distinguished with respect to polyamine titers under conditions of optimal moisture. This observation suggests that stress induces the preferential increase of polyamine pools in tolerant lines. Moreover, the tolerant lines but not the susceptible lines are induced to produce the uncommon polyamines caldine (norspermidine) and thermine (norspermine) under stress. The tolerant lines show a definite shift to increasing proportions of thermine and spermine (long-chain polyamines), under stress versus control conditions. We confirmed the chemical composition of caldine and thermine in these alfalfa plants by mass spectrometry (Rodriguez-Garay et al. 1989).

Corresponding alfalfa cell cultures show parallel patterns of polyamine accumulations (Kuehn et al. 1990b). Heat-stressed cell cultures of tolerant and susceptible cottons also show these trends, with increased levels of the uncommon long-chain polyamine caldopentamine under stress (but not control) conditions.

Our discovery of caldine and thermine in the higher plant, alfalfa, and the putative identification of uncommon polyamines in cotton, bear three important implications: (i) The induction of caldine and thermine in association with drought tolerance and/or water-use efficiency, and of caldopentamine in association with heat tolerance, suggests that the uncommon polyamines may have specific protective properties against such environmental stresses in plants. (ii) The occurrence of caldine, thermine and caldopentamine implicates a new biosynthetic pathway derived from the substrate 1,3-diaminopropane (DAP) in drought-tolerant and heat-tolerant higher plants, such as has been proposed in acidothermophilic bacteria (DeRosa et al. 1978). (iii) The sole known origin of DAP is from spermine and spermidine through the action of polyamine oxidase (PAO). This enzyme may be pivotal in the stress tolerance pathway by providing the substrate for the formation of all the uncommon polyamines (Kuehn et al. 1990a).

Manipulation of Polyamines by Cell Selection

Selection of plant cell lines resistant to inhibitors of polyamine biosynthesis with subsequent overproduction of targeted polyamines has been reported, but there have been no reports of cell selection aimed at the uncommon polyamine pathway. Polyamine, protein and alkaloid accumulations changed in carrot and tobacco callus cultures due to alteration of ornithine decarboxylase (ODC) activity, developed by resistance to the inhibitor 2-difluoromethylornithine (DFMO) (Bagni and Mengoli 1986; Tiburcio et al. 1987). Another DFMO resistant cell line of tobacco had low ODC activity but a 20-fold increase in arginine decarboxylase (ADC) activity, resulting in overproduction of putrescine and increased tolerance to acidic stress conditions (Hiatt and Malmberg 1988). Floral morphology in regenerated tobacco plants changed due to altered spermidine: spermine ratios, after S-adenosylmethionine decarboxylase (SAM DC) activity was affected by cellular resistance to the inhibitor methylglyoxal bis-(guanylhydrazone) (MGBG)

(Malmberg and McIndoo 1983; Malmberg and Rose 1987; Hiatt and Malmberg 1988). These enzymes do not appear to be limiting in the pathway implicated in drought and heat tolerances.

The critical biosynthetic pathway expressed during abiotic stress tolerances in plants (see Figures 1 and 2) is initiated by the enzyme arginine decarboxylase (ADC) to yield the diamine putrescine (Flores and Galston 1984b; Hiatt et al. 1986; Hiatt and Malmberg 1988), but this step does not appear to be limiting (Kuehn et al. 1990b). It is presumed that putrescine aminopropyltransferase (PAPT), which yields spermidine from putrescine, and spermidine aminopropyltransferase (SAPT), which yields spermine from spermidine, are the next enzymes in the abiotic stress tolerance pathway (Tiburcio et al. 1986a, 1986b). PAPT and SAPT are inhibited by cyclohexylamine (CHA) (Greenberg and Cohen 1985; Balint and Cohen 1985) and by S-methyl-5'-methylthioadenosine (methyl-MTA) (Pegg and Coward 1985, 1987; Porter and Sufrin 1986), respectively. However, it is not clear whether this is a single enzyme or two unique enzymes in higher plants (Flores et al. 1989). Our data suggest that PAPT is present (Figure 2) but that SAPT does not exist in drought-tolerant alfalfa (Bagga et al. 1991b). Elongation of spermidine and spermine from putrescine may be rate limiting steps (Flores et al. 1989; Malmberg and McIndoo 1983).

Polyamine oxidase (PAO) is the next enzyme in this pathway (Figure 2), yielding 1,3-diaminopropane (DAP) from spermine and spermidine (Bagga et al. 1991a). This enzyme appears to be essential in the abiotic stress pathway (Kuehn et al. 1990b; Bagga et al. 1991a). PAO in cereal plants is inhibited by 2-hydroxyethylhydrazine (HEH) (Slocum and Galston 1987; Flores and Filner 1985) and guazatine (Smith 1985b). Our assays in alfalfa for PAO activity demonstrate its presence and its sensitivity to HEH and guazatine (Bagga et al. 1991a). This was the first report of PAO in a dicotyledonous plant.

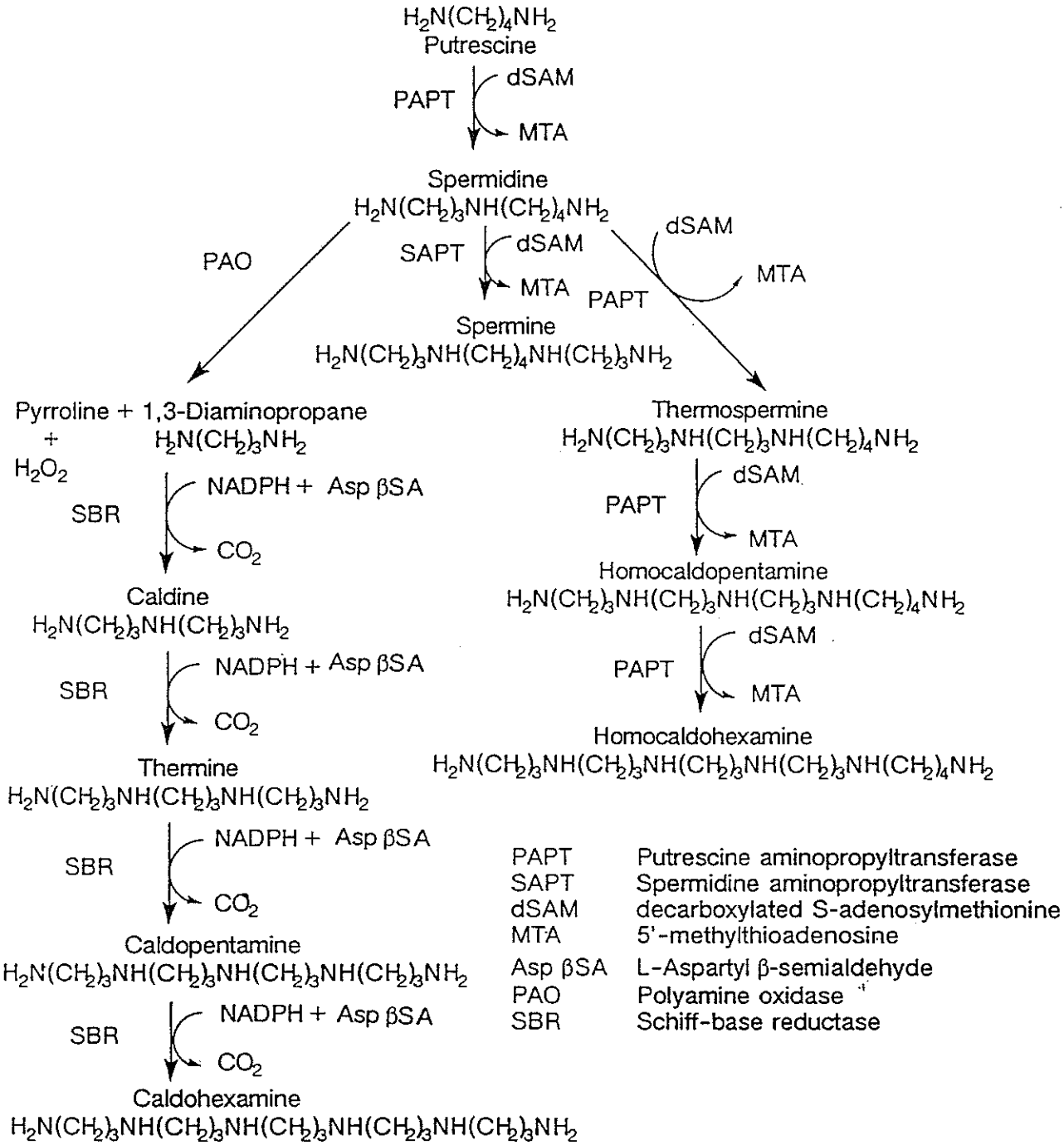

FIGURE 2. Postulated pathway for the biosynthesis of common and uncommon polyamines from the diamine putrescine in alfalfa. Putrescine aminopropyltransferase (PAPT) is inhibited by cyclohexylamine (CHA); polyamine oxidase (PAO) is inhibited by 2-hydroxyethylhydrazine (HEH); schiff-base reductase (SBR) is potentially inhibited by ethylene diamine (ED), caldine or thermine.

The synthesis of caldine and thermine in an extreme acidothermophilic bacterium is catalyzed by a nonspecific aminopropyltransferase (APT) (Cacciapuoti et al. 1986). However, our assays detected only a very low level of APT activity in alfalfa using radiolabeled DAP as substrate (Bagga et al. 1991b). The propylamine donor used for all APT reactions is decarboxylated S-adenosylmethionine (dSAM). The only other known propylamine donor, besides dSAM, is L-aspartyl β-semialdehyde (Phillips and Kuehn 1991), which is used by one plant, *Lathyrus sativus* (Srivenugopal and Adiga 1980), and a few bacteria (Tait 1985) in an alternative route to spermidine synthesis. In this alternative pathway, L-aspartyl β-semialdehyde condenses with putrescine to form a schiff-base complex, which is then reduced by a NADPH-dependent reductase and decarboxylated by a pyridoxal-dependent decarboxylase. We tested for the existence of this schiff-base reductase/decarboxylase (SBR) reaction system with radiolabeled DAP substrate and found very high specific activities for production of caldine and thermine, and sometimes a product corresponding to putative caldopentamine (for which we do not yet have an authentic standard for comparison) (Bagga et al. 1991b). We propose that SBR is the next critical enzyme in the uncommon polyamine biosynthetic pathway (Figure 2). We further propose that SBR may be feedback inhibited by caldine and/or thermine, based on evidence that spermidine can serve as a feedback inhibitor of PAPT (Hartmann et al. 1988; Phillips et al. 1992).

METHODOLOGY

Objective 1

The first objective was to initiate cell suspension cultures from the genetic source materials of alfalfa and cotton, and to perform the baseline experiments necessary to characterize the genetic source materials as a zero-time set of controls.

Four genetic source materials of alfalfa were used. Alfalfa cultivar 'Mesilla', referred to in this study as MES0, was the control because of its inefficient water use and sensitivity to drought stress (Currier et al. 1987). The population resulting from three cycles of phenotypic recurrent selection for water-use efficiency and productivity under limiting water conditions, MES3 (Currier et al. 1987), was used for genetic and physiological comparison. Two efficient regenerator genotypes maintained in the greenhouse as clones, designated 9-D-16 and 77-77-79 (Phillips 1983), also were used. The regenerator lines should provide additional assurance that plants may be recovered from selected cell lines. Three genetic source materials of cotton were used. The cotton control was the cultivar 'Paymaster 404', susceptible to high temperatures (Rodriguez-Garay and Barrow 1988). The heat tolerant BC3 generation resulting from the cross of the recurrent susceptible line with the heat tolerance gene donor line '7456' (Rodriguez-Garay and Barrow 1988) was used for comparison. The efficiently regenerating cultivar 'Coker 312' (Trolinder and Goodin 1987) also was used.

Procedures for cell culture of these alfalfa (Phillips 1983, 1987) and cotton materials (Rodriguez-Garay 1985; Trolinder and Goodin 1987) are well defined. The standard culture medium used for alfalfa is designated SH6, consisting of SH basal formula supplemented with 30 g/l sucrose, 0.06 mg/l picloram and 0.1 mg/l 6-benzylaminopurine (BAP). The culture medium

used for cotton is designated LB1G, consisting of L2 basal formula supplemented with 30 g/l glucose, 0.2 mg/l picloram and 0.1 mg/l BAP. In some experiments involving inhibitor compounds, the culture media were stabilized at pH 5.7 using 0.1 M 2-(N-morpholino)-ethanesulfonic acid (MES) buffer. Each of the seven cell lines were replicated in five petri dishes (callus cultures on agar solidified medium) or in three culture flasks (liquid medium for cell suspension cultures), and incubated at 25° C under diffuse continuous light as control conditions. Water deficit stress was simulated in alfalfa cell cultures by the addition of MW 8000 polyethylene glycol (PEG) at variable rates to an osmotic potential of -2.5 MPa (20% PEG), versus -0.5 MPa (0% PEG) for the control (Phillips 1987; Kuehn et al. 1990b). Heat stress was induced in cotton cell cultures by incubation at various temperatures up to 40° C (Rodriguez-Garay 1985; Kuehn et al. 1990b).

Growth rates in response to control and stress conditions were determined for characterization of the lines before selections, using callus fresh weight or cell suspension packed cell volume measurements (Phillips 1987). For baseline response experiments, polyamine biosynthetic enzyme activity analyses were performed using 10-ml aliquot samples from cell suspension cultures or 2 g callus samples (Bagga et al. 1991a, 1991b; Saavedra 1994). PAPT was extracted and assayed using the method described by Bagga et al. (1991b). Radiolabeled putrescine was used as substrate for the PAPT reaction. The reaction products were separated by paper electrophoresis and located using standards for putrescine, spermidine and spermine. Individual reaction products from PAPT assays were then quantitated by scintillation counting. Extraction and assay conditions for determination of PAO specific activities were by colorimetric determination of 1-pyrroline, one of the oxidation products of spermidine used as substrate, as described by Bagga et al. (1991a). Extraction of SBR was the same as for PAPT. SBR assay

conditions were as described by Bagga et al. (1991b), using radiolabeled DAP as substrate. The reaction products were separated by paper electrophoresis and located using standards for DAP, caldine and thermine. Individual reaction products from SBR assays were then quantitated by scintillation counting.

Objective 2

The second objective was to initiate and sustain cell selections to isolate mutant cell lines resistant to specific inhibitors of the three critical biosynthetic enzymes in the polyamine pathway of interest. The first targeted enzyme is PAPT (Figure 2), which is inhibited effectively in a competitive manner by CHA (Bagga et al. 1991b). The second targeted enzyme is PAO (Figure 2), which is inhibited effectively in a competitive manner by HEH (Bagga et al. 1991a; Kuehn et al. 1990b). The third targeted enzyme is SBR (Figure 2), for which specific inhibitors are not known. We tested caldine and thermine as potential feedback inhibitors, and in some experiments ethylene diamine (ED) was used as a potential competitive inhibitor of SBR. CHA and HEH were added to the culture media prior to autoclaving. Caldine, thermine and ED were added from sterile stock solutions to autoclaved media after cooling.

Each inhibitor was evaluated to determine the appropriate level for initiation of selection (likely between 0.1-10 mM), by determining their effects on growth in each test system. The effects of the inhibitors on polyamine biosynthetic enzyme activities also were determined. Polyamine biosynthetic inhibitor treatments were applied at sublethal levels, and increased in a stepwise fashion during cellular adaptation to normally lethal levels (Flick 1983). Selection was then based on enhanced growth (relative to nonadapted cells) in the presence of the inhibitor. Because enzyme activity may be a function of stress induction, each inhibitor treatment was applied in the presence or absence of the appropriate stress (PEG-induced osmotic stress in the

case of alfalfa, high temperature in the case of cotton). Selection pressures were maintained for at least 12 months for variant isolation. Small, homogeneous regions of callus of putative variants were recovered and proliferated following the methods of Flick (1983). Nonselected control cell lines also were maintained.

Unfortunately, a devastating fire destroyed the plant cell culture facility during this period of the project, December, 1992. All alfalfa cultures and some cotton cultures were lost. In addition, laboratory notebooks and other documentations were lost in the fire. As a result, the data regarding the alfalfa experiments were greatly limited, as reflected in this report. Much of the cotton data were salvaged, but subsequent experiments were altered due to the loss of materials. More than six months were required to renovate the laboratory. It was not possible to reinitiate the alfalfa experiments considering the proposed termination date for the project during 1993.

The responses of recovered selected and control (nonselected) cotton cell lines were compared. Selected cell lines were removed from selection pressure for six months. Each selected and nonselected cell line was then returned to the respective previous selection pressure (to test stability of adaptation) and challenged with high temperature. Growth response and polyamine biosynthetic enzyme activities under stress and control growth conditions were generated, indicating which cell lines exhibited enhanced stress tolerance.

Objective 3

The third objective was to regenerate plants from as many selected and control cell lines as possible. Cotton plant regeneration procedures followed the methods of Trolinder and Goodin (1987). Then, the regenerated plants were to be evaluated for altered performance under stress and optimal growth conditions. Unfortunately, no regenerated plants were recovered for testing.

EXPERIMENTAL RESULTS

Growth of Nonselected Cell Lines under Control and Stress Conditions

The initial experiments were designed to characterize the growth of the callus and cell cultures of the different genotypes under study before cell selection was initiated. Cell suspension cultures of cotton and alfalfa both grew well. However, callus could not be recovered effectively from the cotton cell suspension cultures, which could lead to complications during later stages of the project. Accordingly, these experiments utilized callus cultures of cotton, and cell suspension cultures of alfalfa.

Callus of the three cotton genotypes were cultured under increasing temperatures over time (Table 1). Growth of all three genotypes at 30° C (Table 1) was equivalent to growth rates observed previously at 25° C (data not shown). At lower temperatures (30-33° C) the three genotypes showed equivalent growth rates. Coker 312 showed sensitivity to higher temperatures (36° C) earlier than the other genotypes. At 38° C and higher temperatures, the three genotypes showed statistically different growth responses; BC3 was most tolerant, Paymaster 404 was more susceptible, and Coker 312 was most susceptible to heat stress. These results correlated well with the known whole plant responses of the former two genotypes (Rodriguez-Garay 1985). BC3 showed a significant decline in growth at 38° C in this study, and a further decline at 40° C. The 40° C growth temperature appeared to be too great of a stress, because all three genotypes showed irreversible growth suppression when returned to 38° C. We concluded that 38° C was sufficient to clearly distinguish the pre-existing degree of tolerance among the genotypes.

Cell suspension cultures of alfalfa were grown on several levels of PEG to simulate water-deficit stress (Table 2). The PEG levels were chosen based on earlier experimental results

TABLE 1. Mean final fresh weight (grams) of nonselected cotton callus cultures after 30 days incubation with monthly increases in growth temperature

Genotype	Growth Temperature and Age of Culture						LSD 0.05
	30° C/ 30 days	33° C/ 60 days	36° C/ 90 days	38° C/ 120 days	40° C/ 150 days	38° C/ 180 days	
BC3	5.2 A[1]	4.7 A	4.3 A	3.3 A	1.6 A	1.3 A	0.9
Paymaster 404	4.7 A	4.6 A	4.2 A	1.7 B	1.2 B	0.9 B	0.7
Coker312	4.7 A	4.3 A	3.0 B	0.9 C	0.5 C	0.4 C	0.6

[1]Values within columns followed by the same letter are not significantly different at P<0.05 using Fisher's Protected LSD Test. Each of three replications were initiated with approximately 0.6 g callus and grown for 30 days on LB1G+MES medium at the temperature indicated, weighed for final fresh weight, then transferred to fresh media for incubation at the next temperature treatment.

TABLE 2. Mean final packed cell volume (ml/5 ml aliquot) of nonselected alfalfa cell suspension cultures after 14 days incubation with different concentrations of polyethylene glycol (PEG) used to simulate water-deficit stress

Genotype	Culture Medium, SH6 With:				LSD 0.05
	0% PEG	4% PEG	8% PEG	20% PEG	
MES3	3.4 A[1]	3.1 A	3.7 A	2.6 A	0.5
MES0	2.1 C	2.9 A	1.0 C	0.5 C	0.4
9-D-16	3.0 AB	3.2 A	3.4 A	2.4 A	0.5
77-77-79	2.7 B	3.1 A	1.9 B	1.1 B	0.3

[1]Values within columns followed by the same letter are not significantly different at P<0.05 using Fisher's Protected LSD Test. Each of three replications were initiated with approximately 0.5 ml packed cell volume per 5 ml culture.

(Phillips 1987). In this study, the four genotypes showed different growth rates under control conditions, but all four were equivalent using 4% PEG. This result suggests that mild osmotic stress may stimulate growth of some genotypes. Stress susceptible genotypes, MES0 and 77-77-79, showed a significant decline in growth rate using 8% PEG, and a further decline using 20% PEG. Stress tolerant genotypes, MES3 and 9-D-16, showed a decline in growth rate using 20% PEG. The growth responses for MES0 and MES3 observed here are similar, but not identical, to previous results (Phillips 1987). The two regenerator lines had not been characterized previously for stress response. Line 9-D-16 appeared to be comparable to MES3 in cellular stress tolerance, while line 77-77-79 showed an intermediate level of tolerance to stress conditions. Having this range of stress tolerance in the initial genetic lines is desirable for the purposes of the subsequent cell selection. The growth of MES0 was too low using 20% PEG, but 8% PEG was not sufficient to provide a sublethal stress for MES3. Both dosages were considered potentially useful for cell selections.

Polyamine Biosynthetic Enzyme Activities in Nonselected Cell Lines

These experiments were designed to establish the activity levels of the key polyamine biosynthetic enzymes of interest before cell selection was initiated. Due to the fire in the laboratory, the samples and the data for the alfalfa experiment were lost. At the time the cotton experiment was conducted, an assay for SBR activity was not yet available. Data for PAO and PAPT activities in cotton callus are presented below. In related experiments, activity levels in callus cultures generally were lower than in corresponding whole plants (Bagga et al. 1991b).

PAO activities tended to be higher in cotton callus grown at 38° C than in callus grown at 25° C (Table 3). The levels of PAO activity detected in this experiment were comparable to those observed with whole plants of alfalfa (Bagga et al. 1991a). Cotton is only the second

TABLE 3. Polyamine oxidase specific activities (nmol/min/mg protein) in nonselected cotton callus cultures grown at control and high temperatures

Genotype	Growth Temperature[1]	
	25° C	38° C
BC3	1.9	ND
Paymaster404	5.1	7.8
Coker312	1.3	7.0

[1]Callus was grown on LB1G+MES medium for 14 days. ND = not detected because no protein was recovered.

dicotyledonous plant shown to have PAO activity. PAPT activities were detected sporadically in cotton callus cultures (Table 4). The levels of PAPT activity observed here were one to two orders of magnitude lower than those observed previously in plants of alfalfa (Bagga et al. 1991b).

Growth of Nonselected Cell Lines Treated with Polyamine Biosynthetic Inhibitors

Preliminary experiments were conducted on a qualitative basis to characterize growth of cell lines in response to different dosages of the various inhibitors of interest and to determine dosages suitable for cell selection. Alfalfa and cotton had different sensitivities to the various inhibitors. Based on the results of those experiments, a single dosage of each inhibitor was tested with each system to characterize inhibitor effects on growth in a quantitiative manner.

Growth in the presence of each inhibitor was generally lower in cotton callus grown at 38° C than in callus grown at 25° C (Table 5). The only exception was Paymaster 404 grown on 0.1 mM caldine, which grew better at the higher temperature. One possible interpretation of this result is that the the production or presence of caldine can ameliorate the effects of stress in the susceptible genotype. This interpretation may be supported by the results with Coker 312, which grew best on media supplemented with caldine compared to media containing the other inhibitor treatments or the control, at each temperature. One potential disadvantage of using caldine or thermine as feedback inhibitors may be that they replace any beneficial effects of endogenous production of the uncommon polyamines of interest. Caldine may not enhance growth of BC3 because it, perhaps, already produces a sufficient amount of caldine endogenously, and the added exogenous caldine may be excessive for optimal growth. HEH had stronger suppressive effects on growth at the higher temperature in both BC3 and especially Paymaster 404. This result may suggest that inhibition of PAO by HEH increases sensitivity to high temperature. In Coker 312,

TABLE 4. Putrescine aminopropyltransferase specific activities (pmol/min/mg protein) in nonselected cotton callus cultures grown at control and high temperatures

Genotype	Assay Products	Growth Temperature[1]	
		25° C	38° C
BC3	Spermidine Spermine	ND ND	0 2.9
Paymaster404	Spermidine Spermine	4.7 1.8	0 0
Coker312	Spermidine Spermine	0 0.2	0 4.9

[1]Callus was grown on LB1G+MES medium for 14 days. ND = not detected because no protein was recovered.

TABLE 5. Effects of polyamine biosynthetic inhibitors on fresh weight growth (relative to control medium at 25° C) of nonselected cotton callus cultures grown at 25° C and 38° C

Culture Medium, LB1G+MES Plus:	Genotype					
	BC3		Paymaster404		Coker312	
	25° C	38° C	25° C	38° C	25° C	38° C
Control	100% A[1]	63% E	100% A	36% FG	100% C	36% E
5 mM HEH	73% D	56% F	40% EF	26% G	63% D	36% E
5 mM CHA	85% B	63% E	83% B	52% D	104% B	13% F
0.1 mM Caldine	70% D	58% EF	35%FG	72% C	133% A	43% E
0.1 mM Thermine	79% C	58% EF	69% C	50% DE	110% B	15% F

[1]Values within genotypes followed by the same letter are not significantly different at $P<0.05$ using Fisher's Protected LSD Test (three replications). Control growth at 25° C was recorded at beginning of experiment. Control growth at 38° C and growth on inhibitors at both temperatures was recorded after 120 days treatment.

HEH was the only inhibitor that had a strong suppressive effect on growth at the lower temperature. At the higher temperature, CHA and thermine had stronger growth suppressive effects than did HEH in Coker 312. Coker 312 may have multiple limitations in its polyamine biosynthetic pathway compared to the other genotypes.

Growth on the selected dosage of each inhibitor was generally lower in alfalfa cell suspensions grown on 8% PEG than in cell suspensions grown without PEG (Table 6). In the absence of PEG, CHA and caldine treatments may have enhanced the growth of the tolerant line MES3. In the presence of PEG, both genotypes showed greatest growth suppression on CHA and HEH treatments. Growth suppression by the inhibitor dosages used was not made as stringent in alfalfa as it was in cotton, because cell suspension cultures are more sensitive than callus to irreversible growth suppression. In both alfalfa and cotton, there appears to be a synergistically inhibitory effect on growth caused by the stress treatment in combination with most inhibitor treatments.

Polyamine Biosynthetic Enzyme Activities in Nonselected Cell Lines Treated with Inhibitors

These experiments were designed to establish the effects of the inhibitors on activity levels of three key polyamine biosynthetic enzymes of interest as cell selection was being initiated. Due to the fire in the laboratory, most of the samples and the data for the alfalfa experiment were lost. However, the data for one major experiment involving the alfalfa materials survived, as did all the data for the cotton materials.

PAO activities in cotton callus did not appear to be strongly affected by the different inhibitor treatments (Table 7). The levels of PAO activities in this experiment generally were comparable to those observed in the zero-time controls (Table 3), although Paymaster 404 control

TABLE 6. Effects of polyamine biosynthetic inhibitors on cell volume growth (relative to control medium) of nonselected alfalfa cell suspension cultures grown with and without polyethylene glycol (PEG) for 28 days

Culture Medium, SH6+MES Plus:	Genotype	
	MES3	MES0
Control	100% B[1]	100% A
2.5 mM HEH	96% B	78% BC
2.5 mM CHA	110% A	82% BC
0.25 mM Caldine	106% AB	80% BC
6% PEG	98% B	92% AB
6% PEG + 2.5 mM HEH	89% C	53% E
6% PEG + 2.5 mM CHA	90% C	61% DE
6% PEG + 0.25 mM Caldine	104% AB	70% CD

[1]Values within columns followed by the same letter are not significantly different at $P<0.05$ using Fisher's Protected LSD Test (three replications).

TABLE 7. Effects of polyamine biosynthetic inhibitors on polyamine oxidase specific activities (nmol/min/mg protein) in nonselected cotton callus cultures grown for 14 days at 25° C

Culture Medium, LB1G+MES Plus:	Genotype		
	BC3	Paymaster404	Coker312
Control	2.4	2.0	1.3
5 mM HEH	1.7	2.9	0
5 mM CHA	0	1.6	1.9
0.1 mM Caldine	0.02	9.1	1.5
0.1 mM Thermine	0.03	0	0.5

callus PAO activity was lower in the present experiment (Table 7). It may be of interest that caldine suppressed PAO activity in the tolerant line BC3, perhaps because it did serve as a feedback inhibitor of PAO. However, caldine enhanced PAO activity in Paymaster 404. HEH did not have the anticipated effect of suppressing PAO activity in cotton callus in this study. Nevertheless, the extracted enzyme is inhibited when HEH is included in the reaction assay (Bagga et al. 1991a).

PAPT activities were very low in cotton callus during this experiment (Table 8), about one order of magnitude lower than in the zero-time controls (Table 4). There was no apparent effect of the inhibitor treatments on PAPT activity (Table 8). CHA did not have the anticipated effect of suppressing PAPT activity. Two higher molecular weight polyamine products were observed in this experiment which were not detected during the zero-time controls. These compounds migrated during paper electrophoresis to the positions corresponding to putative homocaldopentamine and homocaldohexamine. However, authentic standards for these compounds were not available commercially, so we were not able to confirm their identities.

SBR activities were detected in cotton callus (Table 9). The levels of SBR activity in cotton callus were about three orders of magnitude lower than that observed previously in whole plants of alfalfa (Bagga et al. 1991b). Polyamine biosynthetic enzyme activities generally are lower in cultured cells compared to whole plants of alfalfa (Kuehn et al. 1990b; Bagga et al. 1991a). Control assays withholding enzyme extract or withholding L-aspartyl β-semialdehyde as propylamine donor confirmed the enzyme activity was present. Cotton is only the second organism, after alfalfa, shown to have SBR activity related to caldine and thermine synthesis. Caldine treatment appeared to increase SBR activity in all three genotypes relative to the respective controls. Either caldine does not serve as a feedback inhibitor of this enzyme, or it is

TABLE 8. Effects of polyamine biosynthetic inhibitors on putrescine aminopropyltransferase specific activities (pmol/min/mg protein) in nonselected cotton callus cultures grown for 14 days at 25° C

Culture Medium, LB1G+MES Plus:	Assay Products	Genotype		
		BC3	Paymaster404	Coker312
Control	Spermidine	0	0	0.4
	Spermine	0	0	0
	Unknowns[1]	0	0.1	0.1
5 mM HEH	Spermidine	0.1	0.2	0
	Spermine	0.2	0.1	0
	Unknowns	0.5	0.1	0.1
5 mM CHA	Spermidine	0.3	0.1	0
	Spermine	0.1	0	0
	Unknowns	0.1	0.02	0
0.1 mM Caldine	Spermidine	0.4	0	0
	Spermine	0	0	0.3
	Unknowns	0	0.1	0.02
0.1 mM Thermine	Spermidine	0	0.1	0
	Spermine	0	0	0
	Unknowns	0.2	0.1	0.04

[1]Two high molecular weight products were obtained in some assays, corresponding to putative homocaldopentamine and/or homocaldohexamine. Their identity is not yet confirmed.

TABLE 9. Effects of polyamine biosynthetic inhibitors on schiff-base reductase specific activities (pmol/min/mg protein) in nonselected cotton callus cultures grown for 14 days at 25° C

Culture Medium, LB1G+MES Plus:	Assay Products	Genotype		
		BC3	Paymaster404	Coker312
Control	Caldine	11.1	6.1	40.3
	Thermine	20.7	7.5	19.1
	Unknowns[1]	2.8	5.4	20.6
5 mM HEH	Caldine	0	23.2	75.7
	Thermine	0	0	56.5
	Unknowns	8.7	16.6	48.8
5 mM CHA	Caldine	25.7	18.1	27.7
	Thermine	0	12.6	19.9
	Unknowns	8.5	4.7	13.6
0.1 mM Caldine	Caldine	21.5	81.2	66.7
	Thermine	20.4	30.7	25.7
	Unknowns	18.3	0	27.4
0.1 mM Thermine	Caldine	15.2	14.7	31.2
	Thermine	22.3	3.5	26.0
	Unknowns	16.1	3.7	16.1

[1]High molecular weight products were obtained in some assays, corresponding to putative caldopentamine and/or caldohexamine. Their identities are not yet confirmed.

being used as an alternative substrate and in doing so is stimulating activity. Thermine also failed to suppress SBR activity. HEH did suppress SBR activity in BC3, while CHA enhanced SBR activity. All inhibitor treatments stimulated SBR activity in Paymaster 404. HEH stimulated SBR activity in Coker 312.

PAO activity was stimulated by 2.25- to 2.5-fold by 20% PEG treatment in both genotypes of alfalfa cell suspension cultures (Table 10). HEH stimulated PAO activity by 3-fold in the tolerant line MES3, but not at all in the susceptible line MES0. A combination of HEH and PEG treatment stimulated PAO activity by 6-fold in MES3, but suppressed PAO activity in MES0. These results indicate there is a complex interaction among genotype, stress treatment and inhibitor treatment. These results illustrate how competitive inhibitors can stimulate the apparent activity of the enzyme it inhibits; by inhibiting the enzyme, there is a selection pressure placed on the cells to overproduce the enzyme as a means to compensate for the inhibition, which in turn leads to a higher amount of enzyme and higher activity level in the assay. This compensatory effect leading to higher enzyme activity as a result of competitive inhibitor treatment may also explain the results in cotton callus with respect to SBR activity following treatment with caldine.

Selection of Cell Lines for Adaptation to Inhibitors and Stress Conditions

Sublethal dosages of the inhibitors were used to initiate cell selections. Cell selections were conducted with and without concomitant stress. Five independent cell lines were maintained on each selection scheme. As cell lines exhibited enhanced growth relative to initial growth rates on those dosages, the dosage rates were increased. Cell lines exhibiting irreversible growth suppression were eliminated from the experiment and replaced with new lines. Selection was

TABLE 10. Effects of 2-hydroxyethylhydrazine (HEH) and polyethylene glycol (PEG) on polyamine oxidase specific activities (nmol/min/mg protein) in nonselected alfalfa cell suspension cultures grown for 14 days

Genotype	Culture Medium			
	SH6	SH6 + 2.5 mM HEH	SH6 + 20% PEG	SH6 + 2.5 mM HEH + 20% PEG
MES3	4.4	12.2	9.9	27.1
MES0	4.4	4.9	10.2	3.7

maintained for at least 12 months. Cell lines exhibiting stable adaptation for at least three months under the highest selection pressure were identified as putative variant cell lines.

Cotton cell lines showing adaptation to sustained selection pressure were recovered from all of the selection schemes (Table 11). All selected cell lines showed at least 50 percent growth inhibition on the terminal selection pressure. Long-term culture on the inhibitors resulted in cumulative growth suppressive effects even at lower dosages. Thus, adaptation to high dosage rates of the inhibitors was not feasible.

Alfalfa cell lines adapted to inhibitors in the presence of high (20%) levels of PEG could not be recovered. Adaptation occurred slowly in cell selections treated with 8% PEG. MES3 cells adapted to 25 mM HEH and MES0 cells adapted to 12.5 mM HEH were recovered in the absence of PEG stress. Adaptation to CHA or caldine occurred more slowly. All alfalfa cell selections were lost as a result of the fire.

Growth of Selected Cell Lines Treated with Inhibitors and Challenged with Stress Growth Conditions

Selected cell lines were removed from selection pressure for six months, then returned to the respective previous selection pressure to test the stability of the adapatations. The selected cell lines of BC3 and Coker 312 generally showed similar or better growth rates at 25° C when returned to the inhibitors (Table 12) compared to growth rates at the termination of selection (Table 11). This result suggests that the adaptations were stable. The improved growth rates may indicate that there was insufficient time for cumulative growth suppression effects to reoccur. In contrast, selected cell lines of Paymaster 404 showed similar or lower growth rates when returned to the inhibitors (Table 12) compared to growth at the end of selection (Table 11). However, the Paymaster 404 cell line growth rates were high enough to consider the lines to be

TABLE 11. Cell lines of cotton selected for adaptation to different polyamine biosynthetic inhibitors after one year of cell selection

Inhibitor	Characteristics of Selected Cell Lines					
	BC3		Paymaster404		Coker312	
	Adapted to [mM] of Inhibitor	% Growth Inhibition[1]	Adapted to [mM] of Inhibitor	% Growth Inhibition	Adapted to [mM] of Inhibitor	% Growth Inhibition
HEH	10	57	5	55	10	60
CHA	10	58	20	54	10	55
Caldine	0.25	59	0.25	55	0.25	90
Thermine	0.25	55	0.25	57	0.25	73
ED	10	90	25	65	25	58

[1]Growth inhibition of selected cell lines on the indicated concentration of inhibitor is relative to 0% growth inhibition of the same cell line cultured on control medium LB1G+MES for 14 days at 25° C.

TABLE 12. Effects of polyamine biosynthetic inhibitors on fresh weight growth (relative to the control at 25° C) of callus from selected cotton cell lines grown for 14 days at two temperatures

Culture Medium, LB1G+MES Plus:[1]	Genotype and Growth Temperature					
	BC3		Paymaster404		Coker312	
	25° C	38° C	25° C	38° C	25° C	38° C
Control	100%	69%	100%	36%	100%	43%
HEH	41%	36%	43%	37%	62%	54%
CHA	65%	33%	42%	34%	59%	44%
Caldine	64%	29%	43%	27%	113%	51%
Thermine	69%	29%	50%	39%	60%	52%
ED	43%	35%	45%	35%	58%	48%

[1]The concentration of inhibitor used for each genotype is indicated in Table 11.

stably adapted (Table 12). In all selected cell lines of the three genotypes growth was lower at 38° C than at 25° C.

Polyamine Biosynthetic Enzyme Activities in Selected Cell Lines

Activities of the three key polyamine biosynthetic enzymes of interest were determined in the selected cell lines. PAO activity was higher in cells selected for adaptation to 38° C compared to 25° C on the control medium in all three genotypes (Table 13). Comparisons of PAO activities in selected cell lines (Table 13) relative to nonselected cell lines challenged with high temperature (Tables 3and 13) revealed that PAO activities were at least doubled in the selected cell lines adapted to high temperature. This result suggests there is a relationship between PAO activity and adaptation to high temperature. Comparison of PAO activities in the selected cell lines (Table 13) relative to nonselected cell lines treated with the inhibitors (Tables 7and 13) revealed that PAO activities were dramatically altered in selected cell lines of BC3 adapted to HEH at 25° C, CHA at 38° C, and thermine at 38° C; Paymaster 404 adapted to CHA and possibly thermine at 25° C; and Coker 312 adapted to CHA at 38° C, and ED at both temperatures. One pattern in these results is that adaptation to CHA, which should lead to overproduction of spermidine and spermine, also results frequently in overexpression of PAO. The PAO may be needed to oxidize the excess spermidine and spermine.

PAPT activity was higher in cells selected for adaptation to 38° C compared to 25° C on the control medium in all three genotypes (Table 14). Comparison of PAPT activities in the selected cell lines (Table 14) relative to nonselected cell lines challenged with high temperature (Tables 4 and 14) revealed that PAPT activities were generally one to two orders of magnitude greater in the selected cell lines adapted to high temperature. This result suggests there is a relationship between PAPT activity and adaptation to high temperature. Comparison of PAPT

TABLE 13. Effects of polyamine biosynthetic inhibitors on polyamine oxidase specific activities (nmol/min/mg protein) in callus of selected cotton cell lines grown for 14 days at two temperatures

Culture Medium, LB1G+MES Plus:[1]	Genotype and Growth Temperature					
	BC3		Paymaster404		Coker312	
	25°C	38°C	25°C	38°C	25°C	38°C
Control	4.0	12.7	6.7	13.6	0	34.1
HEH	43.5	0	0	0	0.2	1.7
CHA	0.5	37.7	10.0	0.9	1.9	19.7
Caldine	0	6.7	8.8	0	0	0
Thermine	1.1	68.3	6.3	0.8	0	0
ED	ND	3.6	ND	1.1	26.5	17.4

[1]The concentration of inhibitor used for each genotype is indicated in Table 11. ND = not detected because no protein was recovered.

TABLE 14. Effects of polyamine biosynthetic inhibitors on putrescine aminopropyltransferase specific activities (pmol/min/mg protein) in callus of selected cotton cell lines grown for 14 days at two temperatures

Culture Medium, LB1G+ MES Plus:[1]	Assay Products	Genotype and Growth Temperature					
		BC3		Paymaster404		Coker312	
		25°C	38°C	25°C	38°C	25°C	38°C
Control	Spermidine Spermine Unknowns[2]	19.5 0.8 1.8	130.5 144.2 17.4	0 0 22.9	0 72.5 481.5	0 0 0	47.5 14.5 11.8
HEH	Spermidine Spermine Unknowns	925.0 300.2 499.7	0 0 166.7	0 0 96.2	501.1 73.3 195.3	0 0 0	102.9 18.3 31.5
CHA	Spermidine Spermine Unknowns	0 0.03 0.04	40.7 103.0 19.1	26.9 13.6 137.2	0 0 0	0 18.5 28.0	0 0 16.9
Caldine	Spermidine Spermine Unknowns	3.8 1.6 2.9	0 0 64.9	0 0 996.6	51.6 126.9 184.0	7.5 0 2.5	0 3.1 30.7
Thermine	Spermidine Spermine Unknowns	3.3 4.0 1.3	191.1 202.2 91.1	4.8 0.03 0.01	0 3.7 4.9	0 0 0	0 0 0
ED	Spermidine Spermine Unknowns	ND	516.5 430.2 140.1	ND	0 0 656.5	0 0 1.2	0 0 0

[1]The concentration of inhibitor used for each genotype is indicated in Table 11.

[2]High molecular weight products were detected in some assays, corresponding to putative homocaldopentamine and/or homocaldohexamine. Their identities are not yet confirmed. ND = not detected because no protein was recovered.

activities in the selected cell lines (Table 14) relative to nonselected cell lines treated with the inhibitors (Tables 8 and 14) revealed that PAPT activities were most notably altered in selected cell lines of BC3 adapted to HEH at 25° C, CHA at 38° C, thermine at 38° C, and ED at 38° C; Paymaster 404 adapted to HEH at 38° C, CHA at 25° C, and caldine at 38° C; and Coker 312 adapted to HEH at 38° C, and CHA at 25° C. Two patterns are apparent in these results. First, adaptation to CHA results frequently in overexpression of PAPT. This was a predicted result of the selection strategy. Second, adaptation to HEH, which results in overexpression of PAO which oxidizes spermidine and spermine, results frequently in overexpression of PAPT which generates spermidine and spermine.

SBR activity was higher in cells selected for adaptation to 38° C compared to 25° C on the control medium in both genotypes analyzed, BC3 and Paymaster 404 (Table 15). This result suggests there is a relationship between SBR activity and adaptation to high temperature. SBR assays were not determined for Coker 312 selected cell lines because of the depletion of the radiolabeled DAP substrate, which must be special ordered. This experiment will be completed in the future. Comparison of SBR activities in the selected cell lines (Table 15) relative to nonselected cell lines treated with the inhibitors (Tables 9 and 15) revealed that SBR activities were one to three orders of magnitude greater in many of the selected cell lines. SBR activities were most notably altered in selected cell lines of BC3 adapted to HEH at both temperatures, and all of the other inhibitors at 38° C; and Paymaster 404 adapted to HEH, CHA and caldine at both temperatures. Two patterns are apparent in these results. First, adaptation to any inhibitor potentially influencing overexpression of PAO or PAPT, which should ultimately lead to overproduction of DAP, results frequently in overexpression of SBR which produces long chain

TABLE 15. Effects of polyamine biosynthetic inhibitors on schiff-base reductase specific activities (nmol/min/mg protein) in callus of selected cotton cell lines grown for 14 days at two temperatures

Culture Medium, LB1G+MES Plus:[1]	Assay Products	Genotype and Growth Temperature			
		BC3		Paymaster404	
		25° C	38° C	25° C	38° C
Control	Caldine Thermine Unknowns[2]	0.2 0.01 0.01	2.5 0.9 1.1	0 0 0	34.0 5.7 8.3
HEH	Caldine Thermine Unknowns	16.2 0 1.7	5.3 5.2 2.2	3.9 3.9 1.2	2.8 2.3 2.8
CHA	Caldine Thermine Unknowns	0 0.01 0.02	0.9 0.9 0.3	0 0.2 0.04	0.3 0.5 1.4
Caldine	Caldine Thermine Unknowns	0 0 0.002	0.3 0.5 0.2	0.01 0.2 0.1	0.5 1.0 0.8
Thermine	Caldine Thermine Unknowns	0.01 0.002 0.1	2.4 2.3 0.8	0 0.04 0.01	0 0.01 0.02
ED	Caldine Thermine Unknowns	ND	8.0 11.0 7.6	ND	0.1 0.001 0.5

[1]The concentration of inhibitor used for each genotype is indicated in Table 11.

[2]High molecular weight products were detected in some assays, corresponding to putative caldopentamine and/or caldohexamine. Their identities are not yet confirmed. ND = not detected because no protein was recovered.

uncommon polyamines from DAP. Second, SBR may be limiting at both temperatures for Paymaster 404, but may be limiting in BC3 only at the high temperature.

Taken together, the enzyme activity analyses indicate that alterations in polyamine metabolism were achieved by cell selections. Inhibitors of the polyamine biosynthetic enzymes resulted in their respective overexpression, but not necessarily in the predicted manner. This result may be due to a lack of specificity on the part of the inhibitors, or because inhibition of one enzyme could influence how other enzymes responded to adaptational pressures. The three enzymes in the polyamine pathway of interest may be coordinately regulated, because selected cells that were adapted to a single inhibitor showed dramatic alterations in the activities of all three enzymes (e.g., BC3 adapted to HEH at 25° C, CHA at 38° C, and thermine at 38° C). Also, all three enzymes showed increased activity upon adaptation to high temperature, and adaptation to high temperature was sufficient to result in enhanced uncommon polyamine biosynthetic activities.

Plant Regeneration from Selected Cell Lines

The final set of experiments were designed to regenerate plants from selected cell lines, and evaluate the plants for tolerance to the respective stress condition and for water-use efficiency. Selected cell lines of cotton have been cultured on regeneration media for over nine months without success. Compared to other crop species cultured *in vitro*, cotton is generally slow to respond to regeneration treatments (Trolinder and Goodin 1987). In addition, it is well known that long-term cell cultures tend to lose their ability to regenerate plants, and the adverse conditions of cell selection may aggravate this situation (Flick 1983).

Attempts at plant regeneration will continue. Any plants regenerated from selected cells in the future will be tested for field performance under control and stress conditions. Any recovered

plants demonstrating improved performance and/or water-use efficiency under conditions of limiting water or high temperature will be retained for subsequent heritability studies and genetic analysis. Genetic transmission of the trait(s) conferring crop productivity under conditions of limiting water or high temperature, as a result of these experiments, would demonstrate the validity of this cell selection approach as a general strategy for crop improvement.

SUMMARY AND CONCLUSIONS

Several cotton and alfalfa genotypes were chosen for this study because they were known to be tolerant or susceptible to heat or drought stress conditions, respectively. The cotton genotypes could be distinguished with respect to their degree of heat tolerance when grown as callus cultures at 38° C. The alfalfa genotypes could be distinguished with respect to their degree of water-deficit stress tolerance when grown as cell suspension cultures on 8% PEG. Known regenerator lines of each system were characterized similarly. This result confirms that, under certain growth conditions, cell cultures can express stress tolerance traits that correlate with whole plant stress tolerance.

Cotton cells exhibit PAPT, PAO and SBR activities. This result indicates that cotton has a biosynthetic pathway similar to that of alfalfa (Figure 2) for the production of the uncommon polyamines, such as caldine and thermine. Cotton is only the second dicotyledonous plant, after alfalfa, confirmed to have PAO and SBR activities.

The polyamine biosynthetic enzyme inhibitors used in this study did suppress growth of cultured cells, a prerequisite for applying them for cell selection. However, they did not necessarily inhibit the specific activity of the targeted enzyme in cultured cells, even though they are effective inhibitors of the isolated enzymes. Under certain experimental conditions, competitive inhibitors could stimulate the overexpression of the targeted enzyme, possibly as a means of compensation for the inhibition. This result suggests that cell selection for adaptations to such inhibitors would not be straightforward but should be feasible.

Selected cell lines of cotton were recovered that exhibited stable adaptations to the inhibitors and/or high temperature growth conditions from all attempted cell selection schemes. This result indicated that the cell selection strategy was fundamentally successful. Adaptation in

alfalfa cell lines appeared to represent a more complex situation. However, all the alfalfa cell lines were lost in a devastating fire in the laboratory during the cell selection experiment.

Many of the selected cell lines of cotton with adaptations to the inhibitors did exhibit enhancements in the activity of one or more of the targeted enzymes. This result indicated that the cell selection strategy was successful as a means to manipulate polyamine biosynthesis. Several of the selected cell lines showed dramatic increases in the activities of all three enzymes in the uncommon polyamine biosynthetic pathway. This result suggests that the three enzymes may act in a coordinate manner to regulate polyamine accumulation.

The activities of all three enzymes increased in response to cotton cell adaptation to high temperature alone, without inhibitor treatment. This result suggests that there is a relationship between adaptation to high temperature and uncommon polyamine biosynthesis. The relationship between stress tolerance and water-use efficiency, which needs to be studied further, awaits the regeneration of plants from selected cell lines.

Taken together, the results of the experiments presented here provide evidences in support of the hypothesis that uncommon polyamines are involved in mechanisms of plant tolerance to heat stress. Further study of the genetic and physiological bases of this relationship are warranted.

The cell selection technique developed in this project may represent a general strategy for developing crops with altered polyamine biosynthesis and tolerance to abiotic stresses. However, cell selection is a lengthy and complicated technique which must be repeated with each targeted crop. An alternative strategy would be to identify and isolate the genetic coding sequences corresponding to the key enzymes in the polyamine pathway, e.g. from drought-tolerant alfalfa or from the selected cell lines of cotton that show overexpression of the enzyme activities. The

genetic coding sequences then could be transferred into cells of various targeted crops for transgenic expression, using either the native promoter sequences or chimeric promoter sequences derived from other sources (such as the heat shock protein genetic promoters, e.g. see Huess-LaRosa et al. 1987). Recombinant DNA delivery into plant cells and recovery of transgenic plants are rapidly becoming routine techniques for many crops, and this approach promises to be more precise than cell selection for introducing specific traits into agronomically productive varieties. Our research team also is exploring the direct gene isolation and delivery strategy for improvement of crops for drought and heat tolerances, based on the additional evidences generated through this cell selection research project that the uncommon polyamines have a direct relationship to mechanisms of abiotic stress tolerance phenotypes in plant cells.

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