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Rice protein mutant expressed in liquid suspension cultures: chitinases, β-glucanases and other proteins *

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Summary. A rice mutant with unique protein expression/transport properties has been established as cells in liquid suspension and partially characterized. Mutants were originally recovered from anther calli grown for three cycles at inhibitory levels of lysine + threonine and one cycle of *S*-(2-aminoethyl)cysteine. Cell suspension cultures were started from high lysine-containing seeds regenerated from the inhibitor selections. Cultures of the mutant produce 2 times as much protein per unit weight as is produced by the control. Significant portions of the proteins are exported from the cells into the surrounding medium. The mutant also has 20% greater lysine content in the exported protein than the control. This cell suspension line should be particularly useful for biochemical and molecular studies on protein synthesis and processing phenomena in cereals.

Key words: Lysine – Rice – Mutant – Protein – β-glucanases

Introduction

Cells suspended in liquid growth medium are particularly useful for kinetic and biochemical studies. The concentration of nutrients and hormones in the medium can be precisely controlled, and the growing cells exposed to growth factors without the imposition of support media such as agar. The range of cell aggregation in liquid culture varies widely depending upon the source of the explants the level of nutrients and hormones in the medium as well as vessel size and aeration, conditioned in part by shaking speed. Aggregate size may be stabilized under defined conditions and maintained during repeated subculture. One of the most important determinants is the genotype of the cells. Hence, mutants for growth rates, growth promoters and inhibitors as well as cell size and aggregation can be selected and recovered in many species including rice, *Oryza sativa* L., both indica and japonica subspecies. For example, we have in the past selected rice mutants of a near-indica subspecies that were resistant to *S*-(aminoethyl)-cysteine (S-AEC) (Schaeffer and Sharpe 1981) and which formed small aggregates in liquid suspension cultures. These lines have been in culture for nearly a decade. However, such cells normally lose the potential to differentiate into whole plants quickly unless experiments are specifically designed to prevent irreversible dedifferentiation. Hence, we chose to recover mutants along the lysine pathway, in our search for endosperm with improved nutritional quality, from anther callus tissue derived from the cv "Calrose 76". This cultivar, of the subspecies japonica, regenerates from callus for at least ten subcultures on agar and permits long-term experiments. Plants were regenerated that produced seed from calli resistant to inhibitory levels of lysine + threonine (L + T) and S-AEC (Schaeffer and Sharpe 1987). Seeds from R0 plants as well as selected progeny from crosses had increased lysine in endosperm proteins and increased protein over the initial cultivar (Schaeffer and Sharpe 1987). Cell suspension cultures derived from the scutellum of mature embryos from selected lines now provide useful material for biochemical, genetic and molecular studies. In early 1991 we demonstrated the presence of chitinases and glucanases in the cell suspension medium of rice cultures (Schaeffer and Ueng 1991). Additionally, cell suspension cultures are excellent materials for organelle isolations, particular

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mitochondria, for protein and nuclei acid isolation and for the isolation of other biochemicals of both large and small molecular weights.

The purpose of the work reported here is to further define and characterize liquid cell suspension cultures of "Calrose 76" (controls) and L + T mutants (4C) in specific comparisons. Our goals were to establish cell lines as suspenion cultures and to define growth rates, aggregation characteristics and protein content of the mutants and control cells. Protein partitioning between cells and the surrounding medium and enzyme activities are defined. Further analyses were designed to compare the activity of stress-related enzymes, including the endochitinases and β-glucanases. We propose the use of the mutant cell line for efficient and controlled studies of the genetics and biochemistry of protein synthesis and transport phenomena in rice and, ultimately, the application of the technology to industrial processes.

Materials and methods [1]

Preparation of germ plasm

Plants from anther calli resistant to L+T and S-AEC were recovered as described earlier (Schaeffer and Shape 1987). These lines were selfed 3 times before the embryos were excised from mature seeds and grown as callus. Mature seeds were surface sterilized for 15 min with household Chlorox (sodium hypochlorite) diluted 1–5 (1:4, v/v) with distilled water, rinsed 3 times to remove Chlorox and soaked for 24 h in distilled water. At the end of the soak period the seeds again were surface sterilized and rinsed 3 times with sterile distilled water.

Embryo isolation and callus formation

Embryos were isolated under a dissecting microscope from surface-sterilized seeds, soaked in water for 24 h, and the embryo explants placed on agar slants in 25-mm test tubes containing 20 ml Blaydes II medium (Blaydes 1966). The medium was supplemented with 3% sucrose as well as with 50 ml/l coconut milk, 0.5 gm/l yeast extract and 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Callus was grown for 3–4 weeks, subcultured 3 times on the same medium at 28-day intervals and then used as source material for cell suspension cultures.

Maintenance and suspension medium

The liquid cell suspension medium consisted of Murashige-Skoog (Murashige and Skoog 1962) inorganic salts and vitamins, 3% sucrose, 0.1 mg/l 2-isopentyladenosine, m*M* glutamine and 0.5 mg/l 2,4-D. Cell lines were subcultured every 28 days over 3 years. Fourteen-day-old inoculum was used for most of the experiments described. Cell aggregates were sized at each subculture. The aggregates were sieved through a 20-mesh (860 µm) screen, collected on 60-mesh (230 µm) stainless screens and then washed with 400 ml of complete cell suspension medium to remove debris.

Approximately 1.5 g of the washed cells were spooned from the 60-mesh screen and subcultured in 20 ml liquid medium in 125 ml Erlenmeyer flasks. For specific experiments the source material was weighed wet aseptically. Cells were maintained on gyrotary shakers at 105 rpm.

Cell volume determinations

Packed cell volume/20 ml of medium was determined in graduated centrifuge tubes spun at approximately 1000 rpm for 1 min in a clinical centrifuge.

Cell weight determinations

Dry and fresh cell weights were routinely determined by filtering cells through preweighed Whatman #2 filter paper under suction and weights recorded before and after drying.

Protein purification and determinations

Proteins were precipitated from solutions with either aqueous acetone (acetone: water, 4:1, v/v) or 75% saturated $(NH4)_2SO_4$ at 4 °C. Protein quantitations were done spectrophotometrically at 562 nm with the Pierce BCA protein assay kit according to the vender's specifications. The chitinases and glucanases were assayed from acetone precipitations of the total proteins. The proteins were pelleted by centrifugation in a Sorval refrigerated centrifuge at 15 000 g for 15 min. Acetone was gently decanted, and the pellet air dried. Proteins were then resuspended in 20 m*M* sodium acetate (NaOAc).

Chitinase {poly[1,4-*N*-acetyl-β-D-glucosaminide)]} glycanhydrolase, E.C. 3.2.1.14 assays were done using the synthetic substrate 4-methylumbelliferyl-β-D-*N*′*N*′-diacetyl-chitobioside (MDC), which forms a fluorescent hydrolysis product (Kuranda and Robbins 1987). Fluorescence readings were converted to nmoles from standard curves of 4-methyl-umbelliferone. Typical reaction mixtures patterned after the conditions described by Legrand et al. (1987) were modified and had 50 µl enzyme in NaOAc buffer, 50 µl 4.1×10^{-4} *M* MDC and 400 µl 25 m*M* NaOAc, adjusted to pH 5.2. This reaction mixture was incubated for 1 h at 37 °C. The reaction was stopped with 0.5 ml of 125 m*M* glycine. Fluorescence was read with a flow through Aminco fluoro-monitor.

β-1,3-Glucanase or laminarinase (E.C. 3.2.1.39) activity was determined by measuring the reducing sugars hydrolyzed from the substrate, laminarin (Cote et al. 1989; Mohnen et al. 1985). The 2-cyanoacetamide spectrophotometric method was used for the quantitation of reducing sugars (Gross 1982).

Replications and statistical methods

Data for statistical analyses were collected from a rice mutant recovered from inhibitor selections and compared with wild-type cells. Both cell lines were derived from the cv "Calrose 76", a Californian cultivar from the subspecies japonica. All response variables were fitted to a full two by four (tissue culture lines by days in culture) analysis of variance (ANOVA) model. The parameters in the ANOVA model were estimated by weighted least squares to account for inequality of variance. For all responses except percent protein in media, the standard deviations were assumed to be proportional to the means of the responses (constant coefficient of variation). The variance of the percent protein in media response was assumed to be proportional to the product of the mean response and 100 minus the mean response. The weights were taken as proportional to the reciprocal of the variances. Individual mean comparisons and orthogonal polynomial contrasts were tested with *t* or *F* statistics.

[1] Mention of a trademark or proprietary product does not constitute a guarantee or a warranty of the product by the USDA, and does not imply its approval to the exclusion of other products that may be suitable.

Table 1. Cell volume, fresh weight and protein content of rice, mutant and wild-type control cells grown in liquid suspension cultures. Percent protein in cells represents the water-extractable proteins of ground cells divided by total protein in cells plus suspension medium $\times 100$. Cells and media were collected at 3, 5, 7, 10, 13 and 21 days after transfer to fresh medium. The transfer inoculum was 14 days old

Cell volume (ml)			Fresh weight (gm)		Protein (% in cells)	
Day \ Line	Mutant	Control	Mutant	Control	Mutant	Control
Estimates of the population means [a]						
3	2.6	2.6	2.6	2.7	61	66
5	3.2 **	3.6	3.3	3.4	65 **	78
7	4.9	5.0	4.7	4.5	50 **	74
10	5.7	5.3	5.8 **	5.1	35 **	57
13	5.6	5.2	5.4 *	5.1	38	45
21	5.6	6.1	5.4	5.7	30 **	40
Orthogonal polynomial trends [b]						
Linear	14.69 (0.000)	15.16 (0.000)	24.49 (0.000)	24.50 (0.000)	−12.56 (0.000)	−13.56 (0.000)
Quadratic	−7.39 (0.000)	−11.02 (0.000)	−12.73 (0.000)	−20.02 (0.000)	4.64 (0.000)	0.97 (0.337)

[a] A mutant line mean followed by one or two asterisks denotes that the population mean is significantly different from the corresponding control line population mean at the 5% ot 1% level, respectively. Means are from two experiments with three replications within each experiment, $n = 6$

[b] The entries for the linear and quadratic polynomial trends are the t-statistics and P-values (beneath and in parentheses) corresponding to the null hypothesis that the regression coefficient equals zero. The sign of the t-statistic is the same as the sign of the regression coefficient

Results

Growth rate

The growth of mutant and wild-type cells in liquid suspension was characterized by measurements of cell volume, fresh and dry weights and % dry matter. The two cell types were very similar in fresh weight until 10 days after inoculation (Table 1). The fresh cell weight of the mutant at 10 days was significantly greater ($P = 0.01$) than that of the control. This tendency was also expressed at 13 days ($P = 0.05$). The growth rates were very similar at all other time periods (Table 1). Packed cell volumes after centrifugation were also similar but showed some differences. The cell mass of the mutant at 5 days was significantly less than that of the control but not significantly different at other time periods. The mean cell volumes for the mutant and control over all time periods were identical. The mutant and control had similar but not identical dry matter at 3, 5, 7, 10, 13 and 21 days. The % dry matter of the mutant was 14,1%, 15.3%, 12.8%, 9.5%, 9.3% and 8.6%, respectively for those time periods. The % dry matter for the control for 3, 5, 7, 10, 13, and 21 days was 13.6%, 12.8%, 11.9%, 10.0%, 9.5% and 8.0%, respectively. At 5 days the % dry matter was significantly greater in the mutant than in the control. However, the means across all time periods were similar in the mutant and control. Protein probably contributes significantly to higher % dry matter at 5 days. Early growth rates between 1 and 3 days, expressed as freeze-dried weights, also showed higher weights for the mutant at 3 days. The growth response to different amounts of starting inocula fits the predicted pattern, showing more rapid growth at elevated inoculum. The difference between the mutant and control in total dry matter with 0.25, 0.50, 1.0 and 2 g of starting inocula and 1, 2 and 3 days of growth is illustrated in Fig. 1. The % dry matter for mutant and control inoculated with 2 g of cells after 3 days of growth was 14.7% and 12.7% for the mutant and control, respectively.

Protein content and export

The soluble protein content of the cells and of the surrounding cell suspension medium was higher in the mutant than in the corresponding control (Fig. 2). This is a striking feature and was expressed throughout the growth period. The differences in the protein content of the cells are significant after 3 days of culture, but highly significant at 7, 10, 13, and 21 days of culture. Figure 2 (top) depicts the total protein recovered from cells and media throughout the 21-day cycle. Clearly the mutant has the greater protein content even at 5 days after the start of the new cycle, and the difference increases with time. The total protein levels of the control are similar between 3 and 10 days and increase between 13 and 21 days. The % protein in the medium changes during

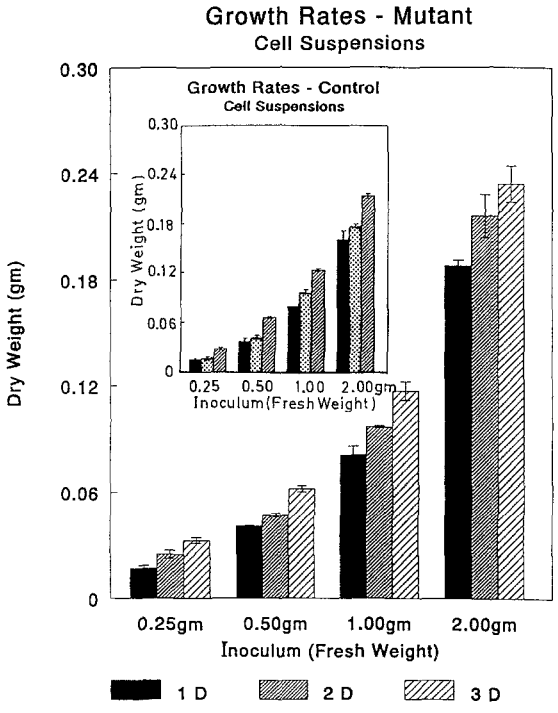

Fig. 1. Effect of inoculum wet weight upon the initial growth rates expressed as dry weights of rice mutant and wild-type control cells (*inset*). Population means are from two experiments, each having three replications. $n=6$ for all data points. *Line symbols above* graphs represent the standard error of means

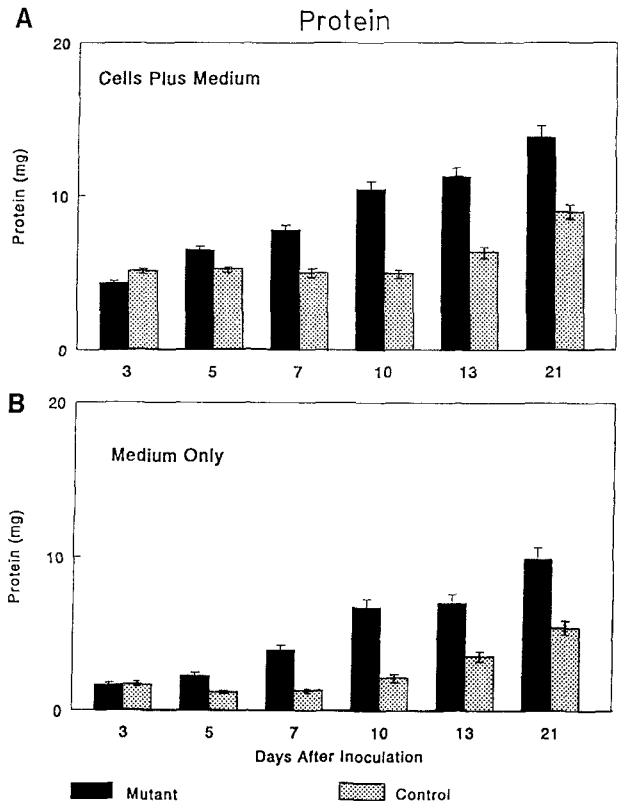

Fig. 2A, B. Relative distribution of water soluble proteins in cells and media at several time periods after subculture: protein in cells plus medium (**A**) and in exported proteins in surrounding medium only (**B**) at different times after subculture. Data from rice mutants and wild-type (control) cell lines represent means of two experiments, with three replications per experiment. The error *bars* represent the standard error of the means of six determinations. Mean total proteins (mg) represent extracts from the entire cell mass and the entire suspension medium

the 21-day cycle. The difference in total protein between the mutant and control is greatest at 5 and 7 days after the start of the culture cycle (Fig. 2). For example, at 7 days the % protein in the medium was over 50% for the mutant and only 26% for the control. The amount of protein exported from the cells into the medium is illustrated for both the mutant and control in Fig. 2.

In a separate set of two experiments the total activities of chitinase in crude extracts of the cells only were 7.3, 7.3, 7.1, 6.3, 6.8 and 7.2 nmol/h for 3, 5, 7, 10, 13 and 21 days of culture, respectively, for the mutant. The corresponding values for the controls were 7.2, 8.6, 8.9, 9.3, 9.2 and 9.2 nmol/h for 3, 5, 7, 10, 13 and 21 days, respectively. These mutant/control ratios for chitinases are 1.01, 0.85, 0.80, 0.67, 0.74 and 0.78 for 3, 5, 7, 10, 13 and 21 days of culture, respectively. The total glucanase activities in the crude extracts of the cell only and extrapolated from the tables and figures were 7447, 9280, 11 407, 9960, 9696 and 9602 nmol/h for cells in culture for 3, 5, 7, 10, 13, and 21 days, respectively, in the mutant, but in the control the activities were 7938, 11 680, 10 697, 8032, 8260 and 9819 nmol/h for cells in culture 3, 5, 7, 10, 13 and 21 days, respectively. The mutant/control ratio for the cellular glucanases are 0.94, 0.79, 1.07, 1.24, 1.17 and 0.98 for cells 3, 5, 7, 10, 13, and 21 days old, respectively. These results on the enzyme activities in the crude cell extracts agree with the data presented and analyzed in Tables 2 and 3 and Fig. 3. The mutant clearly exports a greater quantity of protein than similar cells of the non-selected wild-type control cells. Currently we do not distinguish between proteins in the medium due to cell death and wall rupture and active transport through cell membranes.

Enzyme activity-total and specific activity in suspension medium

There is a near-linear increase in chitinase activity in the medium with time in culture, at least for the controls, whereas the mutant has less activity than the control at 10, 13 and 21 days of culture (Fig. 3). Expressed on a specific activity basis, activity/mg protein (Table 2), the patterns are very different. The activity of the mutant is less than that of the control and is nearly constant throughout the culture cycle. In contrast, the specific activity of the controls is much higher than that of the mutant and is at a maximum 7 days after transfer. However, the difference is due primarily to the greater protein content of the mutant depicted in Fig. 2 without the pref-

Table 2. Chitinase activity recovered from mutant and wild-type control cells of rice grown in liquid cell suspension medium. Enzyme activity is expressed as total activity in nmol 4-methylumbelliferone released from the substrate, 4-methylumbelliferyl-β-*D*-*N*′,*N*′ diacetyl chitobioside (MDC), per hour. Specific activity is the activity/mg protein

Total activity			Specific activity (nmol/mg protein)			
	Cells + medium		Cells + medium		Medium only	
Day \ Line	Mutant	Control	Mutant	Control	Mutant	Control
3	44	42	10 *	8	23	20
5	72	81	11 **	15	27 **	59
7	107	111	14 **	22	25 **	78
10	119 **	151	11 **	30	17 **	67
13	142 *	169	13 **	26	19 **	44
21	162 *	199	12 **	23	16 **	37
Orthogonal polynomial trends						
Linear [a]	0.000 (+)	0.000 (+)	0.352 (+)	0.000 (+)	0.000 (−)	0.070 (−)
Quadratic [a]	0.000 (−)	0.000 (−)	0.009 (−)	0.000 (−)	0.303 (+)	0.000 (−)

* Mutation significantly different from control at probability $P=0.05$. Means represent data from two experiments with three replications within each experiment, $n=6$

** Significantly different at $P=0.01$

[a] P value corresponding to linear and quadratic orthogonal polynomial contrasts. Sign of regression coefficients are indicated in parentheses

Table 3. β-1,3-Glucanase activity recovered from mutant and wild-type control cells of rice grown in liquid cell suspension medium. Enzyme activity is expressed as total activity in nmol of glucose reducing equivalents/h from extracts of cells and/or from the surrounding medium. Specific activity is the activity/mg protein

Total activity			Specific activity (nmol/mg protein)			
	Cells + medium		Cells + medium		Medium only	
Day \ Line	Mutant	Control	Mutant	Control	Mutant	Control
3	12 140	12 505	2804	2441	2805	2679
5	17 029	17 035	2589 *	3165	3307 *	4641
7	23 219 **	16 304	2970	3242	3036 *	4507
10	28 104 **	11 686	2705	2362	2715 **	1738
13	27 304 **	13 363	2420	2078	2499 **	1446
21	30 234 **	16 434	2187	1871	2154 **	1315
Orhtogonal polynomial trends						
Linear [a]	0.000 (+)	0.022 (+)	0.001 (−)	0.000 (−)	0.005 (−)	0.000 (−)
Quadratic [a]	0.000 (−)	0.011 (+)	0.448 (−)	0.410 (−)	0.758 (−)	0.208 (+)

* Mutation significantly different from control at probability $P=0.05$. Means represent data from two experiments with three replications within each experiment, $n=6$

** Significantly different at $P=0.01$

[a] P value corresponding to linear and quadratic orthogonal polynomial contrasts. Sign of regression coefficients is shown in parenthesis

erential increase in the chitinase enzyme activity illustrated in Table 2 and Fig. 3. Most of the chitinases were exported from the cells into the medium in both the mutant and the controls. The pattern for glucanase activity is quite different from that of the chitinase (Fig. 3 and Table 3). The glucanase activity of the mutant medium increases steadily throughout the first 10 days of culture; thereafter, there is little increase. On the other hand, glucanase activity in the controls is not greatly changed throughout the culture period. Expressed on a specific activity basis the patterns again reflect low specific activity due to the high levels of total protein in the mutant. Nonetheless, the glucanase specific activity was higher in the mutant at 10, 13, and 21 days (Table 3), whereas the chitinase activities were less than those of the control at all time periods, except day 3.

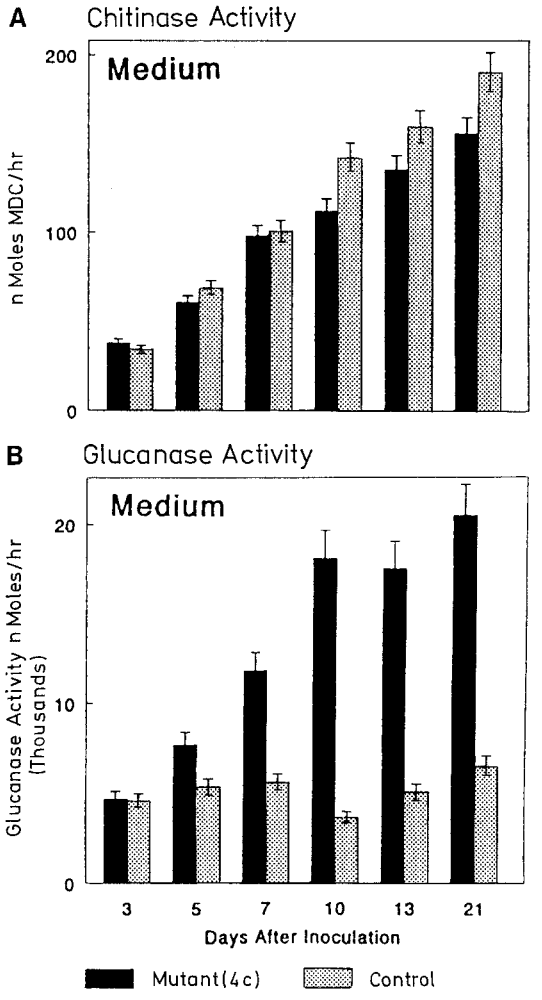

Fig. 3A, B. Chitinase and β-glucanase activities in cell-free suspension medium of rice mutant and control at different times after subculture: **A** Total chitinase activity; **B** total β-glucanase activity. Enzyme activity represents mean of two experiments, with three replications (1 flask per replication) per experiment. Data represents mean total activity from 20 ml medium per flask. Error *bars* represent standard error of the means

Discussion

The selection of rice cells insensitive to inhibitory levels of L+T and S-AEC produced plants with enhanced endosperm protein and % lysine in protein (Schaeffer and Sharpe 1987). The discovery reported here that cell suspension cultures of selected mutants transport more of some proteins from the cells into the suspension medium than wild-type cells provides biochemists and physiologists with new materials for the efficient study of protein synthesis, transport and processing. We consider this cell line important because its phenotype is enhanced protein in cell suspension media. This may reflect the enhanced protein levels observed in the genotype from which the mutant cell line was developed. The cell line now serves as a source of accessible material for kinetic and biochemical studies. It is easier and more precise to do isotope uptake und nucleic acid tagging and isolation procedures with cells in liquid suspension than with whole plants. The nature of the mutation is under active investigation and gene(s) associated with the phenotype(s) will be isolated. Although additional work needs to be done, a correlation between the level of endosperm protein in seeds and the biochemistry of extracellular products in cell suspensions might be extremely useful for molecular studies.

The differences in the activities of chitinases and glucanases were unexpected. Chitinases and glucanases have very different substrates, and hence the regulation of activity/expression at different developmental periods may be different.

Certainly the packaging of enzymes within organelles would alter the movement and effect the intra-organellar hydrolytic degradation or alter the transport of the enzymes to the surface of the cell aggregates where they could be released into the medium. Our prediction is that enzyme packaging or alterations in protein processing and transport phenomena determine, at least in a significant way, the expression of the mutant phenotype.

Statistical analyses for mutant and controls show a good fit for linearity of growth across time for both mutant and controll cells. The polynomial trends for the quadratic equations also show very similar patterns for the mutant and controls. Our conclusions are that the cell lines have similar growth rates over the 21 day growth period and hence are suitable for many biochemical comparisons. The difference in protein exported from the cells is highly significant at all time periods except the 3-day period. The slope of the quadratic expression is negative with time in the mutant but positive with time in the control, reflecting more extensive export during early stages of the growth cycle in the mutant. These export characteristics may be useful for several enzyme purifications. For example, chitinase enzyme activity was recovered primarily in the cell medium for both the mutant and the control. Even at 3 days approximately 90% of the activity was in the medium, and at later stages over 95% of the chitinase activity was in the medium. However, the pattern for the glucanases was quite different, indicating different cellular localizations or transport characteristics, which have functional implications (Mauch and Staehelin 1989). The chitinases and glucanases are considered pathogenesis- and/or stress-related proteins (Kombrink et al. 1988), and we note that these cell lines were grown aseptically and without the application of external stress or fungal phytoalexins. The glucanase gene(s) are expressed or possibly regulated differently in the mutant than in the control. The mutant described here should be useful for evaluating the up or down regulation of expression of these enzymes and to distinguish pathogenic and non-pathogenic plant cell re-

sponses. One striking feature in the enzyme analyses was that the specific activity of both the chitinases and the glucanases was less in the mutant than in the control at 5 and 7 days after subculture, even though the absolute levels of enzyme activities/unit volume suspension medium were similar for the chitinases and much higher for the glucanases in the mutant than in the control. The specific activity was lower because the overall protein content of the mutant was much higher, producing arithmetically a lower specific activity. This means that other proteins are preferentially synthesized or protected against degradation or more proteins are produced in the mutant than the controls. We have not identified any proteins, other than the enzymes mentioned, nor studied the role of proteases or their inhibitors in either the cell extracts or the media. Clearly, the cell lines are useful for such studies. The recent work of Kragh et al. (1991) with barley parallels closely our observations with rice cells (Schaeffer and Ueng 1991) and demonstrates the value of cell suspensions in biochemical studies. These two studies support the hypothesis that a relationship exists between the secretion of chitinases and glucanases into cell media and the expression of the proteins in the developing grain. Inasmuch as our cell culture mutants were derived from seeds with enhanced lysine/protein, a possible link between L+T inhibitor selections and the constitutive expression of stress proteins is indicated. The value of these cell lines for biochemical studies was recently demonstrated with our work on the modification of an 18-kDal polypeptide by spermidine (Mehta et al. 1991). The mutant cell line gives us another tool to examine the regulators associated with protein synthesis in the cereals, the release of proteins from the polysomes and the transport of specific proteins out of the cell.

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