Theor Appl Genet (1996) 92:255–262

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Export of β-1,3-glucanase from mutant rice cells rechallenged and stressed with lysine plus threonine

Received: 28 June 1995 / Accepted: 14 July 1995

Abstract Mutant rice cells (*Oryza sativa* L.) grown in liquid suspension cultures exported greater quantities of protein and β-glucanases than controls. These mutants were isolated from anther calli resistant to 1 m*M* lysine plus threonine (LT), regenerated and reestablished as cell suspension cultures from seeds. Cellular protein levels are genetically conditioned, and the levels of extracellular proteins and enzyme activities are inversely related to that of the cellular portions. The rechallenge of cells with 1 m*M* LT inhibited the expression of both β-1,3-glucanases and β-1,4-glucosidases but had no significant effect upon the levels of chitinase activity. Mutant cells were more sensitive than controls to stress caused by exogenous LT. In general, under exogenous LT stress the mutant/control ratio for extracellular glucanases increased as the assay conditions were changed from a basic to an acidic pH. The specific activity of β-glucanases was highest in media and lowest in cells. Both the mutant and control cells exported β-glucanases into the suspension medium, but the level of activity in media was greater in that in which the mutant was suspended. The export was probably modulated by the internal protein levels which were highest in mutant cells without LT. Seedlings from mutants with enhanced lysine also had enhanced acidic β-glucanase activity.

Key words Rice · β-1,3 glucanases · Mutant · Protein export · Extracellular · Cell culture · Stress · Pathogenesis-related

Introduction

The inhibition of plant cells in culture by amino acids of the lysine pathway has been of interest for many years. Exogenously added lysine plus threonine (LT) functions synergistically to inhibit plant growth, particularly in vitro (Furuhaski and Yatazawa 1970; Gengenbach et al. 1978). Purification methodologies and the cloning of pathway genes along with the molecular dissections of specific enzymes in amino acid biosynthetic pathways were the subjects of a review in 1988 (Matthews et al. 1988).

Rice mutants recovered from inhibitory levels of LT have several unique properties (Schaeffer and Sharpe 1987). Grains of mutant plants recovered from callus culture have chalky/opaque endosperm with 10–15% enhanced lysine and enhanced protein in normal-sized but highly chalky seed (Schaeffer et al. 1989). Biochemically, these mutants, when grown as suspension cultures started from mature embryos of high lysine seed, are characterized by enhanced protein export characteristics (Schaeffer et al. 1992). The proteins exported include stress-related glucanases and chitinases, even in the absence of exogenous stress; hence, these cells in culture are constitutive for components of stress biochemistry.

The response of plant cells both in vitro and in planta to challenges with biotic and abiotic stress is the rapid production of 'pathogenesis-related' (PR) proteins. Divergent aspects of this response have been extensively reviewed and interpreted (Boller 1987; Dixon and Harrison 1990; Dixon and Lamb 1990; Lamb et al. 1989; Linthorst 1991). The co-occurrence and regulation of these enzymes was recognized several decades ago (Abeles et al. 1971) and is still of interest. The PR proteins from numerous plant families include the hydrolases, β-1,3-glucanases and endochitinases, which cooperatively inhibit fungal growth and reduce the consequences of pathogen infections. Boller (1987) summarized the role of hydrolytic enzymes in plant disease resistance. Both chitinases and β-glucanases are components of several endogenous developmental systems including those responsible for callose production in sieve tubes, pollen-tube growth and cell-wall growth. Both enzymes accumulate in bean vacuoles, but only the

Communicated by H. F. Linskens

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β-1,3-glucanases have been demonstrated to be extracellular (Much and Staehelin 1989). Chitinases do, however, occur as exported products in the medium of suspension-cultured rice cells (Schaeffer et al. 1992). It is clear that infections with pathogenic microorganisms cause a massive accumulation of chitinases and glucanases (Boller et al. 1983) and that this response is coordinate with the products acting synergistically (Mauch et al. 1988; Vogeli et al. 1988). Large numbers of genes are associated with plant/pathogen fitness, and may reactions occur rapidly (Lamb et al. 1989). For example, some small but highly potent β-linked molecules elicit stress responses at nanomolar concentrations (Sharp et al. 1984). An analysis of cDNA clones has demonstrated the expression of small families of glucanases (Linthorst 1991). Hormones are able to alter the expression of the glucanases as evidenced by the blockage of synthesis in the presence of auxin and cytokinins and their accumulation in hormone-free media (Mohnen 1985) and in the presence of ethylene (Flex and Meins 1987). β-glucanases are widely expressed and have been reported in barley (Kragh et al. 1991) and rice suspension cultures (Schaeffer and Ueng 1991; Schaeffer et al. 1992). Activity has also been recovered in vegetative tissues (Memelink et al. 1987) and in seeds during maturation and germination (Jacobsen et al. 1990) as well as in flowering tissues (Neale et al. 1990; Lotan et al. 1989). One of the unique features of some PR proteins is their stability and solubility at pH 3.0 (Gianinazzi et al. 1977; Van Loon et al. 1987), thus the isolation of PR hydrolases at low pH facilitates purification by denaturation and precipitation of numerous contaminating proteins. The acid-soluble proteins are partially resistant to proteolytic enzymes and are often present as exported proteins in intercellular fluids, while basic glucanases accumulate more typically in the vacuole (Van den Bulcke et al. 1989). Three acidic PR proteins and one basic PR protein have been identified as β-glucanases (Kauffmann et al. 1987); similarly, two acidic PR proteins and two basic PR proteins have been characterized as endochitinases (Legrand et al. 1987)

The purpose of the investigation reported in this paper was to define the sensitivity of mutant and control rice cells rechallenged with exogenous LT and to identify shifts in protein processing or synthesis in cells grown with LT and without (WO). LT was one of the selection inhibitors used by Schaeffer and Sharpe (1987) in tissue culture from which plants were regenerated and seeds collected. The cells in liquid suspension used in the present experiments were initiated from mature embryos. The extraction and assay for glucanases were done at different pHs to define pH/activity profiles in mutant and wild-type cell lines. Our purpose was also to establish the effect of LT upon β-glucanase activity and protein export properties in mutant and control cells in suspension cultures and to test the hypothesis that LT stress increases the expression of glucanases with acidic pH optima. The work was extended to estimate glucanase activity in seedlings of mutant and wild-type cells and to establish correlations in phenotype between cells in culture and intact plants at the seedling stage. The purpose is also to suggest that specific amino acids such as LT with their demonstrated effects upon hydrolases may play an important role in the well-known carbohydrate/nitrogen metabolic balance with a shift towards reduced carbohydrate metabolism under stress conditions.

Materials and methods

Sources of germ plasm

Two lines of rice were utilized for the investigations on cells in liquid suspension and three lines for enzyme studies in seedlings. Cells of mutant 2K41 were isolated and suspension cultures established as described earlier (Schaeffer et al. 1992). The mutant designation in cell studies refers to 2K41. The control line in both the cell culture and seedling studies was 'Calrose 76' (Rutger et al. 1977), the parent cultivar used for the in vitro selections. The mutant 4C represents unimproved germ plasm recovered from in vitro selections and selfed more than six times in the greenhouse. The mutant 2K41, backcrossed two times to 'Calrose 76', is described in the germ plasm registration published earlier (Schaeffer et al. 1994).

Cell suspension cultures of rice were started from the scutellum of mature embryos and the cell cultures maintained for 6 years (Schaeffer et al. 1992). Cells in suspension were subcultured 1:5, v/v (media with cells/fresh media) every 28 days. Fourteen-day-old cultures were used as starting materials for most of the experiments described.

Lysine plus threonine (LT) treatments of cells in liquid suspension

Stock solutions of 20 m*M* LT were filter-sterilized through 0.22-μm Nalgene filter units, and 1-ml aliquots were added aspectically to 20 ml of cells growing in liquid suspension. Cells were harvested after 24 h except during time-dependent experiments in which all cells were harvested at 0, 24 and 96 h after media replacements. The cells were initially washed with complete medium and 1.5 g wet cells spooned into 20-ml aliquots of fresh medium. Protein quantitations were done spectrophotometrically as optical density at 280 nm or colorimetrically with Bradford/BioRad or Pierce assay kits according to vender's specifications.

Purifications of extracellular proteins

Enzymes were collected from the media after filtration through Whatman no. 2 paper, precipitated from 80% aqueous acetone for 4–6 h and centrifuged at 15,000 *g* for 15 min. Pellets were solubilized in distilled deionized water and recentrifuged to pellet-insoluble material. This insoluble fraction was washed once with water to separate water-soluble and water-insoluble extracellular components.

Purification of cellular proteins

Cells were ground in mortar and pestle in 20 m*M* sodium acetate (NaOAc) buffer, pH 5.2, pH 5.8 or pH 6.8, containing 50 m*M* dithiothreitol, 90 mg polyvinylpyrolidone (PVP) and approximately 0.8 g of acid-washed sand. Three grams of wet cells were ground in 5 ml buffer, extracted and washed 1 × with 5 ml buffered solution. Ground materials were centrifuged 15 min at 15,000 *g*, and the supernatant precipitated with 4 volumes cold acetone for 6 h. Acetone pellets were solubilized in 3 ml water, washed once and the water-soluble portions combined for enzyme and protein assays. For the enzyme assays at varying pHs, cells were ground as described above, centrifuged to remove debri and the supernatant dialyzed for 3

h against 20 m*M* NaOAc, pH 5.2 or pH 6.8. The dialyzed solution was brought to 75% saturation with $(NH_4)_2SO_4$ at room temperature to precipitate proteins and then held for several additional hours at 4 °C. After centrifugation precipitated proteins were solubilized in water and reprecipitated from 80% acetone overnight. Pellets were solublized in water, the remaining insoluble fractions washed again and the water-soluble portions combined for assay at pHs 4.8, 5.2, 5.8, 6.4, 7.0, 7.6 and 8.2. The assay buffer, 100 m*M* NaOAc, pH 8.2, was adjusted down with acetic acid.

Materials extracted with pH 3.0 buffer were processed the same as those for pH 5.2. The buffer consisted of 20 m*M* Na_3citrate. $2H_2O$ adjusted with citric acid, 5% mercaptoethanol, 10 m*M* $MgCl_2$ and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged and acetone precipitated as described above.

β-1,3-glucanase or laminarinase (EC 3.2.1.39) activity was measured as reducing sugars, which were hydrolyzed from the substrate, laminarin (Cote et al. 1989; Mohnen et al. 1985). Reducing sugars were determined colorimetrically using the 2-cyanoacetamide method described by Gross (1982). Chitinases {poly[1,4-*N*-acetyl-β-D-glucosaminide]} and glycan hydrolyase (EC 3.2.1.14) were assayed fluorometrically using the synthetic substrate 4-methylumbelliferyl-*B*-D-*N*′*N*′-diacetylchitobioside(MDC) (Kuranda and Robbins 1987). Fluorescence readings were converted to nanomoles (nmols) from standard curves of 4-methyl-umbelliferone. Reaction mixtures were patterned after Legrand et al. (1987), but modified for flow-through instrumentation. The reaction mixture consisted of NaOAc buffer, 50 ul 4.1 $\times$ 10^{-4} MDC and 400 ul 25 m*M* NaOAc adjusted to pH 5.2. The reaction mixture was incubated for 1 h at 37 °C and then stopped with 0.5 ml of 125 m*M* glycine. Fluorescence was read with a flow-through Aminco fluroro-monitor. β-1,4-glucosidases were assayed spectrophotometrically at 276 nm. The enzyme activity was quantified by the release of *p*-nitrophenol from its β-1,4-glucoside. Data were extrapolated from standard curves of basic *p*-nitrophenol.

Statistical analyses

The 2 $\times$ 2 fractorial (genotype $\times$ treatment) was analyzed as a balanced completely randomized design with four replications. For each of the three enzymes ten response variables were analyzed; three were measured variables and seven were derived variables. Measured variables include: (1) enzyme activities in media; (2) enzyme activities in cell extracts precipitated with acetone; (3) enzyme activities in cell extracts precipitated with $(NH_4)_2SO_4$. Derived variables include: sum of media and cells for acetone, sum of media and cells $(NH_4)_2SO_4$, percent exported = 100 (media)/(media) + (cells)-acetone precipitated, percent exported = 100(media)/(media) + (cells)-$(NH_4)_2SO_4$ precipitated, specific activity-acetone precipitated = (media)/protein, specific activity-acetone precipitated = (cell)/Protein, specific activity-$(NH_4)_2SO_4$ precipitated = (cells)/protein. In addition, responses were analyzed for protein levels. Comparisons between treatments for each genotype and comparisons of treatments between genotypes were made using *t*-tests. $(NH_4)_2SO_4$ data are not presented in the tables because the precipitation patterns were similar to those obtained with acetone.

Results

Extracellular proteins are present at higher concentrations in the cell suspension media of the mutants than in media from the wild-type controls (Schaeffer et al. 1992 and Fig. 1). The data shown in Fig. 1 presents a typical profile. Cellular protein levels are similar or slightly lower in the mutant than in the control probably due to the transport of mutant proteins out of cells. Although different in scale and statistically not comparable, there is an inverse relationship in extracellular and cellular proteins, particularly in the mutant line. The quantity of

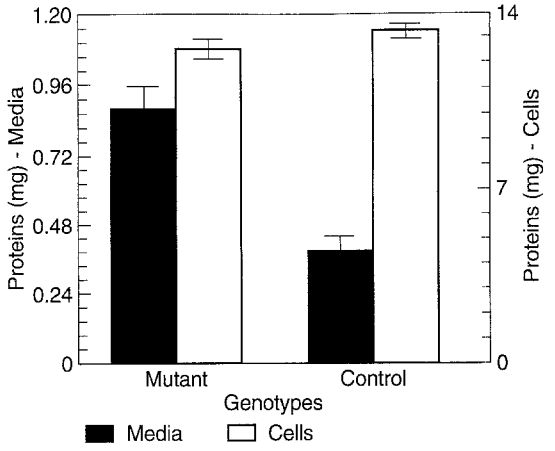


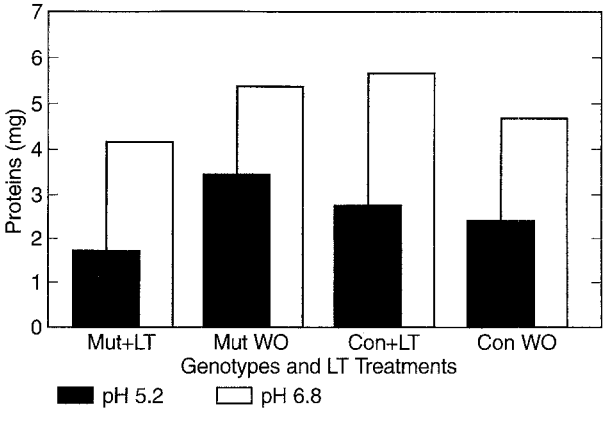
Fig. 1 Proteins recovered from mutant and control rice cells and suspension medium 5 days after cell transfer to fresh media. Values represent water-soluble proteins from acetone-precipitated macromolecules of whole media and ground cellular extracts. Proteins are expressed as milligrams per gram fresh cells. *Bar* symbols represent standard error of means, $n = 20$

proteins exported is linked to total cellular protein content even when disturbed by inhibitory levels of LT.

LT applied exogenously as an inhibitor affects the mutant differently than the control (Fig. 2). LT treatments decrease the level of pH 5.2-extracted cellular proteins of the mutant but increase that of cellular proteins in the control. The sensitivity of mutant cells to millimolar concentrations of LT is expressed in both pH 5.2 as well as 6.8 extractions.

The inhibition of glucanase activity by exogenous LT is time-dependent and severe at 24 h for the mutant and less so for the control cells (Fig. 3). Control cells are inhibited approximately 13% at 24 h, whereas the mutant was inhibited over 36% at 24 h (Fig. 3). Control

Fig. 2 Effect of lysine plus threonine (*LT*) and the absence of amino acids (*WO*) upon the quantity of water-soluble cellular proteins extracted at pH 5.2 and 6.8. Proteins are expressed as milligrams proteins per gram fresh cells and quantified with Bradford reagents. Extracts were dialyzed, sequentially precipitated with $(NH_4)_2SO_4$ and acetone and resolublized for enzyme assays and protein determinations. (Detailed in Materials and methods). *Mut* Mutant, *Con* controls



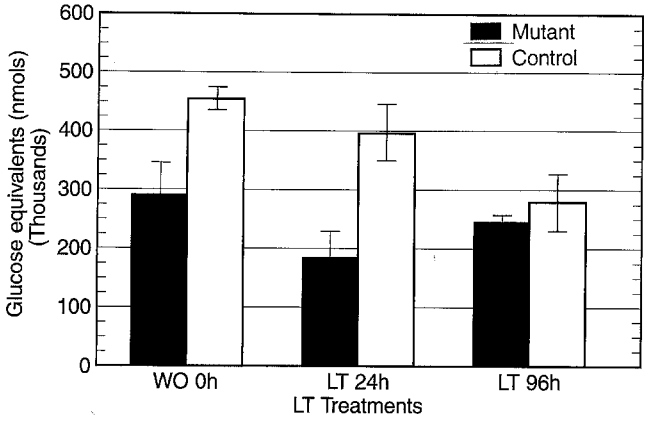
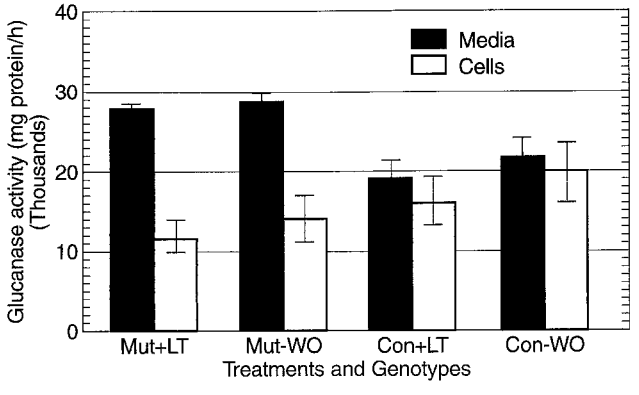


Fig. 3 Comparative effect of m*M* lysine + threonine (*LT*) upon total level of cellular β-1,3-glucanases at 24 and 96 h after start of LT treatments. 0 h represents cells without (*WO*) amino acid treatments. Total β-1,3-glucanase activity is expressed as glucose reducing equivalents (nmols) per gram fresh tissue recovered from pH 6.8 Tris buffer. Extracts were dialyzed and sequentially precipitated with $(NH_4)_2SO_4$ and acetone and resolublized in water

cells are less sensitive initially to LT than mutant cells. The low enzyme activity in the mutant at 0 h is extreme in this experiment but may be caused by the export of proteins from the cells promoted in part by the a pre-treatment rinse.

Glucanase specific activities, expressed on a per unit protein basis, with LT and WO were not greatly different between the mutant and the control (Fig. 4). Since specific activity is dependent upon the level of enzyme activity and is inversely proportional to protein levels in crude extracts, the effect of enhanced levels of undefined proteins would arithmetically produce the lower specific activites observed in the mutant media and LT-treated controls. In this case part of the reduction was due to inhibition by LT (Fig. 4).

Fig. 4 β-1,3-glucanase specific activity, glucose reducing equivalents (nmols) per milligram protein per hour, recovered in media and cells of mutant and control rice cell suspension cultures grown with (*LT*) and without (*WO*) lysine plus threonine. Symbols signifiy standard error of means, $n = 4$



Statistical values assigned to data are summarized in Tables 1–4 for the effects of LT selections upon the activities of chitinase, β-1,3-glucanase, β-1,4-glucosidase and the corresponding protein values, both extracellular and cellular. The percentage of β-1,3-glucanase activity exported into the medium was the greatest with the LT-treated mutant (Table 1). Levels of 15.8% and 12.2% glucanase activity were exported from mutant cells, with LT and WO, respectively while only 7.9% and 6.7% activity was exported from the controls, with LT and WO, respectively. Even though mutant cells exported over 15% of the glucanase activity, only 3.5% of the total portein was exported. The highest levels of cellular glucanase activities were recovered from the WO treatments, both mutant and control. Conversely, the lowest cellular glucanase levels were observed in LT-treated mutants, reflecting the 15.8% export level and the sensitivity of the mutant to inhibition with LT (Table 1). However, the level of extracellular glucanases was the highest in media recovered from mutant cells, both LT-teated and WO.

Interestingly, the mutant/control ratio for percentage extracellular glucanase activity recovered in media with LT was 2.0 and WO was 1.8, whereas the ratio for percentage protein in media was between 1.0 and 1.03. This suggests that LT does not extensively promote the export of proteins and that this export is genotype-conditioned and modulated by protein levels. This is not surprising since the export of protein in plants is not domain-targeted but is a default function. Although many proteins are exported by default, the acidic glucanases were among those prominently exported in these experiments. The probability values presented in Tables 1–4 illustrate clearly the genotype effects in these experiments, particularly in the media fractions. The activity of exported β-glucanases, as percentage of total, was also highly genotype-responsive. On the other hand, the *P* values of cells were not greatly different.

Chitinase specific activity in media was significantly lower in the mutant than in the control when the cells were WO exogeneous amino acids, $P = 0.016$ (Table 2). However, there were no differences in cellular chitinases in response to LT nor could differences be demonstrated between the two genotypes. The expression of chitinases and glucanases was different, which means that the two enzymes are not as tightly co-regulated in liquid cultures as they are in bean leaves (Vogeli et al. 1988).

β-1,3-glucanases (Table 1) and β-1,4-glucosidases (Table 3) had similar acitvity patterns and this is as expected. Evenso, the percentage of β-1,4-glucosidase activity exported in the mutant grown LT was lower than that of the corresponding β-1,3-glucanases, 9.2% for the glucosidases versus 15.8% for the glucanases. There was no significant reduction due to LT in the activity of either hydrolase recovered from cells, although, β-1,3-glucanase activity in mutant cells was close to being significantly different from that in cells grown WO, $P = 0.057$. The probability for the corresponding β-1,4-glucosidase is $P = 0.164$.

Table 1 β-1,3-glucanase activities expressed as glucose reducing equivalents (nmols) recovered from media (extracellular) and cell (cellular) portions of rice suspension cultures grown with lysine plus threonine (LT) and without (WO) exogenous amino acids. Response variables were fitted to a full two by two (genotypes × treatments) analysis of variance (ANOVA) model. Comparisons are considered significantly different at probability values (P values) < 0.051. Mutant versus control values are averaged across LT and WO; LT versus WO values are averaged across mutant and controls (*M* mutant, *C* control, *nmols* nanomoles, *Trt* treatments)

Genotypes	Trt	Media	Cells	Media + cells	Activity exported	Media specific activity	Cells specific activity
		nmols × 10^3	nmols × 10^4	nmols × 10^4	%	× 10^3	× 10^3
M	LT	49	30	34	15.8	28	17
	WO	63	46	53	12.2	28	21
C	LT	35	41	44	7.9	19	24
	WO	36	51	55	6.7	21	29
Comparisons		*P* value					
M: LT vs WO		0.019	0.057	0.050	0.116	0.810	0.400
C: LT vs WO		0.782	0.224	0.236	0.606	0.388	0.212
LT: M vs C		0.018	0.190	0.267	0.003	0.011	0.146
WO: M vs C		<0.001	0.582	0.824	0.024	0.038	0.068

Table 2 Chitinase activities expressed as units of fluorescence converted to nanomoles from standard curves of 4-methyl-umbelliferone. See heading of Table 1 for abbreviations, statisttical methods, treatment and descriptions of cell fractions

Genotypes	Trt	Media	Cells	Media + cells	Activity exported	Media specific activity	Cells specific activity
		nmols	nmols	nmols	%		
M	LT	25	25	50	51	14	1.5
	WO	26	32	57	46	12	1.4
C	LT	29	29	57	50	15	1.7
	WO	33	31	64	51	20	1.8
Comparisons		*P* value					
M: LT vs WO		0.920	0.352	0.227	0.629	0.340	0.825
C: LT vs WO		0.351	0.799	0.260	0.849	0.165	0.778
LT: M vs C		0.488	0.612	0.227	0.912	0.753	0.632
WO: M vs C		0.139	0.855	0.260	0.575	0.016	0.334

Table 3 β-1,4-glucosidase activities expressed as nanomoles *p*-nitrophenol released from the susbtracte, *p*-nitrophenol-β-,D-glucoside. See Table 1 for abbreviations, statical methods, treatments and description of cell fractions

Genotypes	Trt	Media	Cells	Media + cells	Activity exported	Media specific activity	Cells specific activity
		nmols	nmols × 10^3	nmols × 10^3	%		
M	LT	1900	22	24	9.2	1100	1300
	WO	3300	31	34	11.2	1500	1400
C	LT	1700	21	23	7.5	910	1200
	WO	2100	24	26	8.7	1200	1400
Comparisons		*P* value					
M: LT vs WO		<0.001	0.164	0.101	0.493	0.033	0.745
C: LT vs WO		0.204	0.718	0.658	0.735	0.078	0.709
LT: M vs C		0.594	0.937	0.912	0.571	0.383	0.887
WO: M vs C		0.003	0.255	0.177	0.364	0.189	0.924

Protein levels were distinctly lower in the mutant media with LT-treated cells versus WO, $P < 0.001$ (Table 4). Percentage proteins exported was not significantly different. The lowest cellular levels were recorded with LT-treated mutant cells, reflecting strong LT inhibition.

Stress biochemistry may shift the acidic/basic ratio of proteins, including that of some isozymes. The results of our assays for β-glucanases over a range of pHs reflected the shift toward acidic glucanases during LT stress. The regression line for the matuant/control ratio of enzyme activity versus assay pH produced a

Table 4 Protein levels from media cells of rice suspension cultures. Values applied to specific activities in Table 1, 2 and 3. See Table 1 for abbreviations, statistical methods, treatments and descriptions of cell fractions

Genotypes	Trt	Media	Cells	Media + cells	Protein exported
		mg	mg	mg	%
M	LT	1.8	16	52	3.5
	WO	2.2	22	66	3.3
C	LT	1.9	18	53	3.6
	WO	1.7	18	52	3.3
Comparisons		*P* value			
M: LT vs WO		<0.001	0.043	0.032	0.834
C: LT vs WO		0.107	0.936	0.790	0.579
LT: M vs C		0.381	0.597	0.808	0.761
WO: M vs C		<0.001	0.127	0.031	0.965

regression value of 0.78 for LT and 0.89 for WO. The 0.78 regression value reflects higher values at the acid end of the assay profile with LT. The 0.89 value for assays without LT is closer to 1.0, the expected value if the ratio is unchanged over the range of pHs assayed. The steeper slope in the LT-treated material reflects a shift in favor of glucanases with acidic activity optima in mutant cells. The mean mutant/control ratio across the range of pHs was 2.17 for LT-treated and 1.40 for untreated cells. The mutant/control ratio as used here is a complex parameter and may be caused by either elevated mutant values or lowered control values. Also, a lower ratio could be conditioned by an increase in basic glucanases in control cells or other components affecting protein export. The profile does not distinguish between specific LT effects such as feedback inhibition and inhibition of protein synthesis and the induction of other stress responses due to LT membrane effects/toxicity. Other reasearchers have reported changes in the cellular processing of acidic and basic isoforms during stress challenge (Van den Bulcke et al. 1989). This work represents the first report, to our knowledge, of such shifts by specific amino acids, namely LT. We have demonstrated here and observed in numerous other experiments that the mutant is genetically conditioned for increased protein synthesis. This increased capacity provided more protein for export, including glucanases with acidic optima. The pH response was enhanced by exogenous LT stress, which was most strongly expressed at pH 4.8 and 5.2.

The activities of glucanases in media and cells at different pHs is profiled in Fig. 5. The pH optimum for the assay of extracellular glucanases can be observed to be near pH 5.2 but the response curves are broad for both extracellular as well as cellular glucanases. Media from mutant cells grown with LT and WO had higher glucanase levels, whereas the levels of the controls were the lowest in the pH range 4.8–6.4. LT reduced the level of extracellular enzyme activity in both the mutant and controls. Conversely, cellular activities and proteins from the controls, both LT-treated and WO, were the highest and the mutant values the lowest. These levels reflect the active export of glucanases from mutant cells, both LT-treated and WO. The activity in the media was

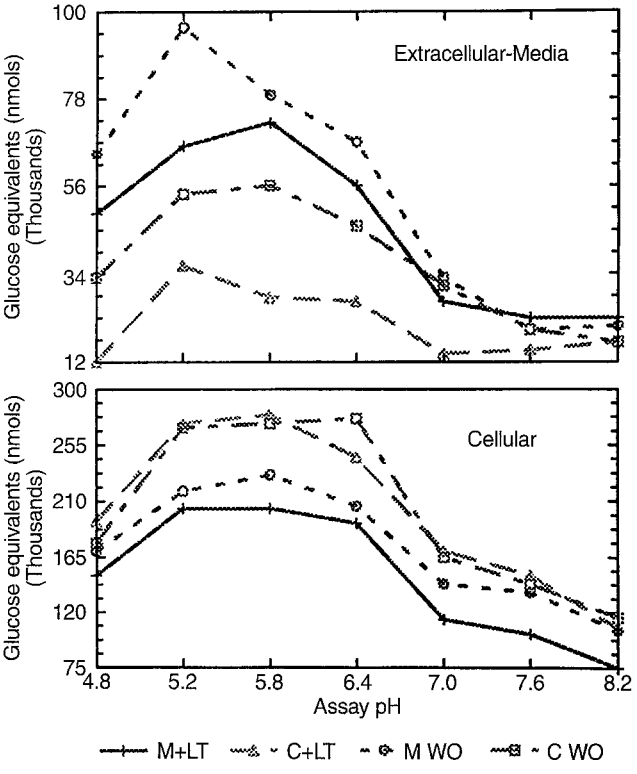


Fig. 5 β-1,3-glucanase response profiles, pH 4.8–8.2, for total extra cellular (*top*) and cellular (*bottom*) from rice cells grown with lysine plus threonine(*LT*) and without (*WO*) amino acids stress. Comparative activity is expressed as glucose reducing equivalents (nmols)/20 ml media (extracellular) and total activity in nanamoles from 1.5 gm fresh cells (*cellular*). *M* Mutant, *C* control

greatly reduced above pH 7.0. The pH response curve was flatter with cellular glucanases, reduced at pH 4.8 compared with media glucanases and has shifted to higher pH, most obvious at pH 6.4. The shift probably reflects the export of acidic intercellular glucanases and the retention of cytosolic glucanases.

There is a fundamental question concerning the relevance of cell suspension data to applications in intact rice plants. Preliminary data suggests that some pH 3.0-extractable isoforms of β-1,3-glucanase are different in mutant and the control. Activity from the pH 3.0-extractions of 10-day-old mutant seedlings have a

broader anion exchange profile and mutant components elute later from the column with NaCl than analogous controls (data not presented). However, there were no significant differences in total enzyme activities in crude extracts between mutants and controls extracted with pH 3.0-, 6.8- or 8.2-buffered solutions. Chitinases in crude extracts were also similar, but the activities of chitinases recovered from DEAE exchange columns were nearly twice as high in the mutant as in the control. Perhaps inhibitors were removed during the DEAE purifications. The mutant phenotype is genetically conditioned for stress biochemistry, as illustrated by the production and export of acidic glucanases in cell suspension cultures in vitro; however, the stress biochemistry is apparently expressed in a different manner in whole plants.

Discussion

The mutant cell line was affected more strongly by LT than the corresponding control. This was not expected since the cells were originally recovered from selections grown in media with 1 m*M* LT, and the expection was a reduced sensitivity to LT. We did not evaluate the role of endogenous modifiers or inhibitors in the expression of the enzymes reported here. One possibility might be that the processing biochemistry, including glycosylation, is modified in the mutant, thereby leading to increased protein synthesis by pathway deregulation. Changes in carbohydrate processing due to changes in soluble hydrolases in and between cells could alter sensitivity to LT. Such a phenotype might be masked in the carbohydrate-rich cell culture environment. Unimproved plants of this mutant, such as 4C, are more intensely abnormal than controls (bent node, incomplete flowers and infertility) when grown under suboptimal greenhouse conditions of temperature and photoperiod in the winter. However, growth of the imporved line, 2K41 backcrossed to 'Calrose 76', and the control cultivar is similar under optimal conditions. A logical hypothesis might be that β-glucanases specifically alter glycosylation patterns, producing changes in membrane stability and/or DNA binding properties.

The low glucanase values in the mutant WO amino acids at 0 h in Fig. 3 was not anticipated. We expected this value to be equal to that of the control or only slightly less. This depression of the mutant value is probably caused by the pre-treatment rinse that was designed to place all the treatments under equal conditions. The rinse with fresh media removed the free amino acids, complex carbohydrates and the preexisting proteins from the suspension medium. The removal of media products and the continued export of cellular proteins could shift the diffusion equilibrium toward the full export potential, which would be greater in the mutant than the controls. This mutant genotype has value in deciphering the role of hydrolases in protein processing and export phenomena and evaluating the biological stress caused by inhibitory levels of indigenous metabolites.

Acknowledgements The authors thank Dorothy Roach for clerical help with the tables and gratefully acknowledge the statisticans W. Potts and A. Wilcox for their help in analysis and interpretation of the results. We also thank Barbara Robinson for contributions from studies associated with the high abilities program.

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