Regulation of maltose utilization in *Saccharomyces cerevisiae* by genes of the RAS/protein kinase A pathway

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Abstract In *Saccharomyces cerevisiae* maltose utilization requires a functional *MAL* locus, each composed of three genes: *MALR* (gene 3) encoding a regulatory protein, *MALT* (gene 1) encoding maltose permease and *MALS* (gene 2) encoding maltase. We show that constitutive activation of the RAS/protein kinase A pathway severely reduces growth of *MAL1* strains on maltose. This may be a consequence of reduction in *MALT* mRNA, reduced V_{max} and increased catabolite inactivation of the *MALT*-encoded maltose transporter in the *MAL1* strain. Mutations in the *GGS1/TPS1* gene, which restricts glucose influx and possibly affects signalling, relieve carbon catabolite repression on both maltase and maltose permease and reduce maltose permease inactivation.

Key words: Catabolite inactivation; Catabolite repression; Gene expression; Maltase; Maltose permease

1. Introduction

The budding yeast *Saccharomyces cerevisiae* can utilize different sugars as a sole carbon source, depending on the presence of specific genes encoding enzymes required for sugar uptake and/or metabolism [1]. Glucose is the preferred carbon source and complex regulatory circuits have evolved to ensure that expression of gluconeogenetic and alternative sugar utilizing enzymes is repressed when glucose is present in the growth medium. These mechanisms turn off gene expression at the transcriptional level – a mechanism known as glucose or catabolite repression [2] – and at the post-translational level by modulation of activity and/or enzyme degradation – a mechanism known as glucose or catabolite inactivation [3]. Although catabolite repression has been associated mostly with glucose, other sugars including sucrose and maltose are known to elicit it, but to a lesser extent.

In addition to glucose derepression, full expression of certain genes, notably those required for galactose and maltose utilization [4,5] requires the presence of an inducer. Maltose utilization is dependent upon a multigene family. Each functional *MAL* locus is composed of three genes: *MALS* (gene 2) encoding maltase, *MALT* (gene 1) encoding maltose permease and *MALR* (gene 3), encoding a transcriptional activator specifically activating expression of the *MALS* and *MALT* genes. Expression of both *MALS* and *MALT* is carbon catabolite repressed by glucose and coordinately induced by maltose; in addition, maltose permease is catabolite inactivated by glucose and – to a lesser extent – by maltose [5,6].

In *S. cerevisiae* RAS proteins exert their effects primarily – if not exclusively – through the adenylyl cyclase/protein kinase A (PKA) pathway [7,8]. So far, few and conflicting reports have been published regarding interactions between genes of the RAS pathway and genes involved in – and subject to – catabolite repression. Here we show that constitutive activation of the RAS/PKA pathway inhibits maltose utilization, mostly by reducing maltose permease activity. A mutation in the *GGS1/TPS1* gene, encoding the catalytic trehalose-6-phosphate synthase subunit of the trehalose synthase complex, and which has been proposed to play a role in restricting glucose influx and possibly glucose sensing (reviewed in [9]) severely reduces glucose repression of *MALS* and *MALT* expression and strongly reduces catabolite inactivation of maltose permease.

2. Materials and methods

2.1. Strains, media and growth conditions

Escherichia coli strain JM101 was used as a recipient strain for plasmid transformation. *E. coli* cells were grown in LB medium supplemented with 50 mg/l ampicillin [10]. The *S. cerevisiae MAL1* strain JM2763-14 (*MATa ura3-52 leu2-3 leu2-112 MAL1*) has been described [11]. Strain MVY99 (*MATα ura3 his3 lys2 fdp1*) was constructed by crossing JM2763-14 with strain LVA1531 (*MATα ura3 his3 lys2 fdp1*) [12]. YEP and synthetic (S) [13] media – with appropriate amino acids and bases (50 mg/l) were supplemented with the appropriate carbon source as detailed below. Solid media contained 2% agar. [] indicates plasmid-carrying cells.

2.2. Enzyme assays

Specific activities of maltase, β-galactosidase and [^{14}C]maltose uptake were determined essentially as described previously [14–16] except that 5 s of incubation were used for measuring [^{14}C]maltose uptake. Kinetic parameters of maltose permease were determined with 5 different maltose concentrations: 20, 10, 4, 2, and 1 mM. Protein content was measured by Bio-Rad protein assay reagent (Biorad). Inactivation of maltose permease was determined by measuring [^{14}C]maltose uptake of cells grown on synthetic medium – containing 0.1% glucose+2% maltose – washed with sterile double-distilled water and placed into synthetic medium containing 5% glucose at time zero. Maltose permease activity of the culture was followed for at least 60 min.

2.3. Recombinant DNA techniques and plasmids

Standard procedures for DNA subcloning and plasmid construction were as described in [10]. Yeast transformation was performed using the lithium acetate method described by Ito et al. [17] or the DMSO lithium acetate method of Hill et al. [18]. Plasmids pRAS2,

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This paper is dedicated to the memory of Julius Marmur.

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pRAS2^{val19} [19] and pTPK1 [20] were used to obtain strains JM2763-14 [pRAS2], JM2763-14 [pRAS2^{val19}] and JM2763-14 [pTPK1]. Plasmids pFDP1 (carrying *GGS1/TPS1*) and pFPS1 (carrying *FPS1*) [12] were used to obtain strains MVY99 [pFDP1] and MVY99 [pFPS1]. Strain JM2763-14 *bcy1::URA3* was obtained by the one-step gene disruption method [21] using *Bam*HI-cut plasmid *pbcy1::URA3* [22] selecting for Ura$^+$ transformants. Disruption of the *BCY1* gene was confirmed by Southern blotting [10]. *MALS*- and *MALT-lacZ* fusions were constructed by subcloning the same *Eco*RI(blunted)-*Bcl*I fragment encompassing nucleotides 1–17 of *MAL6S*, nucleotides 1–92 of *MAL6T* and the complete region between the divergently transcribed genes into appropriate *lacZ* fusion vectors [23]. Cloning of the *Eco*RI(blunted)-*Bcl*I into *Sma*I-*Bam*HI-cut YEp368 and of the *Bcl*I-*Eco*RI(blunted) into *Bam*HI-*Sma*I-cut YEp366R yielded plasmids pMS*lac* and pMT*lac*, expressing *MAL6S*- and *MAL6T*-fusions, respectively.

2.4. Hybridization techniques

Total yeast RNA for Northern blot analysis was isolated, electrophoresed, blotted and hybridised with random primed probes essentially as described previously [24]. Probes for *MALR*, *MALS* and *MALT* were the *Eco*RI-*Eco*RI fragment of *MAL6R*, the *Bgl*II-*Bgl*II fragment of *MAL6S* and the *Nco*I-*Pvu*II fragment of *MAL6T*, respectively.

3. Results

3.1. Maltose utilization is inhibited in strains with an activated RAS/protein kinase A pathway

Constitutive activation of the RAS/PKA pathway inhibits growth on maltose of the JM2763-14 strain regardless of whether activation is brought about by deregulated RAS activity – strain JM2763-14 [pRAS2^{val19}] – or by deregulated protein kinase activity – strains JM2763-14 *bcy1::URA3* lacking PKA regulatory subunit – or JM2763-14 [pTPK1], overexpressing PKA catalytic subunit. The same results were obtained at either 24, 30 or 37°C in both liquid and solid media and indicate that expression of one or more of the *MAL* genes is directly or indirectly reduced by unbridled PKA activity. Growth on maltose of the JM2763-14 strain with an activated RAS pathway (strain JM2763-14 [pRAS2^{val19}]), but interestingly not of JM2763-14 *bcy1::URA3* was at least partially restored by growing cells in 10% maltose, a feature often associated with mutants in maltose permease, thus suggesting that maltose transport is the major target affected by an overactive RAS pathway (data not shown).

3.2. Constitutive activation of the RAS/PKA pathway preferentially affects maltose permease at both transcriptional and post-transcriptional levels

JM2763-14-derived strains were then analyzed for their ability to synthesize maltase and maltose permease under standard inducing conditions (overnight growth in synthetic medium supplemented with 0.1% glucose+2% maltose). Table 1 shows that JM2763-14 [pRAS2^{val19}] and JM2763-14 *bcy1::URA3* strains synthesize maltase levels close to the wild type strain or to strains expressing a second *RAS2* gene. In contrast, the level of maltose permease was severely reduced in both strains. By growing JM2763-14 [pRAS2^{val19}] cells in 10% maltose nearly normal levels of maltose permease activity were regained. Strain JM2763-14 *bcy1::URA3* grew poorly, if at all.

The expression of each *MAL* gene was examined by Northern blotting using subcloned *MAL* gene fragments as probes. Levels of *MALR* and *MALS* mRNAs were close to wild type as expected. The decrease in *MALT* mRNA was more severe for the JM2763-14 *bcy1::URA3* strain than for the JM2763-14 [pRAS2^{val19}] strain (Fig. 1), in keeping with the more severe phenotype of the former.

Both *MALS*- and *MALT-lacZ* fusions were constructed and their expression examined under steady-state inducing conditions. Table 1 shows that in both JM2763-14 [pRAS2^{val19}] and JM2763-14 *bcy1::URA3* mutant strains, β-galactosidase levels driven by *MALS* promoter are close to the levels found in the wild type. Expression of *MALT-lacZ* fusions is reduced in both strains, but to a significantly higher degree in JM2763-14 *bcy1::URA3*. These results indicate that post-transcriptional regulation of maltose permease is a key factor in the Mal$^-$ phenotype of *MAL1* strains of which the RAS pathway is constitutively activated. The more severe reduction of *MALT-lacZ* expression in strain JM2763-14 *bcy1::URA3* is in keeping with results obtained with maltose permease assays and *MALT* Northern blots.

Kinetic parameters of maltose permease were examined in JM2763-14 [pRAS2^{val19}] and compared with those of its isogenic counterpart JM2763-14 [pRAS2]. The apparent K_m values determined for JM2763-14 [pRAS2] and JM2763-14 [pRAS2^{val19}] agreed within the experimental error (15%), while the apparent V_{max} dropped to less than 50% in

Table 1
Effect of unbalanced RAS/cyclase pathway on maltase, maltose permease and the expression of *MALS* and *MALT -lacZ* fusions

Strain		0.1% D 2% M				10% M			
Genotype	Plasmid	Maltase	*MALS-lacZ*	Maltose permease	*MALT-lacZ*	Maltase	*MALS-lacZ*	Maltose permease	*MALT-lacZ*
MAL1 BCY1	–	1.03	0.9	0.84	1.05	0.97	1.08	0.94	1.1
MAL1 BCY1	pRAS2	1.00	1.0	1.0	1.0	1.00	1.00	1.00	1.00
MAL1 BCY1	pRAS2^{val19}	0.70	0.8	0.15	0.64	1.00	1.04	0.82	0.41
MAL1 bcy1::URA3	–	0.81	0.2	0.15	0.07	NG	NG	NG	NG

NG, no growth. Maltase and maltose permease specific activities of JM2763-14 derivatives grown on 0.1% glucose+2% maltose and on 10% maltose were determined as described in Section 2.2. The values (average of at least two independent experiments, each conducted in duplicate and triplicate for maltase and maltose permease, respectively) are expressed as a fraction of either maltase specific activity (28–32 OD_{405}/min per mg protein) or [^{14}C]maltose uptake (300–400 cpm/min per μg protein) of the JM2763-14 [pRAS2] strain grown under the same conditions. Results of different experiments agreed within 10 and 20% for maltase and maltose permease, respectively. β-Galactosidase specific activities of mutant strains transformed with *MALS*- and *MALT-lacZ* fusions were determined as previously described [16]. The values (average of at least three independent experiments, each assayed in duplicate) are expressed as a fraction of β-galactosidase activity (45–50 and 29–33 OD_{420}/min per mg of protein for *MALS* and *MALT* fusions, respectively) of the JM2763-14 [pRAS2] strain grown under the same conditions. Results of different experiments agreed within 20%.

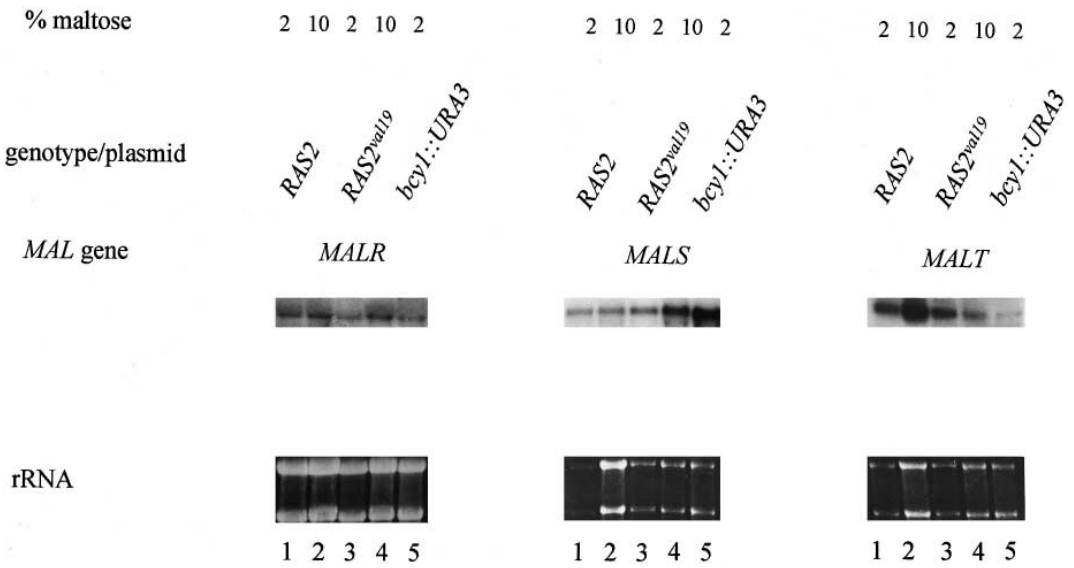

Fig. 1. Top panels show Northern blot analysis of *MAL* gene expression in JM2763-14 strains transformed with the pRAS2 plasmid (lanes 1,2) or with the pRAS2^{val19} plasmid (lanes 3,4) and in JM2763-14 *bcy1::URA3* strain (lane 5). Total RNA was prepared after growth on 0.1% glucose+2% maltose and on 10% maltose. The blots were probed with random primed *MALR*, *MALS* and *MALT* probes. Bottom panels show rRNA corresponding to each lane. 10 μg of total RNA were loaded on each lane for *MALS* and *MALT*, 50 μg for *MALR* analysis.

JM2763-14 [pRAS2^{val19}] compared to its isogenic counterpart. Accumulation of maltose permease may depend not only on its synthesis, but also on its degradation. Unlike maltase, in fact, maltose permease is known to be subject to carbon catabolite inactivation [3,5,25,26]. We thus investigated whether maltose permease inactivation was faster in JM2763-14 [pRAS2^{val19}] than in JM2763-14 [pRAS2]. The decrease in specific activity of maltose permease upon glucose addition is the result of both dilution – due to protein synthesis – and inactivation. Dilution is very different for wild type cells and JM2763-14 [pRAS2^{val19}] since whilst the wild type starts accumulating proteins almost immediately, JM2763-14 [pRAS2^{val19}] cells increase their cell mass only after approx. 30 min. Because of the initial absence of the dilution term, plotting of inactivation data in the usual manner, i.e. as % of initial specific activity, artefactually indicates that inactivation in JM2763-14 [pRAS2^{val19}] is slower than in wild type (Fig. 2A). When data are replotted as a difference between the actual data and those expected for dilution only, it can be appreciated that cells expressing the *RAS2*^{val19} allele inactivate maltose permease faster than cells expressing the wild type allele (Fig. 2A, insert). As a whole, the above-mentioned results indicate that deregulation of the RAS/PKA pathway affects maltose permease at both the transcriptional and post-transcriptional levels.

3.3. *Mutations in the FDP1/GGS1/TPS1 gene relieve carbon catabolite repression and inactivation of maltase and maltose permease*

Mutants in the *GGS1/TPS1* gene have been shown to display reduced glucose signalling [12]. We thus tested whether *ggs1/tsp1* mutations reduce catabolite repression of *MALS* and *MALT* expression in a defined *MAL1* background. Cells of strain MVY99 (*fdp1*, an allele of *ggs1/tps1*) transformed with centromeric plasmids carrying either the wild type *GGS1/TPS1* gene (pFDP1) or a suppressor gene allowing growth on glucose while not relieving the regulatory defects (pFPS1) were grown to mid-exponential phase in synthetic media supplemented with either 2% glucose, 2% maltose or 2% maltose+2% glucose. Table 2 shows that the *ggs1/tps1* mutation strongly decreases glucose repression of both maltase and maltose permease, but only when maltose is present in the medium. Results obtained by analyzing the expression of each *MAL* gene by Northern blotting are shown in Fig. 3 and are consistent with the enzymatic data. Analysis of glucose inactivation of maltose permease indicated that the *ggs1/tps1* mutation strongly stabilizes maltose permease. It was completely stable for at least 15 min, and retained as much as 30–50% of activity after 60 min in the presence of glucose while in the isogenic wild type strain only 0–5% of the initial activity remained (Fig. 2B).

Table 2
Effect of mutations in the *GGS1/TPS1* gene on catabolite repression of maltase and maltose permease

Plasmid	2% glucose		2% glucose+2% maltose		2% maltose	
	Maltase	Maltose permease	Maltase	Maltose permease	Maltase	Maltose permease
pFDP1	0.05	0.05	0.03	0.03	1.0	1.0
pFPS1	0.03	0.05	0.4	0.2	1.0	1.0

Maltase and maltose permease specific activities of MVY99 (relevant genotype *MAL1 ggs1/tps1*) transformants grown on indicated carbon sources were determined as described in Section 2.2. The values (average of at least two independent experiments, each conducted in duplicate and triplicate for maltase and maltose permease, respectively) are expressed as a fraction of either maltase specific activity (50–80 OD_{405}/min per mg protein) or [^{14}C]maltose uptake (800–1100 cpm/min per μg protein) of each strain grown in 2% maltose. Results of different experiments agreed within 10 and 20% for maltase and maltose permease, respectively.

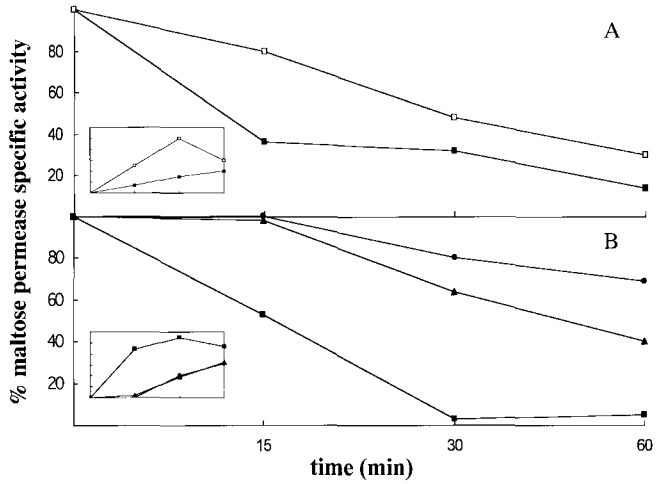

Fig. 2. Catabolite inactivation of maltose permease as a function of the time after addition of glucose (see Section 2) in JM2763-14 [pRAS2] (■), JM2763-14 [pRAS2^{val19}] (□) (A) and in MVY99 (*MAL1 ggs1/tps1*) untransformed (●), transformed with pFDP1 (■) or with pFPS1 (▲) plasmids (B). In the insets in A and B the fractional difference between the theoretical specific activity of maltose permease – expected according to dilution – and the actual measured specific activity is plotted as a function of time after glucose addition (time = 0).

4. Discussion

Since several laboratory strains have a poorly characterized *MAL* genotype and are often Mal$^-$, only few data are available on the role of the RAS/PKA pathway in maltose utilization. In particular, the inability of a *bcy1* strain to grow on maltose plates at 37°C has been reported, but the molecular basis of this defect was not further investigated [27]. We addressed this aspect by assaying enzyme activity and mRNA levels of *MAL* genes, *MALS*- and *MALT-lacZ* gene fusions and catabolite inactivation of maltose permease. Expression of maltase-encoding *MALS* is essentially unaffected by deregulated PKA activity, in keeping with the results of a previous study that failed to detect any effect of the RAS/PKA pathway on maltase expression [28]. *MALT* expression appears to be more affected than *MALS*; these results fit with the previously reported preferential sensitivity of *MALT* expression – compared to *MALS* – to alterations in the bidirectional UAS_{MAL} promoter [29]. In contrast, deregulated PKA severely reduces the V_{max} of maltose permease (as well as expression of *MALT* mRNA and *MALT-lacZ* fusions) under steady-state growth on maltose and decreases its half-life upon glucose addition. Although conflicting results on the role of the RAS/PKA pathway on steady-state levels and catabolite inactivation of sugar transporters have been reported [30,31], our data – being complemented by previously unavailable data on mRNA levels or promoter activity – strongly point towards a role for cAMP in regulation of maltose permease levels. Since maltose itself is a catabolite repressing sugar, maltose permease inactivation may be the primary maltose-related phenomenon brought about by deregulated activation of the RAS/PKA pathway. Because of the reduced levels of maltose permease activity, a secondary reduction in the intracellular maltose pool may arise, resulting in decreased transcription of the *MAL* genes.

A role for cAMP in catabolite inactivation of proteins was previously shown for soluble proteins (see for instance [32]). Our data regarding inactivation of the maltose transporter in the *RAS2*^{val19} mutant indicate a role for the RAS/PKA pathway in maltose permease activity and turnover, and suggest that PKA-dependent phosphorylation could be the first event leading to the reduced activity of maltose permease mediated by glucose and possibly other fermentable sugars. A potential phosphorylation site for PKA is present on maltose permease [5]. Moreover, a recent study reports that maltose permease is present in differentially phosphorylated forms in maltose-grown cells and that the hyperphosphorylated species accumulate in glucose-grown cells [26]. Whether maltose permease itself or an element(s) of the proteolysis machinery is the target of PKA remains to be seen. In at least one well-studied case, in fact, PKA-dependent phosphorylation of FBPase was shown to be dispensable for its eventual proteolysis [33–35].

Recently, it has been reported that glucose affects the expression of *MALT* and *MALS* genes through two independent mechanisms: a MIG1-dependent mechanism is operative in the presence and absence of maltose, whereas another MIG1-independent mechanism is operative only in the presence of maltose and may involve inhibition of the induction process [36]. Our results suggest that *GGS1/TPS1* may act through this second mechanism.

In conclusion, in keeping with data obtained for *SUC2* and *ADH2* expression [37,38], our data indicate that the RAS/

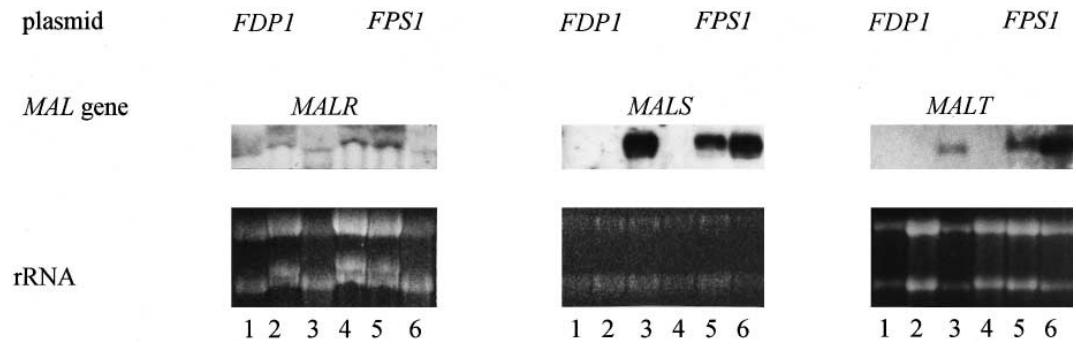

Fig. 3. Top panels show Northern blot analysis of *MAL* gene expression in MVY99 (*MAL1 ggs1/tps1*) transformed with the *GGS1*-bearing pFDP1 plasmid (lanes 1–3) or with the pFPS1 suppressor plasmid (lanes 4–6). Total RNA was prepared after growth on either 2% glucose (lanes 1,4), 2% glucose+2% maltose (lanes 2,5) or 2% maltose (lanes 3,6). The blots were probed with random primed *MALR*, *MALS* and *MALT* probes. Bottom panels show rRNA corresponding to each lane. 10 μg of total RNA were loaded on each lane for *MALS* and *MALT*, 50 μg for *MALR* analysis.

PKA pathway modulates glucose repression of *MAL* gene expression.

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