The Plant Cell, Vol. 7, 1963–1970, November 1995 

# The Lysine-Dependent Stimulation of Lysine Catabolism in Tobacco Seed Requires Calcium and Protein Phosphorylation

**Hagai Karchi, Daphna Miron, Sari Ben-Yaacov, and Gad Galili[1]**

Department of Plant Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

**The accumulation of free lysine in tobacco seed triggers the stimulation of lysine-ketoglutarate reductase, an enzyme that acts in lysine catabolism. The mechanism of lysine-ketoglutarate reductase stimulation was studied in two different systems: (1) developing seeds of wild-type plants in which the low basal lysine-ketoglutarate reductase activity can be stimulated by the exogenous addition of lysine; and (2) developing seeds of transgenic tobacco plants expressing a bacterial dihydrodipicolinate synthase in which lysine-ketoglutarate reductase activity is stimulated by endogenous lysine overproduction. In both systems, the stimulation of lysine-ketoglutarate reductase activity was significantly reduced when treated with the $Ca^{2+}$ chelator EGTA. Moreover, the inhibitory effect of EGTA was overcome by the addition of $Ca^{2+}$ but not $Mg^{2+}$, suggesting that the lysine-dependent activation of lysine-ketoglutarate reductase requires $Ca^{2+}$. This was further confirmed by a significant stimulation of lysine-ketoglutarate reductase activity following the treatment of wild-type seeds with ionomycin (an ionophore that increases $Ca^{2+}$ flow into the cytoplasm). In addition, treatment of wild-type seeds with the protein phosphatase inhibitor okadaic acid triggered a significant induction in lysine-ketoglutarate reductase activity, whereas treatment of the transgenic seeds with the protein kinase inhibitor K-252a caused a significant reduction in its activity. Thus, we conclude that the stimulation of lysine-ketoglutarate reductase activity by lysine in tobacco seed operates through an intracellular signaling cascade mediated by $Ca^{2+}$ and protein phosphorylation.**

## INTRODUCTION

Eukaryotic cells respond to various extracellular and intracellular stimuli by various cascades of signal transduction that generally involve $Ca^{2+}$ and $Ca^{2+}$ binding proteins as well as protein kinases and phosphatases. Such cascades mediate the induction or inhibition of many cellular processes, particularly cell proliferation and various metabolic pathways (Poovaiah and Reddy, 1987; Ferreira et al., 1994; Clapham, 1995). Regulation via signaling cascades is widespread in all organisms and so far has been studied mostly in carbohydrate and lipid metabolism (Nimmo et al., 1990; Alderson et al., 1991; Carter et al., 1991; Bakrim et al., 1992; Baur et al., 1992; Halford et al., 1992; Mackintosh et al., 1992; Jang and Sheen, 1994; Neumann et al., 1994; Saito et al., 1994).

The regulation of amino acid metabolism is also subjected to complex cellular and environmental stimuli (Cohen and Saint-Girons, 1987; Hinnebusch, 1988, 1992; Last, 1993; Galili et al., 1994; Galili, 1995). Among the various amino acids, the regulation of metabolism of the essential amino acid lysine has been studied extensively in bacteria, yeast, animals, and plants. In bacteria, lysine autoregulates its synthesis by two mechanisms: (1) feedback inhibition of the activity of aspartate kinase, the first enzyme in the lysine biosynthetic pathway from aspartate; and (2) repression of the gene encoding aspartate kinase (Cohen and Saint-Girons, 1987). In yeast, lysine regulates its own synthesis by repressing several of its biosynthetic enzymes (Hinnebusch, 1988). In addition, the biosynthesis of lysine and several other amino acids in yeast may also be coordinately regulated by various nutritional conditions (Hinnebusch, 1988, 1992). This general control (GCN) type of regulation in yeast cells operates via a specific transcription factor, GCN4, which binds to the promoters of many genes encoding enzymes in amino acid metabolic pathways (Hinnebusch, 1988, 1992). In plants, lysine autoregulates its synthesis by feedback inhibiting the activity of dihydrodipicolinate synthase (DHPS), the third enzyme in its biosynthetic pathway from aspartate. Yet, it is still not clear whether other regulatory mechanisms, such as gene repression, are also involved in lysine synthesis (Bryan, 1980; Galili, 1995).

We have recently identified a novel regulatory mechanism of lysine metabolism in developing tobacco seed. In this system, lysine regulates its own accumulation by stimulating the activity of lysine-ketoglutarate reductase (LKR), an enzyme that catabolizes lysine into saccharopine (Karchi et al., 1994). This pathway is seemingly restricted to seeds, inasmuch as tobacco leaves can accumulate large amounts of lysine (Shaul and Galili, 1992) and show no detectable LKR activity (Karchi et

[1] To whom correspondence should be addressed.

al., 1994). In this study, we present evidence that this novel regulatory process is mediated by $Ca^{2+}$ as a second messenger as well as by protein phosphorylation.

## RESULTS

In plants, LKR catalyzes the conversion of lysine plus α-ketoglutarate into saccharopine using NADPH. Hence, the mechanism of the lysine-dependent stimulation of LKR activity was studied in two different approaches. Using partially purified extracts from developing tobacco seed, we first assayed the activity of LKR by measuring the rate of conversion of NADPH to NADP. In some experiments throughout the study, the activity of LKR was confirmed by measuring the rate of saccharopine production. Each assay also included a control lacking the LKR substrate, lysine. As shown in Figures 1A and 1B, histograms 1 and 2, both approaches gave comparable results. In all cases, the activity of LKR in seeds of the "high-lysine" transgenic tobacco plant AE26, expressing the bacterial feedback-insensitive DHPS in a seed-specific manner, was substantially higher than that in seeds of wild-type tobacco. LKR from developing maize grains is feedback inhibited by saccharopine with a 50% inhibition ($I_{50}$) of 0.21 mM (Brochetto-Braga et al., 1992). Thus, to confirm that the activity measured in our study was specific for LKR, in some experiments an additional control assay containing 0.2 mM saccharopine was included. As shown in Figure 1A, histogram 3, the addition of saccharopine also inhibited the tobacco LKR activity. Based on the specificity of LKR activity measured by the conversion of NADPH to NADP, and in view of the similar results described in Figures 1A and 1B, we present in the following only the experiments using the NADPH conversion assay.

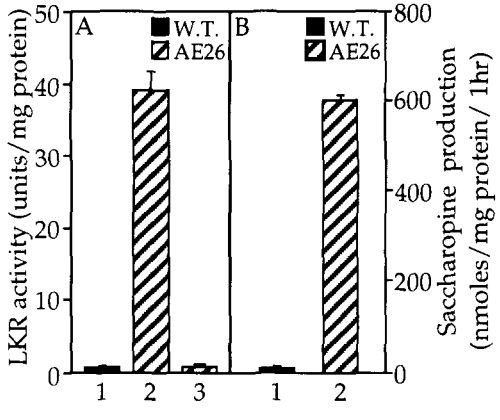


**Figure 1.** Analysis of LKR Activity in Partially Purified Extracts from Developing Tobacco Seeds.

**(A)** Analysis of LKR activity by the NADPH conversion assay.
**(B)** Analysis of LKR activity by the rate of production of its product, saccharopine.
LKR activity was measured in extracts from wild-type plants (histograms 1 in **[A]** and **[B]**) and from the high-lysine AE26 plants expressing bacterial DHPS in a seed-specific manner (histograms 2 and 3 in **[A]** and 2 in **[B]**). One of the reactions (histogram 3 in **[A]**) also included 0.2 mM saccharopine. The data represent four separate measurements of different extracts for all histograms, except for histogram 2 in **(A)**, which included 12 separate measurements. Error bars represent the standard errors of the mean. W.T., wild type.

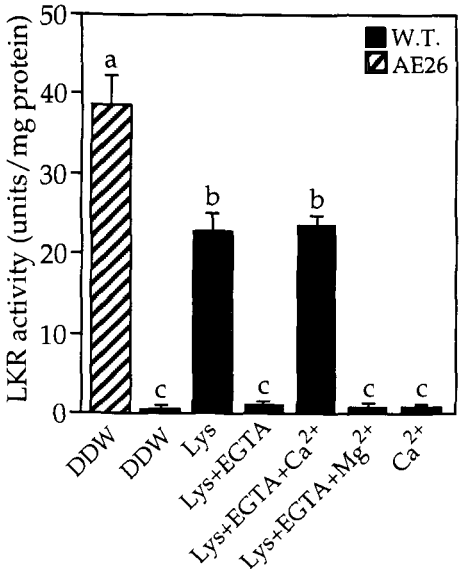


**Figure 2.** Effect of EGTA on the Lysine-Dependent Stimulation of LKR Activity in Wild-Type Seeds.

Developing pods from wild-type plants were injected with double-distilled water (DDW) or with Lys, Lys+EGTA, Lys+EGTA+$Ca^{2+}$, Lys+EGTA+$Mg^{2+}$, or $Ca^{2+}$, as indicated below the histograms. Chemical concentrations were 1 mM lysine, 10 mM EGTA, 50 mM $Ca^{2+}$ or $Mg^{2+}$. Seeds were harvested 24 hr after injection and analyzed for LKR activity. The data represent at least four separate measurements of different extracts for each treatment. LKR activity in seeds of the transgenic AE26 plants treated with double-distilled water is shown as a reference. Error bars represent the standard errors of the mean. Letters above error bars represent significant differences at the 5% level as determined by a Duncan test. W.T., wild type.

### The Role of $Ca^{2+}$ in the Lysine-Dependent Stimulation of LKR Activity

A common approach to studying the role of $Ca^{2+}$ in regulating cellular processes is to reduce its level by chelation with EGTA. Because plant and animal cells store millimolar amounts of $Ca^{2+}$ in their extracellular matrix (Poovaiah and Reddy, 1987, 1993; Clapham, 1995), generally millimolar amounts of EGTA are needed to inhibit $Ca^{2+}$-regulated responses in vivo (Carricarte et al., 1988; Kuznetsov et al., 1992; Raz and Fluhr, 1992). To determine whether EGTA affected LKR activity, we initially injected increasing millimolar concentrations of EGTA

into developing pods of tobacco and analyzed their effect on LKR activity. Whereas 1.0 and 2.5 mM EGTA did not exert any significant effect on LKR activity, 5 and 10 mM EGTA had a significant inhibitory effect (data not shown). We therefore selected the concentration of 10 mM EGTA in all subsequent experiments.

The effect of EGTA on the lysine-dependent stimulation of LKR activity was first studied in developing seeds of wild-type plants in which the stimulatory effect was obtained by exogenous application of the amino acid. As shown in Figure 2, EGTA inhibited significantly the stimulation of LKR activity by lysine. Moreover, the effect of EGTA was completely overcome by the addition of 50 mM $Ca^{2+}$ but not 50 mM $Mg^{2+}$. Treatment of developing seeds of wild-type plants with only 50 mM $Ca^{2+}$ did not cause any stimulatory effect on LKR activity. Next, we studied the effect of EGTA in developing seeds of the transgenic AE26 plant, in which lysine is overproduced endogenously as a result of the activity of the bacterial feedback-insensitive DHPS (Karchi et al., 1994). As shown in Figure 3, EGTA significantly reduced the endogenously stimulated LKR activity to nearly wild-type levels. In this case too, the effect of EGTA was overcome by the addition of 50 mM $Ca^{2+}$ but not 50 mM $Mg^{2+}$. Notwithstanding, although LKR activity in developing seeds

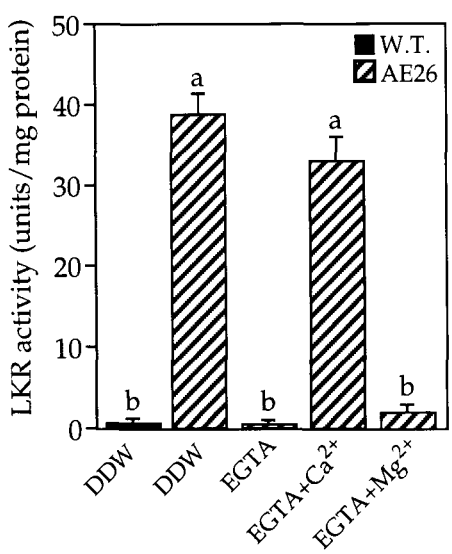


**Figure 3.** Effect of EGTA on LKR Activity in Seeds of the Transgenic AE26 Plant.

Developing pods were injected with double-distilled water (DDW) or with EGTA, EGTA+$Ca^{2+}$, or EGTA+$Mg^{2+}$, as indicated below the histograms. Chemical concentrations were 1 mM lysine, 10 mM EGTA, 50 mM $Ca^{2+}$ or $Mg^{2+}$. Seeds were harvested 24 hr after injection and analyzed for LKR activity. The data represent at least four separate measurements of different extracts for each treatment. LKR activity in wild-type seeds treated with double-distilled water is shown as a reference. Error bars represent the standard errors of the mean. Letters above error bars represent significant differences at the 5% level as determined by a Duncan test. W.T., wild type.

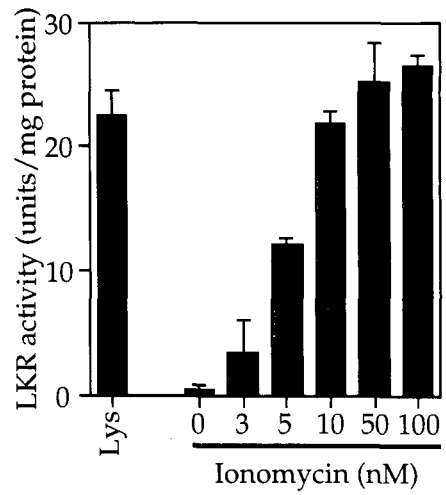


**Figure 4.** Effect of Ionomycin on LKR Activity in Seeds of Wild-Type Plants.

Developing pods were injected with increasing concentrations of ionomycin, as indicated below the histograms. Seeds were harvested 24 hr after injection and analyzed for LKR activity. The data represent at least three separate measurements of different extracts for each treatment. LKR activity in Lys-treated seeds is shown as a reference. Error bars represent the standard errors of the mean.

of wild-type plants could be stimulated by lysine injection, its activity never reached more than ~75% of that obtained in developing seeds of the transgenic AE26 plants (Figure 2). This was observed throughout our study and is discussed later.

The above-mentioned results suggest that the stimulation of LKR activity by lysine requires the release of $Ca^{2+}$ from storage compartments. This was tested by injecting developing pods from wild-type seeds with the $Ca^{2+}$ ionophore ionomycin, which transports $Ca^{2+}$ specifically through the cell membrane and thus increases its intracellular concentration (Liu and Hermann, 1978). As shown in Figure 4, increasing nanomolar concentrations of this ionophore had progressive stimulatory effects on LKR activity.

### The Role of Protein Kinases and Phosphatases in the Lysine-Dependent Stimulation of LKR Activity

Cellular signaling cascades generally involve the activation of various protein kinases or phosphatases (Ma, 1993; Bowler and Chua, 1994; Hunter, 1995). To test whether the stimulation of LKR activity was also operating through these enzymes, we conducted two types of experiments. In the first series of experiments, developing pods of the transgenic AE26 plant (in which LKR activity is stimulated endogenously) were treated with increasing concentrations of the broad protein kinase inhibitor K-252a. As shown in Figure 5, increasing nanomolar concentrations of this inhibitor caused a progressive decrease in the activity of LKR, which was reduced to nearly wild-type

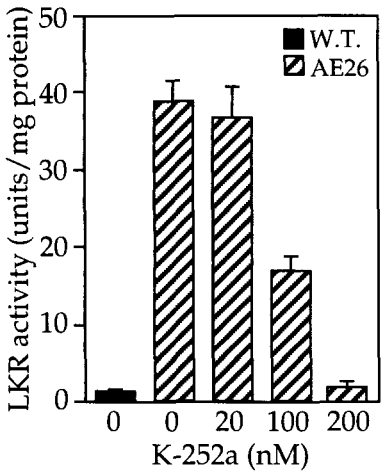


**Figure 5.** Effect of the Protein Kinase Inhibitor K-252a on LKR Activity in Seeds of the Transgenic AE26 Plant.

Developing pods were injected with increasing K-252a concentrations, as indicated below the histograms. Seeds were harvested 24 hr after injection and analyzed for LKR activity. The data represent at least three separate measurements of different extracts for each treatment. LKR activity in wild-type seeds treated with double-distilled water is shown as a reference. Error bars represent the standard errors of the mean. W.T., wild type.

levels by 200 nM K-252a. Thus, the involvement of protein phosphorylation in the lysine-dependent stimulation of LKR activity is evident.

Because the phosphorylation state of proteins is known to be conversely affected by protein kinases and protein phosphatases, we tested whether inhibition of protein phosphatases by itself could stimulate LKR activity. Thus, in the second set of experiments, developing pods of wild-type plants containing low lysine levels and hence possessing low LKR activity were injected with okadaic acid, a broad range inhibitor of PP1- and PP2A-type protein phosphatases (Cohen et al., 1990). As shown in Figure 6, increasing nanomolar concentrations of okadaic acid caused a progressive significant stimulation of LKR activity.

### Interaction of $Ca^{2+}$ and Protein Phosphorylation in the Stimulation of LKR Activity

We next studied whether $Ca^{2+}$ and protein phosphorylation operate independently or in a cooperative manner in regulating the lysine-dependent stimulation of LKR activity. Developing pods of wild-type plants were injected with 200 nM K-252a and 2 hr later with 50 nM ionomycin. Pods were harvested 24 hr after the first injection and analyzed for LKR activity. As shown in Figure 7, K-252a prevented the stimulatory effect of ionomycin on LKR activity.

### Effects of EGTA and K-252a on DHPS Activity

In the transgenic plant AE26, lysine overproduction results from the expression of a chimeric gene encoding the bacterial feedback-insensitive DHPS in a seed-specific manner (Karchi et al., 1994). We have shown previously that in developing seeds of this plant, the bacterial enzyme accounts for >90% of total DHPS activity between ~16 to 20 days after anthesis (Karchi et al., 1994). Thus, it was possible that the inhibitory effects of EGTA and K-252a on the stimulation of LKR activity were indirect, operating via their inhibitory effect on the expression of the bacterial gene encoding the feedback-insensitive DHPS, causing a significant reduction in the level of lysine. To test this hypothesis, we measured the effects of EGTA and K-252a on DHPS and LKR activities in extracts from the same seed batches of AE26 plants. As shown in Figures 8A and 8B, the two compounds significantly inhibited LKR activity but had no detectable effect on DHPS activity.

## DISCUSSION

In bacteria, yeast, and many types of plant cells, lysine controls its own synthesis and accumulation by feedback inhibiting or repressing the expression of specific enzymes involved in its biosynthesis. However, we have recently found that in tobacco seed, lysine controls its own accumulation by an additional novel regulatory mechanism involving the stimulation

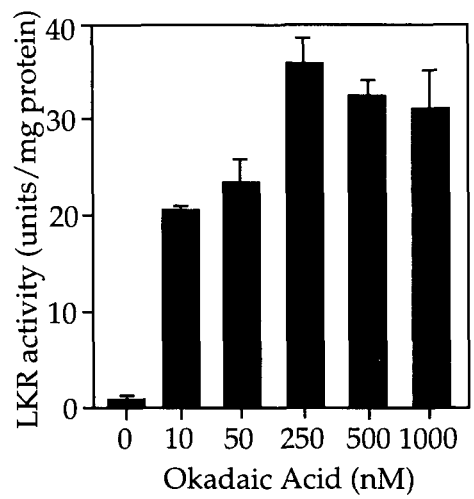


**Figure 6.** Effect of the Protein Phosphatase Inhibitor Okadaic Acid on LKR Activity in Seeds of Wild-Type Plants.

Developing pods were injected with increasing concentrations of okadaic acid, as indicated below the histograms. Seeds were harvested 24 hr after injection and analyzed for LKR activity. The data represent at least three separate measurements of different extracts for each treatment. Error bars represent the standard errors of the mean.

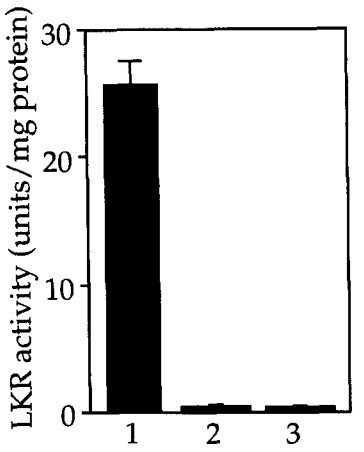


**Figure 7.** Combined Effect of the Protein Kinase Inhibitor K-252a and the $Ca^{2+}$ Ionophore Ionomycin on the Stimulation of LKR Activity in Developing Seeds of Wild-Type Plants.

Developing pods were injected with 50 nM ionomycin (histogram 1), 200 nM K-252a (histogram 2), or 200 nM K-252a and 2 hr later with 50 nM ionomycin (histogram 3). Pods were harvested 24 hr after the first injection and analyzed for LKR activity. The data represent at least four separate measurements for each treatment.

of LKR activity, an enzyme that acts in lysine catabolism. In this study, using the $Ca^{2+}$ chelator EGTA, the $Ca^{2+}$ ionophore ionomycin, the protein kinase inhibitor K-252a, and the protein phosphatase inhibitor okadaic acid, we showed that this stimulation occurs by a specific signaling cascade mediated by $Ca^{2+}$ and protein phosphorylation.

The concentrations of the various chemicals used in this study are within the ranges expected to affect $Ca^{2+}$ as well as protein phosphorylation and dephosphorylation signaling in vivo. The $Ca^{2+}$ level in the cytoplasm of plant cells is generally at submicromolar levels, and it is generally increased to micromolar levels when signaling cascades are activated (Poovaiah and Reddy, 1987, 1993; Clapham, 1995). However, the extracellular $Ca^{2+}$ level in plant cells is in the millimolar range (Poovaiah and Reddy, 1987, 1993; Clapham, 1995). Therefore, millimolar levels of EGTA are needed to chelate the extracellular $Ca^{2+}$ efficiently. Indeed, millimolar concentrations of EGTA are routinely used when studying $Ca^{2+}$-mediated signaling cascades in plants in vivo (Carricarte et al., 1988; Kuznetsov et al., 1992; Raz and Fluhr, 1992). However, ionomycin, which transports $Ca^{2+}$ specifically through the cell membrane, as well as the inhibitors okadaic acid and K-252a were active in our studies in nanomolar or submicromolar concentrations. This is in accord with the effective concentration ranges for these compounds in vivo (Liu and Hermann, 1978; Haystead et al., 1989, 1990; Cohen et al., 1990; Felix et al., 1991; MacKintosh and MacKintosh, 1994).

In all of the experiments involving wild-type seeds, the maximal activity of LKR obtained by all stimulatory treatments reached only ~75% of the level obtained in the transgenic AE26 plants. Possibly, LKR activity in the transgenic plants was turned on slightly before the time of injection, and hence, its basal level of activity was somewhat higher when compared with that of wild-type seeds at the injection time. At the time of injection, LKR activity in transgenic seeds was slightly higher when compared with that in wild-type seeds (data not shown). However, because the two activities were rather low, it was difficult to assess the significance of these differences.

Although many cellular processes are induced by $Ca^{2+}$ and protein phosphorylation, our results suggest that the inhibitory effects of EGTA and K-252a on the stimulation of LKR activity are rather specific. In seeds of the transgenic plants, the synthesis of the bacterial feedback-insensitive DHPS is driven by a phaseolin promoter, whose expression is turned on concomitantly with that of the tobacco gene encoding LKR,

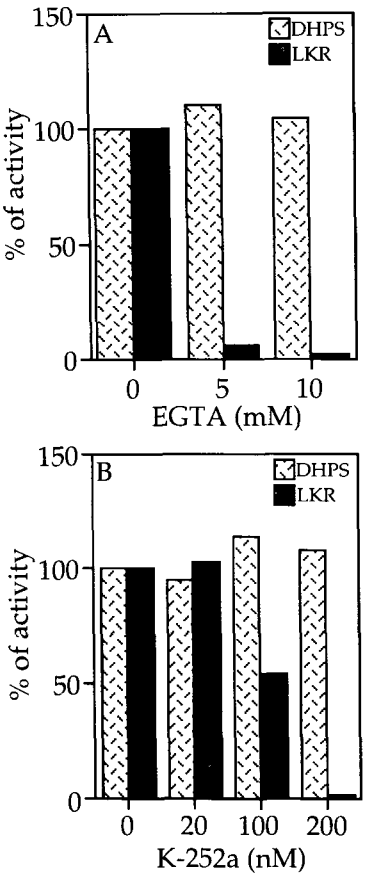


**Figure 8.** Effect of EGTA and K-252a on DHPS and LKR Activities in Developing Seeds of the Transgenic AE26 Plant.

**(A)** Developing pods were injected with increasing concentrations of EGTA, as indicated below the histograms.

**(B)** Developing pods were injected with increasing concentrations of K-252a, as indicated below the histograms.

Seeds were harvested 24 hr after injection. Extracts from each treatment were divided into two aliquots and analyzed for LKR and DHPS activities.

accounting for >90% of total DHPS activity between ~16 and 20 days after anthesis (Karchi et al., 1994). Therefore, if the effects of EGTA and K-252a were nonspecific, these chemicals would also be expected to inhibit the expression of the chimeric gene encoding the bacterial DHPS and, hence, cause significant inhibition of total DHPS activity and lysine level. This, however, was not the case, because neither of these compounds had any major effect on DHPS activity in developing seeds of the transgenic AE26 plant (Figures 8A and 8B). We have also analyzed the effect of EGTA and K-252a on the level of the bacterial DHPS mRNA in developing seeds of the transgenic AE26 plant. Although there was some variation in the level of this mRNA between individual plants, it was comparable between treated and nontreated seeds (data not shown).

The lack of any major influence of EGTA and K-252a on DHPS activity in seeds of the transgenic AE26 plant also suggests that their inhibitory effect on the stimulation of LKR activity was not due to a reduced rate of lysine synthesis. This is also supported by the following: (1) these two compounds had a similar inhibitory effect on LKR stimulation in wild-type seeds supplemented with exogenous lysine; (2) treatments of wild-type seeds, which contain low lysine levels (Karchi et al., 1994), with ionomycin and okadaic acid also stimulated the activity of LKR. Still, ionomycin and okadaic acid did not have any detectable effect on DHPS activity in the transgenic AE26 plant (data not shown).

The mechanism of the lysine-dependent stimulation of LKR activity in tobacco seed is still not known. It may result from direct phosphorylation of LKR by a $Ca^{2+}$-dependent protein kinase or from $Ca^{2+}$-mediated activation of a protein kinase that leads to a signaling cascade resulting in increased expression of the gene encoding LKR. Injections of K-252a 2 hr before that of ionomycin prevented the stimulatory effect of ionomycin on LKR activity, suggesting that $Ca^{2+}$ and protein phosphorylation operate in concert in this regulation. It is thus possible that $Ca^{2+}$ operates upstream to a protein kinase on a single signaling cascade, or that $Ca^{2+}$ and protein phosphorylation operate via two separate cascades that converge at a downstream step. Nonetheless, the stimulation of LKR activity is unlikely to operate via allosteric binding of lysine to the enzyme, because the addition of lysine into LKR assays in vitro had no effect on LKR activity (data not shown). Unraveling the fine mechanism of LKR stimulation awaits the purification of LKR, production of anti-LKR antibodies, and cloning of the gene encoding LKR.

The methodology of application of the various compounds into the developing seeds did not allow an accurate assessment of the time needed from the recognition of lysine as a stimulant to the actual stimulation of LKR. This is due to the apparent extended time lag between injection of the compounds into the pods and their actual penetration into the cells. Therefore, we believe that the duration from recognition to response is much shorter than the 24-hr incubation time used between injection and harvesting of the pods.

The fact that seeds can sense lysine as a stimulant for LKR activity is of particular interest, because it reflects the ability of this organ to regulate metabolic processes by monitoring the levels of metabolites in the cells. In response to nitrogen and sulfur availability, seeds can control the synthesis of specific proteins that are rich in glutamine and sulfur-containing amino acids (Muller and Knudsen, 1993; Morton et al., 1994). In addition, a cDNA encoding a specific protein kinase involved in carbon metabolism in seeds has also been cloned recently (Alderson et al., 1991). Thus, seeds may represent an attractive organ for studying signaling cascades that regulate metabolic processes.

The findings presented in this report are also of a broad practical and scientific interest. Lysine is an important essential amino acid that is most limiting in the major grain crops. Hence, inactivation of LKR activity may be a feasible route in the attempt to elevate lysine levels in plant seeds. LKR is also an important enzyme in mammalian cells. Defective production of LKR prevents the catabolism of free lysine and hence causes its accumulation in mitochondria. This defect is the underlying cause of the human genetic disease familial hyperlysinemia (Markovitz et al., 1984). In yeast cells as opposed to mammalian and plant cells, LKR functions in vivo in the synthesis rather than in the catabolism of lysine (Bhattacharjee, 1985). It is, therefore, of particular interest to determine whether the lysine-dependent stimulation of LKR activity is specific for plants or whether a similar mechanism operates in mammalian and yeast cells.

## METHODS

### Plant Material

The following plants were used in this study: wild-type *Nicotiana tabacum* cv Samsun and a transgenic "high-lysine" *N. tabacum* cv Samsun (previously designated AE26), expressing a bacterial feedback-insensitive dihydrodipicolinate synthase (DHPS) in a seed-specific manner (Karchi et al., 1994). Growth conditions were as described previously (Karchi et al., 1993). Different wild-type and transgenic plants were placed randomly in the greenhouse to minimize variations due to environmental factors.

### Application of the Different Compounds to Developing Pods

The peak of lysine-ketoglutarate reductase (LKR) activity during tobacco seed development varied between ~16 and 20 days after anthesis (Karchi et al., 1994), depending on environmental conditions. Notwithstanding, this peak was highly correlated with the stage at which seed color turned from white to light brown. This "breaking point" in seed color was used as a marker for tracing the peak of LKR activity during seed development. The various chemicals were injected into the developing pods ~24 hr before the breaking point. After an additional 24 hr, the pods were harvested and kept on ice. Seeds were separated from the pods and stored at −70°C until used. All injected compounds were calibrated to pH 7.0, except for ionomycin, which was calibrated to pH 8.0. Pods injected with double-distilled water were used as controls. Lysine, $Ca^{2+}$, and $Mg^{2+}$ were always injected at the respective concentrations of 1, 50, and 50 mM. The concentrations of the rest

of the chemicals are given in the legends of Figures 1 to 3 and 7. Ionomycin (free acid), K-252a, and okadaic acid were purchased from Calbiochem (La Jolla, CA).

### Preparation of Seed Extracts for Measuring Lysine-Ketoglutarate Reductase Activity

Extracts were prepared according to a previous procedure (Brochetto-Braga et al., 1992), with several modifications. All steps were performed at 4°C. Immature seeds were homogenized with a mortar and pestle in buffer A (100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM DTT, 15% glycerol, 1 μg/mL leupeptin). The homogenate was centrifuged for 30 min at 22,000*g*, and the supernatant was collected. Solid ammonium sulfate was slowly added to the supernatant until 60% saturation was reached. The supernatant was equilibrated for 20 min and centrifuged for 20 min at 22,000*g*, and the pellet was resuspended in buffer A. After an additional centrifugation, the supernatant was collected, and the protein concentration was measured according to Bradford (1976).

### Analysis of LKR Activity

LKR activity was performed essentially as previously described (Brochetto-Braga et al., 1992), with several modifications. Assays were performed at 30°C in a 0.3 mL reaction mixture containing 100 mM potassium phosphate buffer, pH 7.0, 20 mM L-lysine, 10 mM α-ketoglutarate, 0.1 mM NADPH, and 50 μg of protein extract. Conversion of NADPH to NADP was monitored by the reduction in $A_{340}$ at the linear range. One unit of LKR activity was defined as the amount of enzyme that catalyzed the oxidation of 1 nmol of NADPH per min at 30°C. Each extract was divided into two to provide for a control assay lacking the LKR substrate, lysine. Activity of each extract was calculated by subtracting the values of the control assay from the values in the assay that included lysine.

### Measurement of Saccharopine Content after LKR Activity Assays

After termination of the LKR assays, the reaction mixtures were used for measuring saccharopine content according to Simonson and Eckel (1985), with several modifications. Reactions (0.3 mL) contained 0.5 M glycine/0.4 M hydrazine, pH 9.0, 21 mL of 27 mM $NAD^+$, and 129 μL of the LKR assay. After a 5-min incubation at 30°C, 0.5 units (as defined by the manufacturer) of commercial yeast saccharopine dehydrogenase (lysine forming) was added, and the reaction proceeded until no additional saccharopine dehydrogenase activity was detected (Simonson and Eckel, 1985). The total amount of NADH formed was determined by absorbance at 340 nm. Saccharopine content in each sample was calculated from the amount of NADH formed, using 6.22 $mM^{-1}$ $cm^{-1}$ as the nanomolar extinction coefficient for NADH.

### Analysis of DHPS Activity

Developing seeds were homogenized with a mortar and pestle at 4°C in buffer B (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1.4% sodium ascorbate, 1 mM phenylmethylsulphonyl fluoride, and 0.5 μg/mL leupeptin). The homogenate was centrifuged for 30 min at 21,000*g* at 4°C, and the supernatant was collected and analyzed for protein concentration according to Bradford (1976). DHPS activity was assayed in crude seed extracts containing 100 μg of protein, using the *O*-aminobenzaldehyde method (Yugari and Gilvarg, 1965).

## ACKNOWLEDGMENTS

We thank Dr. Hillel From and Yigal Avivi for critical reading of the manuscript. This work was supported in part by a grant from the National Council for Research and Development, Israel, and the Gesellschaft für Biotechnologische Forschung MBH, Braunschweig, Germany; a grant from the Israel Science Foundation administrated by the Israel Academy of Sciences and Humanities; and the Leo and Julia Forchheimer Center for Molecular Genetics at the Weizmann Institute of Science. G.G. is an incumbent of the Bronfman Chair in Plant Sciences.

Received July 19, 1995; accepted September 18, 1995.

## REFERENCES

**Alderson, A., Sabelli, P.A., Dickinson, J.R., Cole, D., Richardson, M., Kreis, M., Shewry, P.R., and Halford, N.G.** (1991). Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA. Proc. Natl. Acad. Sci. USA **88,** 8602–8605.

**Bakrim, N., Echevarria, C., Cretin, C., Arrio-Dupont, M., Pierre, J.N., Vidal, J., Chollet, R., and Gadal, P.** (1992). Regulatory phosphorylation of sorghum leaf phosphoenolpyruvate carboxylase. Identification of the protein-serine kinase and some elements of the signal-transduction cascade. Eur. J. Biochem. **204,** 821–830.

**Baur, B., Dietz, K.J., and Winter, K.** (1992). Regulatory protein phosphorylation of phosphoenolpyruvate carboxylase in the facultative crassulacean-acid-metabolism plant *Mesembryanthemum crystallinum* L. Eur. J. Biochem. **209,** 95–101.

**Bhattacharjee, J.K.** (1985). α-Amino-adipate pathway for the biosynthesis of lysine in lower eukaryotes. Crit. Rev. Microbiol. **12,** 131–151.

**Bowler, C., and Chua, N.-H.** (1994). Emerging themes of plant signal transduction. Plant Cell **6,** 1529–1541.

**Bradford, M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72,** 248–254.

**Brochetto-Braga, M., Leite, A., and Arruda, P.** (1992). Partial purification and characterization of lysine-ketoglutarate reductase in normal and opaque-2 maize endosperms. Plant Physiol. **98,** 1139–1147.

**Bryan, J.K.** (1980). Synthesis of the aspartate family and branched-chain amino acids. In The Biochemistry of Plants, Vol. 5, B.J. Miflin, ed (New York: Academic Press), pp. 403–452.

**Carricarte, V.C., Bianchini, G.M., Muschietti, J.P., Téllez-inón, M.T., Perticari, A., Torres, N., and Flawia, M.M.** (1988). Adenylate cyclase activity in a higher plant, alfalfa (*Medicago sativa*). Biochem. J. **249,** 807–811.

**Carter, P.J., Nimmo, H.G., Fewson, C.A., and Wilkins, M.B.** (1991). Circadian rhythms in the activity of a plant protein kinase. EMBO J. **10,** 2063–2068.

**Clapham, D.E.** (1995). Calcium signaling. Cell **80,** 259–268.

**Cohen, G.N., and Saint-Girons, I.** (1987). Biosynthesis of threonine, lysine and methionine. In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, F.C. Neidhardt, ed (Washington, DC: American Society for Microbiology), pp. 429–444.

**Cohen, P., Holmes, C.F.B., and Tsukitani, Y.** (1990). Okadaic acid: A new probe for the study of cellular regulation. Trends Biochem. Sci. **15,** 98–102.

**Felix, G., Grosskopf, D.G., Regenass, M., and Boller, T.** (1991). Rapid changes of protein phosphorylation are involved in transduction of the elicitor signal in plant cells. Proc. Natl. Acad. Sci. USA **88,** 8831–8834.

**Ferreira, P., Hemerly, A., Van Montagu, M., and Inzé, D.** (1994). Control of cell proliferation during plant development. Plant Mol. Biol. **26,** 1289–1303.

**Galili, G.** (1995). Regulation of lysine and threonine synthesis. Plant Cell **7,** 899–906.

**Galili, G., Shaul, O., Perl, A., and Karchi, H.** (1994). Synthesis and accumulation of the essential amino acids lysine and threonine in seeds. In Seed Development and Germination, H. Kigel and G. Galili, eds (New York: Marcel Dekker), pp. 811–831.

**Halford, N.G., Vicenete-Carbajosa, J., Sabelli, P.A., Shewry, P.R., Hannappel, U., and Kreis, M.** (1992). Molecular analysis of a barley multigene family homologous to the yeast protein kinase gene *SNF1*. Plant J. **2,** 791–797.

**Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P., and Hardie, D.G.** (1989). Effects of the tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature **337,** 78–81.

**Haystead, T.A.J., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H., and Krebs, E.G.** (1990). Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. J. Biol. Chem. **265,** 16571–16580.

**Hinnebusch, A.G.** (1988). Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52,** 248–273.

**Hinnebusch, A.G.** (1992). General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*: Gene expression. In The Molecular and Cellular Biology of the Yeast *Saccharomyces*, E.W. Jones, J.R. Pringle, and J.R. Broach, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), pp. 319–419.

**Hunter, T.** (1995). Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. Cell **80,** 225–236.

**Jang, J.C., and Sheen, J.** (1994). Sugar sensing in plants. Plant Cell **6,** 1665–1679.

**Karchi, H., Shaul, O., and Galili, G.** (1993). Seed specific expression of a bacterial desensitized aspartate kinase increases the production of seed threonine and methionine in transgenic tobacco. Plant J. **3,** 721–727.

**Karchi, H., Shaul, O., and Galili, G.** (1994). Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. Proc. Natl. Acad. Sci. USA **91,** 2577–2581.

**Kuznetsov, G., Brostrom, M.A., and Brostrom, C.O.** (1992). Demonstration of a calcium requirement for secretory protein processing and export. J. Biol. Chem. **267,** 3932–3939.

**Last, R.L.** (1993). The genetics of nitrogen assimilation and amino acid biosynthesis in plants: Progress and prospects. In International Review of Cytology, Vol. 143, K.W. Jeon and M. Friedlander, eds (San Diego: Academic Press), pp. 297–330.

**Liu, C.-m., and Hermann, T.E.** (1978). Characterization of ionomycin as a calcium ionophore. J. Biol. Chem. **253,** 5892–5894.

**Ma, H.** (1993). Protein phosphorylation in plants: Enzymes, substrates and regulators. Trends Genet. **9,** 228–230.

**MacKintosh, C., and MacKintosh, R.W.** (1994). Inhibitors of protein kinases and phosphatases. Trends Biochem. Sci. **19,** 444–447.

**Mackintosh, R.W., Davies, S.P., Clarke, P.R., Weekes, J., Gillespie, J.G., Gibb, B.J., and Hardie, D.G.** (1992). Evidence for a protein kinase cascade in higher plants: 3-Hydroxy-3-methylglutaryl-CoA reductase kinase. Eur. J. Biochem. **209,** 923–931.

**Markovitz, P.J., Chuang, D.T., and Cox, R.P.** (1984). Familial hyperlysinemias. J. Biol. Chem. **259,** 11643–11646.

**Morton, R.L., Quiggin, D., and Higgins, T.J.V.** (1994). Regulation of seed storage protein gene expression. In Seed Development and Germination, H. Kigel and G. Galili, eds (New York: Marcel Dekker), pp. 103–138.

**Muller, M., and Knudsen, S.** (1993). The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. Plant J. **4,** 343–355.

**Neumann, G.M., Condron, R., Thomas, I., and Polya, G.M.** (1994). Purification and sequencing of a family of wheat lipid transfer protein homologues phosphorylated by plant calcium-dependent protein kinase. Biochim. Biophys. Acta **1209,** 183–190.

**Nimmo, H.G., Carter, P.J., Fewson, P.A., McNaughton, G.A., Nimmo, G.A., and Wilkins, M.B.** (1990). Regulation of phosphoenolpyruvate carboxylase: An example of signal transduction via protein phosphorylation in higher plants. Adv. Enzyme Regul. **30,** 121–131.

**Poovaiah, B.W., and Reddy, A.S.N.** (1987). Calcium messenger system in plants. Crit. Rev. Plant Sci. **6,** 47–103.

**Poovaiah, B.W., and Reddy, A.S.N.** (1993). Calcium and signal transduction in plants. Crit. Rev. Plant Sci. **12,** 185–211.

**Raz, V., and Fluhr, R.** (1992). Calcium requirement for ethylene-dependent responses. Plant Cell **4,** 1123–1130.

**Saito, Y., Vandenheede, J.R., and Cohen, P.** (1994). The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. Biochem J. **303,** 27–31.

**Shaul, O., and Galili, G.** (1992). Increased lysine synthesis in transgenic tobacco plants expressing a bacterial dihydrodipicolinate synthase in their chloroplasts. Plant J. **2,** 203–209.

**Simonson, M.S., and Eckel, R.E.** (1985). Enzymatic measurement of saccharopine with saccharopine dehydrogenase. Anal. Biochem. **147,** 230–233.

**Yugari, Y., and Gilvarg, C.** (1965). The condensation step in diaminopimelate synthesis. J. Biol. Chem. **240,** 4710–4716.