Expression of the maize *Cat3* catalase gene is under the influence of a circadian rhythm

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ABSTRACT The accumulation of *Cat3* catalase mRNA in the green leaves of maize (*Zea mays*) exhibits an unusual circadian rhythm. The steady-state level of the *Cat3* transcript varies dramatically over the day in seedlings grown on a 12-hr photoperiod. Low or undetectable levels of the *Cat3* mRNA are found late in the dark and early in the light period, while the transcript accumulates to high levels late in the light and early in the dark period. This dramatic diurnal variation in mRNA level does not occur with the maize *Cat1* or *Cat2* catalase transcripts. The diurnal fluctuation in *Cat3* mRNA persists when the seedlings are transferred to continuous light or darkness, which indicates the influence of a circadian rhythm. The lack of influence of red and/or far-red light on this diurnal variation in transcript level indicates that the circadian rhythm is not regulated by phytochrome. Run-on transcription assays show that changes in the rate of *Cat3* gene transcription closely parallel the observed changes in steady-state mRNA levels. This circadian rhythm in *Cat3* gene expression and the resulting high levels of mRNA which accumulate late in the light period suggest that the catalase 3 (CAT-3) isozyme is being synthesized for accumulation in the dark period. This, together with the high level of *Cat3* mRNA and CAT-3 protein accumulation in dark-grown maize shoots, suggests that the activity of the CAT-3 isozyme might be associated with a metabolic process important in shoot cells in the dark.

Among the important rhythmic behaviors which have been described in eukaryotic organisms are the endogenously generated daily cycles known as circadian rhythms (1, 2). Several characteristic properties are shared by all circadian rhythms, with the primary one being a periodicity of about 24 hr. Circadian rhythms are generally insensitive to changes in temperature, often having Q_{10} values near 1. Perhaps the most striking characteristic of a circadian rhythm is the fact that, once entrained by environmental stimuli, the rhythm will persist in the absence of further external cues (e.g., light and dark). Indeed, this feature is often used to establish that an observed diurnal rhythm is circadian in nature (1, 2).

Perhaps because of the fundamental dependence of photosynthesis on light and the resulting differences in plant metabolism in the light and dark, many physiological and biochemical processes in plants exhibit circadian rhythms (2). Ultimately, at least some of these rhythms are the result of periodic changes in plant gene expression (1, 2). This has been shown to be the case for several plant genes, including those encoding the chlorophyll a/b binding protein (Cab), the small subunit of ribulose-bisphosphate carboxylase/oxygenase, subunit II of the photosystem I reaction center, subunit I of the oxygen-evolving enzyme, and nitrate reductase (3–9). Among these genes the circadian rhythm in *Cab* gene expression is the most dramatic and best characterized. In general, the transcripts for these genes begin to accumulate slightly before the start of the light period, and high levels of the

transcripts are present early in the light period. Transcript levels then decline later in the light period to low levels early in the dark. Circadian rhythms with different patterns of transcript accumulation have not been described, especially for the expression of nonphotosynthetic, nonchloroplast-associated genes. The identification and characterization of genes whose expression is governed by circadian rhythms different than those exhibited by photosynthetic genes will give us a better understanding of both rhythmic phenomena and metabolic regulation in plants. In addition, the identification of such genes may aid in the identification and isolation of genes and factors which control the plants' circadian rhythms, such as has been the case in fungi and *Drosophila* (1, 10–12).

In maize (*Zea mays* L.), three biochemically distinct catalase isozymes ($H_2O_2:H_2O_2$ oxidoreductase, EC. 1.11.1.6, denoted CAT-1, CAT-2, and CAT-3) are encoded by the three unlinked structural genes *Cat1*, *Cat2*, and *Cat3*, respectively (13). These three genes have unique patterns of expression in the tissues of the developing maize seedlings, including the dark- and light-grown shoot (13, 14). The *Cat1* gene is "constitutively" expressed in that both the CAT-1 isozyme and *Cat1* transcript can be detected at low levels in all tissues throughout the development of light- and dark-grown seedlings (13–15). *Cat2* gene expression in shoots is positively regulated by light (14, 16). That is, the CAT-2 isozyme and *Cat2* transcript accumulate to high levels only in light-grown, green tissue. In contrast, the highest levels of the CAT-3 isozyme and *Cat3* mRNA are found in dark-grown shoot tissue (14). When maize seedlings are grown in the presence of light, the levels of both the CAT-3 isozyme and *Cat3* transcript decrease with development. Thus, in comparison with the *Cat2* gene, the *Cat3* gene might be viewed as being either positively regulated by darkness or negatively by light.

In this paper, we describe another unique feature in the expression of the maize *Cat3* gene, the characterization of a diurnal fluctuation in the steady-state level of the *Cat3* mRNA.

MATERIALS AND METHODS

Plant Material and Treatments. After an overnight imbibition in water, maize seeds (*Zea mays* L. var. W64A) were sown in a sand/peat mixture and grown in a chamber at 27°C with constant relative humidity of 80%. The plants were watered twice daily with a 1/10-strength Hoagland's solution. Light was provided by cool white fluorescent and incandescent lights (650 $\mu\text{mol}/\text{m}^2\cdot\text{sec}$ between 400 and 700 nm) for a 12-hr photoperiod beginning at 6 a.m. (0600). The design of the chambers was such that plants could be re-

Abbreviation: CAT, catalase.

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moved from a chamber without exposing the remaining seedlings to light. The seedlings were either treated with a continued 12 hr light/12 hr dark photoperiod or transferred to constant darkness or constant light as described in the figure legends beginning on day 6 after imbibition at 6 p.m. (1800). Leaf tissue was harvested 1 hr after the treatment was begun and, subsequently, every 3 hr for the next 72 hr. The tissue was frozen in liquid nitrogen immediately after harvest and stored at -70°C . Each of these treatments (light/dark, continuous light, and continuous darkness) was done on two independent sets of seedlings. Similar results were obtained for the replicate treatments.

Analysis of Catalase Transcript Levels. Total RNA was isolated from the leaf tissue as described (17). S1-nuclease protection analysis was used to determine the steady-state level of the *Cat1*, *Cat2*, and *Cat3* transcripts in 5 μg of total RNA as previously described (14). For each independently repeated treatment, the S1 protection analysis was done for duplicate extractions of total RNA. Data from representative experiments are shown.

Influence of Phytochrome. Maize plants were grown for 6 days after imbibition as described above. At the end of the light period on day 6 (1800), the plants were given a 15-min red-light treatment, a 15-min far-red-light treatment, or a 15-min red followed by a 15-min far-red-light treatment, then transferred to darkness. Plant tissue was harvested at 3-hr intervals over a 24-hr period. *Cat3* mRNA levels were analyzed as described above. Red light was provided by six 4-foot (1.2-m) cool white slimline fluorescent lights above a Rohm and Haas (Philadelphia) red Plexiglas filter. Far-red light was provided by four 150-W incandescent lamps above water and FRF-700 filters (Carolina Biological Supply).

Nuclear Run-on Transcription. Maize seedlings were grown 7 days after imbibition in a cabinet at 25°C with a 12-hr photoperiod provided by four cool-white fluorescent lamps. Nuclei were extracted from leaves by the method of Guilfoyle (18) and filtered through 80-, 50-, and 20- μm -pore nylon nets (18). After purification by centrifugation on an 82%/41%/30% Percoll step gradient, the nuclei were collected from the 82%/41% interface, washed, and stored (ref. 18; M. A. Conkling, personal communication). Run-on transcripts were synthesized in the presence of [^{32}P]CTP (19). The radioactive transcripts were then isolated by gel filtration on Sephadex G-100 in the presence of 10 mM Tris-HCl, pH 8/1 mM EDTA, followed by precipitation with ethanol. The level of specific transcript was determined by hybridization of 5×10^6 cpm/ml run-on transcription products with 5 μg each of

filter-bound pCat1.1 (20), pCat2.1 (21), pCat3.1 (20), or pUC12 plasmid DNA as described (22). Nonspecifically bound transcripts were washed away as described (22) and the hybridized transcripts were visualized by autoradiography.

RESULTS

When the steady-state level of *Cat3* mRNA was examined in 7- to 9-day-old maize leaves grown on a 12-hr light/12-hr dark cycle, the transcript level exhibited a clear pattern of diurnal variation (Fig. 1A). The transcript was present at detectable levels 1 and 4 hr into the dark period (1900 and 2100). The level of transcript declined through the next 9 hr, becoming undetectable by the beginning of the light period (0400 to 0700). The *Cat3* mRNA began to accumulate at 3 to 6 hr into the light period (1000 and 1300), with the highest level of the transcript present at about 10 hr into the light period (1600). This pattern was repeated every 24 hr over the 3-day experimental period.

When the steady-state levels of *Cat1* and *Cat2* transcripts were examined in the same samples, no dramatic variation was observed (Fig. 1B and C). In general, *Cat1* mRNA increased somewhat over the 3-day sampling period but did not vary with a 24-hr periodicity. For the *Cat2* mRNA, the level varied during the sampling period, although no periodic variation is obvious. In neither case did the variation in transcript level resemble the pattern seen for the *Cat3* transcript. It is possible that much of the variation observed in Fig. 1B and C is due to sample-to-sample variation, as it was not reproduced consistently between experiments (data not shown). In contrast, the large variation in transcript levels shown in Fig. 1A was observed without fail for each of four analyses (i.e., two independently derived sets of leaf tissue were analyzed in duplicate). Thus, the diurnal fluctuation in the *Cat3* mRNA was specific to this catalase gene.

To determine if the diurnal variation observed in *Cat3* mRNA levels was governed by a circadian rhythm, plants grown for 6 days on the light/dark cycle were transferred to continuous darkness or continuous light for the 3-day sampling period (Fig. 2). In the leaves of plants transferred to continuous darkness, the steady-state level of the *Cat3* mRNA continued to vary with a periodicity similar to that observed for plants continued on the light/dark cycle (Fig. 2A). However, during the last 36 hr of the sampling period, the *Cat3* transcript level no longer decreased to undetectable between 0100 and 0700 (cf. Figs. 1A and 2A). The accumu-

FIG. 1. Accumulation of catalase transcripts in the green leaves of maize grown with a 12-hr light period. After 6 days after imbibition, maize seedlings were continued on a 12-hr light period as described. Leaves were harvested at the times indicated and the steady-state level of *Cat3* (A), *Cat1* (B), or *Cat2* (C) transcript in 5 μg of total RNA was determined by S1-nuclease protection analysis. The data presented are from a representative experiment. The position of the protected fragment is indicated at the left of the figure. The time of sample harvest (on a 24-hr clock) is indicated above the figure and the harvest of samples during dark or light periods is indicated by a black or white band below the figure.

FIG. 2. Diurnal variation in *Cat3* transcript level in the leaves of maize seedlings transferred to continuous light or darkness. Maize seedlings were grown for 6 days after imbibition. At 1800 on day 6 after imbibition, the seedlings were transferred to a chamber maintained in continuous darkness (A) or continuous light (B). Samples were collected and the level of *Cat3* mRNA was determined by S1-nuclease protection analysis of total RNA. The position of the protected fragment is indicated at the left of the figure. The time of sample harvest (on a 24-hr clock) is indicated above the figure and the harvest of samples during continuous darkness (black band) or continuous light (white band) is indicated below the figure.

lation of *Cat3* mRNA during this period could be due to a "damping" of the rhythm in the absence of external cues (2). Alternatively, nongreen tissue began to accumulate at the base of the leaf blade during the 2 days of dark growth. Since the entire leaf blade was used in the analysis, the rapid damping of the rhythm might be due to the fact that *Cat3* mRNA accumulates to high levels in dark-grown leaves (14). In the leaves of plants transferred to continuous light, the diurnal variation in *Cat3* transcript level also continued for at least 48 hr (Fig. 2B). In these plants, a fairly rapid damping of the 24-hr variation in transcript levels was apparent by the second day of sampling. The *Cat1* transcript levels in plants treated with continuous darkness or light were similar to those of plants maintained on a light/dark cycle. In contrast, the level of *Cat2* mRNA in plants transferred to continuous light decreased slightly and the level of *Cat2* transcript in seedlings placed in continuous darkness decreased substantially over the 72-hr sampling period with no noticeable periodicity (data not shown). The persistence of the variation of *Cat3* mRNA levels in the absence of external light cues indicated that this phenomenon is controlled by a circadian rhythm (1, 2).

When light influences the expression of a plant gene, the question arises whether the plant pigment phytochrome is involved in the regulation of the gene (23). The influence of phytochrome on the circadian rhythm in *Cat3* mRNA accumulation was examined by treating 6-day-after-imbibition maize seedlings with red light, far-red light, or red light followed by far-red light prior to the dark sampling period (Fig. 3). The pattern of *Cat3* transcript accumulation in these three treatments did not differ from that found in plants grown on a 12-hr light/12-hr dark cycle (cf. Fig. 3 with Fig. 1A). There appears to be a small decrease in the *Cat3* mRNA level in the leaves of red-light-treated plants. However, this quantitative difference is small and may well fall within the error of the detection method. Thus, phytochrome did not influence the circadian rhythm in *Cat3* transcript accumulation, although it may have a small negative influence on the level of the mRNA.

To determine whether the changing steady-state levels of *Cat3* mRNA in the experiments described above were reflected in corresponding changes in the rate of gene transcription, the rate of *Cat3* gene transcription in maize leaves was estimated by run-on transcription assays of leaf nuclei isolated at four times during the diurnal period (Fig. 4). The level of *Cat3* transcripts in the run-on mixture was greatest 7 hr into the light period (1300). Subsequently, the level declined at 1 and 7 hr into the dark period. The lowest rate of *Cat3* gene transcription in this experiment was found 1 hr into the light period (0700). A very low level of hybridization with the *Cat1* cDNA was detected in the mixture of run-on transcripts. Longer exposure of the autoradiogram showed

that the level of *Cat1* run-on transcripts was approximately the same at all four times (data not shown). Some minor variation in the level of run-on transcripts hybridizing with *Cat2* cDNA was observed (0700), but, in a replicate experiment, a similar level of *Cat2* transcripts was observed at all four times. Therefore, the rate of transcription of the *Cat3* gene varied specifically in a manner consistent with the observed variation in *Cat3* mRNA levels.

DISCUSSION

The data presented show that the accumulation of *Cat3* mRNA exhibits an unusual diurnal pattern in the leaves of maize seedlings, with high levels of transcript accumulating late in the light period. This pattern of transcript accumulation is specific to *Cat3*, as indicated by the fact that neither *Cat1* nor *Cat2* mRNA levels show a clear diurnal pattern of variation. In addition, this lack of fluctuation in *Cat1* and *Cat2* transcripts suggests that the observed pattern of *Cat3* mRNA accumulation is not due to a general variation in leaf transcript levels during the day. Most, if not all, of this diurnal variation in *Cat3* mRNA levels can be attributed to changes in the rate of transcription of the *Cat3* gene. Here, too, the absence of variation in the relative rates of *Cat1* and *Cat2* transcription implies that the changes shown for *Cat3*

FIG. 3. Effect of phytochrome on the diurnal variation in *Cat3* mRNA levels. At 1800 on day 6 after imbibition maize seedlings were treated with red, far-red, or red followed by far-red light (12). The plants were then transferred to continuous darkness for the 24-hr sampling period. The level of *Cat3* mRNA was determined by S1-nuclease protection analysis of 5 μ g of total RNA. The position of the protected fragment is indicated at the left of the figure. The time of sample harvest (on a 24-hr clock) is indicated above the figure and the harvest of samples during a dark period is indicated by a black band below the figure.

FIG. 4. Rate of *Cat3* gene transcription in the leaves of maize seedlings grown on a 12-hr photoperiod. The relative rate of transcription of the *Cat1*, *Cat2*, and *Cat3* genes in the leaves of maize harvested at 6 hr into the light period (1300), 1 hr into the dark period (1900), 7 hr into the dark period (0100), or 1 hr into the following light period (0700) was determined by run-on transcription analysis. Run-on transcripts did not bind to plasmid DNA (pUC12) at detectable levels.

cannot be attributed entirely to general shifts in leaf transcription rates. In tomato leaves, the relatively invariant rate of transcription of several "constitutive" genes, including those encoding actin, subunit II of the photosystem I reaction center, and a biotin-binding protein of unknown function, also indicates that leaf transcription rates do not vary widely over the day (4).

Continuation of the periodic variation in *Cat3* mRNA accumulation when the seedlings were transferred to continuous darkness or continuous light, under otherwise identical conditions, shows that the transcript level variation has a circadian rhythm (1, 2). The "damping" of the fluctuation in the absence of external light cues is typical of a circadian rhythm (2). A similar, although less rapid, damping was observed for *Cab* mRNA levels under similar conditions (3–5). Another feature of circadian phenomena is that the frequency of the period often varies in the absence of external light cues (2). This was not observed with the rhythm in *Cat3* levels, perhaps due to the spacing of harvest times and the 48- or 72-hr sampling period. Under similar conditions, no change in the frequency of the circadian rhythm in *Cab* gene expression was found (3–5). Like the circadian rhythm in *Cab* gene expression, the *Cat3* rhythm does not appear to be influenced by phytochrome (5).

While the circadian rhythm in *Cat3* expression shares certain features with those for *Cab* and other photosystem-associated genes, the regulation of *Cat3* expression is unique in several ways. First, the patterns of diurnal variation in mRNA level for the *Cat3* and photosystem-associated genes relative to the light cycle are nearly opposite. That is, the *Cab* transcript level increases late in the dark and early in the light period, when the *Cat3* mRNA levels are lowest. In addition, the highest levels of the *Cat3* transcript occur late in the light period, when minimal *Cab* transcript is present. However, it is interesting to note that the mRNA levels for two plastid-encoded genes (*rbcL* and *psbA*), as well as tubulin, show a diurnal variation similar to that for *Cat3* (3). Secondly, the *Cab*, photosynthetic, and nitrate reductase genes are all positively regulated by light and phytochrome (5, 23, 24). These transcripts are present at low levels in etiolated shoots and accumulate in response to red-light treatment. In contrast, the *Cat3* mRNA accumulates to highest levels in dark-grown shoots (14). Although the influence of phytochrome on the expression of *Cat3* in etiolated tissue is not known, the data presented here suggest that phytochrome may have some negative influence on gene expression. When plants grown on a light/dark cycle are transferred to continuous darkness, the steady-state level of the *Cat3* transcript increases, while the *Cab* transcript level decreases dramatically (5). Thus, the light and diurnal period appear to influence the expression of *Cat3* in a manner nearly opposite to that of the *Cab* and most other photosystem-related genes.

By analogy, if the transcripts of these photosynthetically associated genes accumulate such that their encoded proteins are produced for photosynthetic activity in the light, then the CAT-3 isozyme might be thought of as accumulating for a metabolic process important late in the light or early in the dark period.

Light also affects the expression of the three maize catalase genes in different ways (13, 14, 16). While each of the catalases is almost certainly involved with the degradation of intracellular hydrogen peroxide, this differential regulation of the genes by light suggests that each isozyme fulfills a different metabolic role in the maize shoot. There is increasing evidence that the expression of the *Cat1* gene is "constitutive" and the CAT-1 isozyme may be present in the peroxisomes of all maize tissues at low levels (14, 15). Thus, CAT-1 may serve a general role in H_2O_2 degradation. The association of CAT-2 protein with maize leaf bundle-sheath cells (25) and the positive effect of light on *Cat2* gene expression (14, 16) suggest that it is associated with the degradation of H_2O_2 generated during the oxidation of photosynthetically produced glycolate in the shoot (26). In contrast, the CAT-3 protein is localized in the mesophyll of the green maize leaf (25). The tissue- and cell-type-specific distribution of the CAT-3 isozyme and *Cat3* transcript and the unusual temporal pattern of *Cat3* mRNA accumulation suggest that CAT-3 plays a specific, although as yet undefined, role in the degradation of H_2O_2 in the mesophyll of maize shoots in the dark.

An intriguing possibility is that *Cat3* expression is negatively regulated by photosynthetic activity, perhaps through the action of some light-sensitive trans-acting transcription factor. Further study of the molecular events which influence the expression of *Cat3* could provide valuable information on factors and genes that influence light regulation and circadian rhythms in plants.

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