Photoregulation of the Cat2 and Cat3 Catalase Genes in Pigmented and Pigment-Deficient Maize: The Circadian Regulation of Cat3 Is Superimposed on Its Quasi-Constitutive Expression in Maize Leaves

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ABSTRACT

We have investigated the accumulation of Cat2 and Cat3 catalase transcripts in 6–7-day postim- bition leaves of normally pigmented and pigment-deficient maize seedlings under different light regimes. In seedlings of normal inbred maize lines Cat2 mRNA accumulates to significantly higher levels in either continuous light or a diurnal light/dark cycle than in continuous dark. In contrast to the high levels of the Cat2 message observed in their wild-type siblings, carotenoid-deficient mutants accumulate Cat2 mRNA at barely detectable levels. Mutants deficient in chlorophylls, but having normal carotenoid levels, accumulate normal levels of Cat2 mRNA. This suggests that both light and carotenoids are required for the normal accumulation of the Cat2 message. The steady-state level of Cat3 mRNA exhibits a dramatic diurnal variation when seedlings are grown under a 24-hr light/dark cycle. We have previously shown that this variation is at the level of Cat3 gene transcription and is under the control of a novel circadian rhythm. In this study we show that both pigment-deficient mutants and their wild-type siblings exhibit the normal diurnal pattern of Cat3 RNA accumulation. This indicates that photosynthetic pigments, allelic variation, and genetic background do not directly affect the temporal pattern of Cat3 accumulation in leaves. We observed, however, that when normal plants are grown in either continuous light or continuous dark, the Cat3 transcript in leaves is present at uniformly high levels throughout the 24-hr sampling period. Because the Cat3 gene is continually transcribed in leaves in the absence of a cyclic light regime, the normally observed diurnal variation of Cat3 gene expression is apparently the result of a circadian-regulated transcriptional repressor.

CATALASE (H₂O₂:H₂O₂ oxidoreductase, EC 1.11.1.6; CAT) is a tetrameric, heme-containing enzyme that catalyzes the dismutation of H₂O₂ into H₂O and O₂. In maize (Zea mays L.) three unlinked catalase structural genes, Cat1, Cat2 and Cat3, encode three biochemically distinct isozymes, CAT-1, CAT-2 and CAT-3 (SCANDALIOS 1965, 1968; ROUPAKIAS, McMILLIN and SCANDALIOS 1980). In addition to exhibiting differential temporal and spatial patterns of expression (SCANDALIOS 1979; REDINBAUGH, SABRE and SCANDALIOS 1990a), the three maize catalase genes respond differentially to light in developing maize leaves (SCANDALIOS 1979; SKADSEN and SCANDALIOS 1987; REDINBAUGH, SABRE and SCANDALIOS 1990a).

CAT-1 protein and Cat1 transcript accumulate at low levels in the mesophyll of maize leaves throughout development (REDINBAUGH, SABRE and SCANDALIOS 1990a). This accumulation appears to be light independent. In contrast, both Cat2 and Cat3 respond to light, although in opposite ways. The Cat2 gene appears to be positively regulated in a relatively simple manner by light. CAT-2 protein and Cat2 mRNA accumulate in the bundle sheath cells of shoots grown either in a light/dark regime or in constant light (TSAFARIS, BOSABALIDIS and SCANDALIOS 1983). CAT-2 protein and Cat2 mRNA levels are low or not detected in young leaves grown in constant dark. The CAT-2 isozyme is dramatically induced in leaves when dark-grown seedlings are exposed to light (SKADSEN and SCANDALIOS 1987). The positive light response of the Cat2 gene is not phytochrome mediated (SKADSEN and SCANDALIOS 1987). Although some fluctuation in Cat2 mRNA levels is seen during the diurnal cycle, these variations are relatively small and do not exhibit a circadian rhythm (REDINBAUGH, SABRE and SCANDALIOS 1990b).

In contrast, Cat3 gene products are present in mesophyll of young light/dark-grown leaves, and accumulate to high levels in constant dark. We recently described a novel circadian regulation of the Cat3 gene in developing maize leaves (REDINBAUGH, SABRE and SCANDALIOS 1990b). Most circadian phenomena previously described in plants at the molecular level involve the regulation of chloroplast-associated gene products directly involved in photosynthesis (GIULIANO et al. 1988; NAGY, KAY and CHUA 1988; STAYTON, BROSIO and DUNSMUIR 1989; TAYLOR 1989; HERRERA-ESTRELLA and SIMPSON 1990). The Cat3 gene product is not associated with chloroplasts, nor

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does it appear to be regulated positively by phytochrome (REDINBAUGH, SABRE and SCANDALIOS 1990b). The transcriptionally mediated accumulation of Cat3 RNA late in the photoperiod, together with its localization in the leaf mesophyll, suggested that CAT3 protein might play a role in non-photosynthetic reactions in the dark.

Because phytochrome does not seem to have a positive regulatory effect on either Cat2 or Cat3 gene expression, we wanted to determine if the presence or absence of other photosynthetic pigments influence Cat2 or Cat3 RNA accumulation. In addition, because phytochrome does not appear to mediate "setting" the light-regulated circadian rhythm exhibited by Cat3, we would like to know if other photosynthetic pigments are involved. Using S-1 nuclease protection and Northern blot analyses, we examined the accumulation of Cat2 and Cat3 gene products under various light regimes in both pigment-deficient and normal inbred lines of maize. Our results suggest that whereas normal Cat2 mRNA accumulation is dependent on the presence of normal levels of carotenoids and/or chlorophylls, accumulation of Cat3 transcript is normal in the absence of both. In addition, we present evidence that the diurnal variation in Cat3 RNA accumulation is the result of the circadian-regulated cyclic repression of Cat3 gene expression.

MATERIALS AND METHODS

Materials: Zea mays L. inbred line W64A is maintained in our laboratory. Line IDS-28 (Cat3 null) (WADSWORTH and SCANDALIOS 1990) was initially provided by M. GOODMAN and is presently maintained in this laboratory. The lemon white (lw^3) mutant was obtained from Carolina Biological Supply Company, and the albino (w^3) and luteus (l^-195) mutants were provided by M. G. NEUFFFER, University of Missouri. Plasmid pBT515, containing a full-length RbeS cDNA sequence, was a gift of T. NELSON, Yale University.

Plant growth conditions: Plants utilized in this study were grown at the Southeast Plant Environmental Laboratories (Phytotron) at North Carolina State University. Plants were grown in continuous light (140–165 μEms^-1m^-2), in continuous dark or under a 24-hr photoperiod (12-hr light/12-hr dark). Following overnight imbibition in water, seeds to be grown in constant light or constant dark were placed on top of moistened germination pads, and grown in transparent germination boxes. This ensured that leaves were exposed to constant light or constant dark from the earliest stages of growth. For one set of analyses, 6-day postimbibition W64A leaves were grown under a 24-hr photoperiod and collected at 3-hr intervals over a 48-hr period. Samples were collected beginning at 7 p.m. (1900 hr), or one hour into the dark period. For all other analyses samples were collected at four representative time points, spaced at 6-hr intervals over the 24-hr period beginning at noon on day 7. After harvest, leaves were frozen in liquid nitrogen and stored at −70°.

Zymogram analysis: Starch gel electrophoresis using a Tris-Citrate buffer system was performed as previously described (SCANDALIOS 1968), except that insoluble polyvinylpyrrolidone was added to the extraction buffer to adsorb
FIGURE 1.—Accumulation of Cat2 catalase message in the leaves of pigmented and pigment-deficient lines of maize. Relative levels of Cat2 mRNA in the total RNA of leaves of normally pigmented (A) and pigment-deficient mutants and their wild-type siblings (B) were determined by S-1 nuclease protection analysis. Lines used are listed in Table 1. Plants were grown for 7 days in continuous light (LL), continuous dark (DD) or under a 24-hr photoperiod (12-hr light/12-hr dark) (LD) as indicated by the black and/or white bars below each set of samples. Samples were collected every 6 hr over a 24-hr period, beginning at noon on day 7. The time of sampling (on a 24-hr day) is indicated above each lane. Note that Cat2 mRNA accumulated to high steady-state levels in the leaves of all plants containing normal levels of carotenoids (W64A LD, W64A LL, IDS-28 LD, l-195/-195, and the wild-type siblings W3/-, Lu3/- and l-195/-). In contrast, neither leaves of dark-grown W64A nor the carotenoid-deficient mutants w3/w3 and lu3/lu3 accumulate significant levels of Cat2 message.

FIGURE 2.—Accumulation of RbcS mRNA in the leaves of pigmented and pigment-deficient lines of maize. Relative levels of RbcS message in the total RNA of leaves of normally pigmented (A) and pigment-deficient mutants and their wild-type siblings (B) were determined by Northern blot analysis. Seedlings were grown for 7 days postimbibition in either continuous dark (DD) or under a 24-hr photoperiod (12-hr light/12-hr dark) (LD) as indicated by the black and/or white bars below each set of samples. Leaves were collected at 6-hr intervals beginning at noon on day 7. As expected, RbcS message accumulated to high levels in the normally pigmented line W64A and in the wild-type siblings of both carotenoid- and chlorophyll-deficient mutant lines (W3/- and l-195/-, respectively), but at reduced levels in leaves of dark-grown W64A and both pigment-deficient mutants w3/w3 and l-195/l-195.

cient mutant lines of maize were used in this study. The pigment-deficient mutants fall conveniently into two classes: (1) lines completely deficient in both chlorophylls and carotenoids (e.g., lemon white, lu3; and albino, w3) (Mayfield et al. 1986) and (2) lines deficient only in chlorophylls (e.g., luteus, l-195). A summary of the mutant phenotypes used in this study is presented in Table 1. The genes involved in the carotenoid-deficient phenotypes have been mapped to separate chromosomes (Hoisington, Coe and Neuffer 1988), indicating they are the result of independent mutational events. Progeny tests confirmed Mendelian segregation for each mutant (data not shown). Zymogram analysis was used to determine the allelic variant of the CAT-3 isozyme present in each line, and levels of photosynthetic pigments in each line were determined by spectrophotometric analysis (Table 1).

The steady-state level of the Cat2 message is low in the leaves of carotenoid-deficient mutants: To determine if either chlorophylls or carotenoids are required for the normal accumulation of Cat2 mRNA in developing maize leaves we examined steady-state levels of Cat2 message at various times of the day in the leaves of 6-7-day postimbibition mutant and wild-type seedlings. Total RNA was extracted from leaves of the normal inbred line W64A grown under various light regimes, as well as from leaves of the Cat3-null line IDS-28, and various pigment-deficient mutants.
and their wild-type siblings. Relative steady-state levels of Cat2 mRNA were determined by S-1 nuclease protection analysis. Levels of the positively light-regulated RbcS mRNA (Smith and Ellis 1981) were determined by Northern analysis for comparison.

All plants having leaves with normal levels of both chlorophylls and carotenoids (see Table 1) accumulated normal levels of Cat2 and RbcS mRNAs at all sampling points throughout the diurnal cycle (Figures 1 and 2). In contrast, levels of both Cat2 and RbcS mRNAs were barely detectable in leaves of the totally pigment-deficient mutants, w3 and lw3, or in leaves of the dark-grown (etiolated) W64A controls. In contrast, the chlorophyll-deficient mutant l-195, which makes normal levels of carotenoids but reduced levels of chlorophylls, accumulated normal levels of Cat2 mRNA, but reduced levels of RbcS message. The wild-type siblings of all pigment-deficient mutants accumulate normal levels of both Cat2 and RbcS RNA. This suggests that normal biosynthesis or accumulation of carotenoids is in some manner necessary for the normal light-induced accumulation of the Cat2 message.

Both pigment-deficient mutants and their wild-type siblings exhibit normal patterns of Cat3 RNA accumulation in leaves: The steady-state level of Cat3 RNA exhibits a novel circadian rhythm when plants are grown under a 24-hr light/dark cycle. Sampled at eight time points over the 24-hr period, the highest levels of Cat3 RNA are observed late in the light period, with levels thereafter declining to a minimum late in the dark period (Figure 3). This dramatic diurnal variation has been shown to be controlled at the level of transcription (Redinbaugh, Sabre and Scandalios 1990b). For these studies four representative time points, two late in the light period and two late in the dark, were utilized to analyze Cat3 accumulation patterns (Figure 3).

In contrast to the pigment-dependent nature of Cat2 RNA accumulation, steady-state levels of Cat3 RNA were essentially the same for the normal inbred line (Figure 3), as well as the pigment-deficient mutants and their wild-type siblings (Figure 4). As expected, the Cat3 null line IDS-28 showed no detectable Cat3 RNA accumulation (Wadsworth and Scandalios 1990; Figure 5).

The steady-state level of Cat3 is continuously high in leaves of seedlings grown in continuous light or continuous dark: To further define the parameters affecting the circadian regulation of Cat3 gene expression in developing maize leaves, seedlings of the normal inbred line W64A were grown under several different light regimes.

In plants grown under a normal 24-hr photoperiod (12-hr light/12-hr dark) we observed the expected diurnal pattern of Cat3 RNA accumulation, with Cat3 RNA levels reaching a maximum late in the light period (1800/6 p.m.), decreasing thereafter to a minimum late in the dark period (0600/6 a.m.) (Figures 3 and 5 and Redinbaugh, Sabre and Scandalios 1990b). In addition, seedlings grown in constant dark had high steady-state levels of Cat3 RNA throughout the 24-hr sampling period as previously observed (Redinbaugh, Sabre and Scandalios 1990a). When seedlings were grown in constant light, however, Cat3

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**Figure 3.**—Abbreviated sampling regime used to show diurnal variation in Cat3 RNA accumulation in developing maize leaves. The relative steady-state levels of Cat3 transcript in the total RNA of leaves of the normal inbred maize line W64A were determined by S-1 nuclease protection analysis. For one set of analyses, 6-day postinhibition W64A seedlings were grown under a 24-hr photoperiod (12-hr light/12-hr dark) (LD) and leaves collected at 3-hr intervals over a 48-hr period. Samples were collected beginning at 7 p.m. (1900 hr), or one hour into the dark period. Alternatively, samples were collected at four representative time points, spaced at 6-hr intervals over the 24-hr period beginning at noon on day 7. The relative steady-state level of Cat3 RNA exhibited the expected diurnal variation as previously described. Under the abbreviated sampling regime, note that representative time points selected are diagnostic of the presence or absence of the normal diurnal variation in Cat3 RNA levels.

**Figure 4.**—Accumulation of the Cat3 catalase transcript in the leaves of pigmented and pigment-deficient lines of maize. Steady-state levels of Cat3 transcript in the total RNA of leaves of pigment-deficient mutants and their wild-type siblings were determined by S-1 nuclease protection analysis. Plants were grown for 7 days under a 24-hr photoperiod (12-hr light/12-hr dark) (LD) and samples collected every 6 hr, beginning at noon on day 7. Note that the Cat3 catalase transcript accumulated to normal steady-state levels and showed the expected pattern of diurnal variation in the leaves of both pigmented and pigment-deficient seedlings. This indicates that the pattern of Cat3 RNA accumulation is pigment independent.
FIGURE 5.—The effect of light regime on the accumulation of the Cat3 catalase transcript in maize leaves. The steady-state levels of Cat3 transcript in the total RNA of leaves of the normal inbred maize line W64A and the Cat3 null line IDS-28 grown under three different light regimes were determined by S1 nuclease protection analysis. For these analyses, postinhibition W64A seedlings were grown as indicated and leaves collected at four representative time points, spaced at 6-hr intervals over the 24-hr period beginning at noon on day 7. For these analyses, plants were grown in constant light (LL), constant dark (DD) or under a 24-hr photoperiod (LD). Note that leaves of W64A grown under a 24-hr light/dark regime, exhibit the expected large diurnal variation in Cat3 RNA levels. In contrast, plants grown in either constant light or constant dark accumulate constant, high steady-state levels of the Cat3 transcript throughout the sampling period. As expected Cat3 RNA was not detected in leaves of the Cat3 null line IDS-28.

RNA also accumulated to high levels throughout the 24-hr sampling period. In contrast, RbcS RNA showed the expected differential low level of accumulation in constant dark (Figure 2) and high level of accumulation in constant light (data not shown).

**DISCUSSION**

Because the pigment-deficient mutations utilized in these studies are homozygous recessive lethals (Roberts 1975), plants containing mutant alleles are maintained as heterozygotes. Heterozygous plants exhibit normal leaf pigmentation (green) because they carry one wild-type copy of the locus responsible for normal pigment production. Selfing these heterozygotes produces a population of plants that is near isogenic for all loci except that determining leaf pigmentation. This allows us to attribute differences in catalase RNA accumulation in pigment-deficient F1 progeny and their wild-type siblings to single gene differences or their pleiotropic effects.

Analysis of Cat2 mRNA levels in the leaves of various pigment-deficient mutants and their wild-type siblings showed that accumulation of the Cat2 message was very low in carotenoid-deficient leaves, but normal in leaves of a chlorophyll-deficient mutant. Although both of the homozygous carotenoid-deficient mutants, zw3 and w3, synthesize chlorophylls, the lack of protective carotenoids in these mutants allows its rapid photooxidation (Mayfield et al. 1986). Thus, by itself, the lack of Cat2 mRNA accumulation in carotenoid-deficient mutants might have been due either to the lack of carotenoids or to a lack of chlorophylls, or to an indirect effect of either. However, the normal accumulation of Cat2 message observed in the chlorophyll-deficient mutant l-195 (that has <5% normal levels of chlorophyll and much reduced levels of RbcS message) suggests that carotenoids themselves are in some manner necessary for the normal light-induced accumulation of the Cat2 message. That Cat2 mRNA is light-induced, and not directly induced by the carotenoids themselves, was confirmed by the lack of Cat2 message in dark-grown leaves of the normal line W64A that has normal carotenoid levels.

In contrast, Cat3 RNA accumulated to normal levels in the leaves of both pigment-deficient and normally pigmented plants. Both plant types also displayed the normal pattern of diurnal variation in Cat3 RNA accumulation. This suggests that the photosynthetic pigments are not involved in either setting the circadian pattern of Cat3 gene expression or determining the level of accumulation of Cat3 RNA. In addition, the normal and pigment-deficient plants used in these studies are derived from different genetic lines and carry different Cat3 alleles. We can conclude, therefore, that neither Cat3 allelic variation nor genetic background alters the pattern of Cat3 RNA accumulation in maize leaves. This also suggests that the circadian regulation of Cat3 transcription reported for W64A (Redinbaugh, Sabre and Scandalios 1990b) is a general phenomenon in maize.

Finally, these studies revealed an unusual aspect of the circadian regulation of Cat3 gene expression. Normal Cat3 RNA accumulation exhibits a circadian rhythm when plants are grown under a regime of alternating light and dark. If plants are transferred to constant light or constant dark after the circadian rhythm is set, the diurnal variation in Cat3 transcription continues (Redinbaugh, Sabre and Scandalios 1990b). In earlier studies of Cat3 transcript accumulation in developing maize leaves, plants were grown under a 24-hr photoperiod, and leaves were collected on successive days early in the light period (Redinbaugh, Sabre and Scandalios 1990a). Under normal growth conditions (i.e., an alternating 12-hr light/12-hr dark photoperiod) the circadian rhythm becomes entrained shortly after leaves of germinating seedlings emerge from the soil and are first exposed to light. Once under control of the circadian regime, Cat3 RNA levels in the leaves are low early in the light period (Figure 3 and Redinbaugh, Sabre and Scandalios 1990b). Thus, under the sampling conditions used in earlier studies, Cat3 RNA accumulation in developing maize leaves appeared to be light re-
pressed. However, when seedlings were exposed to either constant light or constant dark from imbibition (day 0), we saw that \textit{Cat3} RNA steady-state levels were uniformly and maximally expressed throughout the 24-hr sampling period on day 7 (Figure 5). Thus, normal \textit{Cat3} gene expression is neither the result of simple light induction nor light repression but would be consistent with the mediation of a novel circadian-regulated \textit{Cat3} transcriptional repressor.

All previously reported light-induced circadian rhythms in plants are phytochrome mediated, and are superimposed on the simple positive light induction of gene transcription, \textit{i.e.}, genes are on in constant light and off in constant dark (Guiliano et al. 1988; Nagy, Kay and Chua 1988; Stayton, Brosio and Dunsmuir 1989; Taylor 1989; Herrera-Estrella and Simpson 1990). Our results, in contrast, suggest that expression of the \textit{Cat3} gene appears to have a “default” mode of expression in leaves where, in the absence of an alternating light/dark cycle, the \textit{Cat3} gene is maximally expressed in leaf mesophyll throughout the 24-hr sampling period. Under a normal diurnal light regime, however, a cyclic repression superimposed on top of this quasi-constitutive mode of expression in leaves yields the observed variation in \textit{Cat3} gene transcription. Present models of circadian regulation in plants hypothesize the existence of a transcriptional enhancer that is the product of a cyclic regulatory gene or “clock” gene as has been superimposed on top of this quasi-constitutive mode of expression in leaves where, in the absence of an alternating light/dark cycle, the \textit{Cat3} gene is maximally expressed in leaf mesophyll throughout the 24-hr sampling period. Under a normal diurnal light regime, however, a cyclic repression superimposed on top of this quasi-constitutive mode of expression in leaves yields the observed variation in \textit{Cat3} gene transcription. Present models of circadian regulation in plants hypothesize the existence of a transcriptional enhancer that is the product of a cyclic regulatory gene or “clock” gene as has been described for Neurospora and Drosophila (McClung, Fox and Dunlap 1989; Hall and Rosbash 1988). In our case this clock gene product could be either a specific repressor of \textit{Cat3} gene expression or a regulator of the cyclic expression of a specific \textit{Cat3} gene repressor. This novel type of repressor-mediated circadian regulation has, to our knowledge, not been previously reported.

The tissue-specific distribution and differential response of the three catalase genes to light and other stimuli in maize leaves suggest that each might have a specific metabolic role. Although the catalase isozymes are almost certainly involved in the degradation of intracellular hydrogen peroxide, they have yet to be unambiguously linked with specific metabolic processes or pathways. A more complete elucidation of the developmental and temporal distribution of catalase expression, and the role of controlling factors such as pigments, will enable us to define the specific role(s) of the catalases in cellular development and metabolism.

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