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Gibberellin-Induced Delay of Leaf Senescence of *Alstroemeria* Cut Flowering Stems is *Not* Caused by an Increase in the Endogenous Cytokinin Content

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Abstract. The effects of various chemically pure gibberellins and cytokinins on leaf yellowing of *Alstroemeria* were described. The loss of chlorophyll was measured both in leaves of cut flowering stems and in a model system consisting of detached leaf tips. It was demonstrated that plant growth substances affected chlorophyll loss in both systems to the same extent. Leaf senescence was delayed by various gibberellins and cytokinins. The results demonstrated that some of the gibberellins (GA_4 and GA_7) are far more effective in delaying chlorophyll loss than GA_3, which is commonly used as a postharvest treatment for *Alstroemeria* cut flowering stems. Immunoassays were used to demonstrate that the effect of gibberellins on leaf yellowing does not involve an increase in the endogenous cytokinin concentrations in the leaves as an intermediate step.

A large number of metabolic changes occur during leaf senescence, for example, increases in the activity of proteases, glyoxysomal enzymes, nucleases, and enzymes of chlorophyll catabolism (for recent review see Smart 1994). Chlorophyll loss is the most obvious feature of the leaf senescence process. Various plant growth regulators affect this process. In general, cytokinins and in some systems gibberellins or auxins delay the loss of chlorophyll, whereas ethylene and abscisic acid enhance the loss (Thimann 1980, Smart 1994). Additionally, jasmonic acid can induce senescence of leaves, and for a number of systems it has been shown that polyamines delay leaf senescence (for review see Evans and Malmberg 1989).

Quality loss of *Alstroemeria* cut flowering stems (*Alstroemeria pelegrina* L. cv. Westland) starts with chlorophyll loss of the leaves especially under low light conditions (Dai and Paull 1991, Hicklenton 1991, Van Doorn et al. 1992, Van Doorn and van Lieburg 1993, Jordi et al. 1993, 1994). Recent studies on chlorophyll loss of cut flowering stems of *Alstroemeria* focused on metabolism of GA_3 (Van Doorn et al. 1992), the role of the developing bud (Jordi et al. 1993), and changes in photosynthesis during leaf senescence (Jordi et al. 1994). In addition to GA_3 also kinetin, 6-benzylaminopurine, zeatin riboside, and to a lesser extent indoleacetic acid have been reported to delay the loss of chlorophyll of various *Alstroemeria* cultivars (Dai and Paull 1991, Hicklenton 1991, Van Doorn et al. 1992). In these studies preparations containing mixtures of plant growth regulators (especially for gibberellins) were used. Therefore, no unequivocal conclusions about the efficacy of the individual plant growth regulators could be drawn (see the Discussion section in Van Doorn et al. 1992). Furthermore, it is unclear whether gibberellins acted indirectly on chlorophyll loss by affecting the endogenous cytokinin concentration in the leaves.

We have investigated the effects of various chemically pure plant growth regulators (gibberellins, cytokinins, auxins, and polyamines) on the loss of chlorophyll both of leaves attached to *Alstroemeria* cut flowering stems and in a model system of detached leaf tips. In addition, we investigated the endogenous cytokinin concentrations in the leaves during senescence following exogenous application of gibberellins. The results demonstrated that some of the gibberellin types delayed leaf yellowing far more effectively than GA_3. The mechanism causing this effect of gibberellin does not involve elevation of endogenous cytokinin concentrations.

Abbreviations: GA, gibberellin A; HPLC, high performance liquid chromatography; GA_{3Me}, GA_3-methyl ester; ZR, zeatin riboside; IPAR, isopentenyl adenine riboside.

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Materials and Methods

Gibberellins

Pure preparations of GA_1, GA_4, GA_7, GA_8, GA_9, GA_{19}, and GA_{20} were obtained from Professor L. N. Mander. GA_3 from Sigma contained relatively high percentages of contaminating gibberellins and was purified further by reversed phase HPLC. GA_3-methyl ester (GA_{3Me}) was obtained from Sigma. The purity of all gibberellins used in the experiments was higher than 99%.

Cytokinins

Isopentenyl adenine, D,L-dihydrozeatin, zeatin *trans*-isomer, kinetin, and 6-benzylaminopurine were obtained from Sigma. The purity of the cytokinins was higher than 95%.

Auxins

Indole-3-acetic acid and indole-3-butyric acid were obtained from Sigma. The purity of both compounds was higher than 95%.

Polyamines

Spermine, spermidine, and cadaverine were obtained from Sigma. Putrescine was obtained from Janssen Chimica. The purity of the polyamines was higher than 97%. The effects of polyamines on the chlorophyll content of the leaves of cut flowering stems and leaf tips were tested at concentrations of 10^{-2} to 10^{-7} M (Evans and Mamlberg 1989).

Stock solutions in ethanol were prepared at 10^{-2} M for the various gibberellins and auxins and at 10^{-3} M in ethanol for the cytokinins. Polyamines were dissolved in demineralized water at a concentration of 10^{-2} M. In all experiments the final ethanol concentration was $\leq 1\%$ (v/v). Control experiments demonstrated that this ethanol concentration did not affect the rate of chlorophyll loss (data not shown).

Plants

Cut flowering stems of *Alstroemeria* were obtained from a commercial nursery at a developmental stage in which all buds were closed but petals of the first bud were already colored red. The plants were treated as described previously (Jordi et al. 1993). For experimental use all leaves were removed except for the upper whorl, which consisted of five leaves. The cut flowering stems were placed in demineralized water in the absence or presence of the various plant growth regulators in permanent darkness, a relative humidity of 70%, and a temperature of 20°C.

Detached Leaf Tips

Leaves from the upper 40 cm of the stem were collected, and leaf tips were excised with a sharp knife. Subsequently, the weight of the leaf tips was determined, and the leaf tips (fresh weight approximately 100 mg, area approximately 3 cm^2) were incubated with the cut end in the absence or presence of different concentrations of plant growth regulators (total volume 3 ml) in the dark. Leaf tips exhibit little wound effects compared with leaf discs (Thimann 1980); a leaf tip (average leaf area approximately 3 cm^2) has a cut edge of approximately 1.5 cm, whereas a leaf disc of 3 cm^2 has a wound edge of 6.13 cm. Furthermore, the leaf tips have the advantage that only a small part of the leaf is submerged in the test solution. At different time intervals the chlorophyll content was determined. Control experiments demonstrated that leaf tips obtained from the upper 40 cm of the stem exhibited identical kinetics of chlorophyll loss at a relative humidity of 70% and a temperature of 20°C.

Endogenous Cytokinin Measurements

For one sample, leaves from the upper whorl of three cut flowering stems were collected and frozen in liquid N_2. Subsequently, samples were extracted, treated with phosphatase, and purified essentially as described (Vonk et al. 1986). [^{3}H]Zeatin riboside was added to determine the recovery during the purification procedure. Total recovery after the purification was 82%. Determination of zeatin riboside (ZR) and isopentenyl adenine riboside (IPAR) equivalents was performed by an enzyme immunoassay (Vonk et al. 1986). The ZR and IPAR antibodies exhibit a cross-reactivity of 40.5 and 40% to zeatin and isopentenyl adenine, respectively (Vonk et al. 1986). The experiment was performed in duplicate.

Chlorophyll

Chlorophyll content of the leaves and detached leaf tips was determined after extraction with dimethylformamide, and measurement of the absorption at 647 and 664.5 nm. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll were calculated using the published molar extinction coefficient in dimethylformamide (Inskeep and Bloom 1985).

Statistics

All experiments were performed at least twice with three and five replications for cut flowering stems and leaf tips, respectively. Analysis of variance using the statistics package Genstat 5 (Rothamsted Experimental Station) provided the standard errors of differences.

Results

The average amount of chlorophyll (a + b) in the leaves at the start of the experiment was approximately 1.7 mg of chlorophyll/g fresh weight and comparable for all experiments. During the first 4 days the amount of chlorophyll remained constant

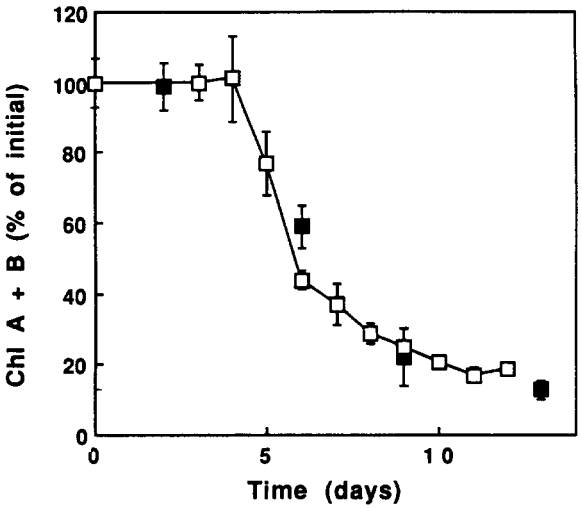

Fig. 1. Chlorophyll (Chl) content of detached leaf tips (*open squares*) and leaves attached to cut flowering stems (*closed squares*) during incubation in the dark. Chlorophyll is expressed as a percentage of the initial chlorophyll content (1.7 mg/g fresh weight). *Vertical bars* denote SE.

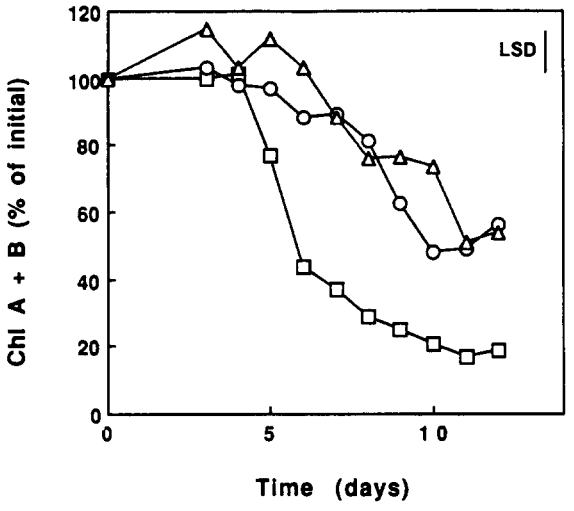

Fig. 2. Effect of 10^{-6} M GA_4 (*circles*) and 10^{-5} M zeatin (*triangles*) on the kinetics of chlorophyll loss from detached leaf tips in the dark as compared with untreated leaf tips (*squares*). The *vertical bar* denotes the least significant difference (LSD) ($p < 0.05$).

after which it decreased rapidly. Chlorophyll loss from leaves attached to cut flowering stems exhibited kinetics of chlorophyll loss similar to those of the detached leaf tips (Fig. 1).

None of the tested auxins and polyamines delayed leaf yellowing in the concentration range tested. Therefore, the effect of these compounds will not be elaborated further. In contrast, some of the gibberellins and cytokinins strongly delayed chlorophyll loss, which became visible between 5 and 8 days of incubation in the dark (Fig. 2). Therefore, we have determined the concentration dependence of the effect of other plant growth regulators after 8 days of incubation in the dark.

Exogenous Gibberellins

Cut flowering stems were treated with one of the following gibberellins: GA_1, GA_3, GA_{3Me}, GA_4, GA_7, GA_8, GA_9, GA_{19}, and GA_{20} at concentrations 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. After 8 days the amount of chlorophyll in untreated cut flowering stems decreased to approximately 10–20% of the initial amount. The various gibberellins exhibited a wide range inefficacy with regard to retention of chlorophyll. Figure 3 shows the results of three examples out of nine tested gibberellins: one with high (GA_4), average (GA_3), and very low potency (GA_{3Me}).

GA_{3Me} exhibited no significant effect on the retention of chlorophyll in the concentration range

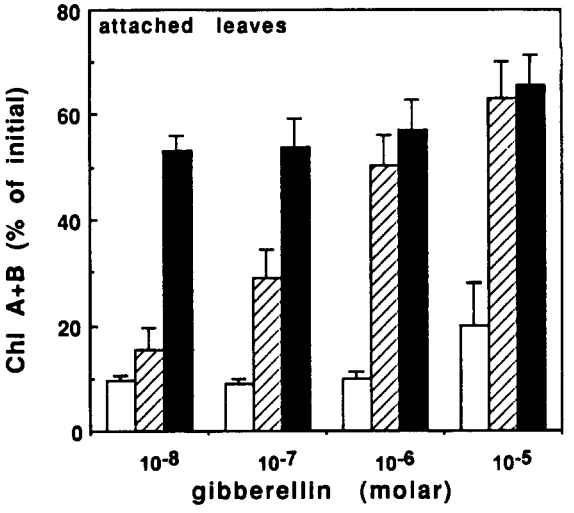

Fig. 3. Effect of various concentrations of GA_4 (*black bars*), GA_3 (*hatched bars*), and GA_{3Me} (*open bars*) on the chlorophyll (Chl) content of leaves attached to cut flowering stems after 8 days in the dark as a percentage of the initial amount. The results are the means of triplicate experiments. *Vertical bars* denote S.E.

tested (Fig. 3). Mixtures of GA_3 and GA_{3Me} showed that the inactive GA_{3Me} did not affect the GA_3-induced retention of chlorophyll even at a tenfold higher concentration. These data suggest that GA_{3Me} is not recognized by the cellular target of GA_3.

Alstroemeria growers use GA_3 in a postharvest treatment to prevent leaf yellowing. GA_3 shows a concentration-dependent effect on the retention of

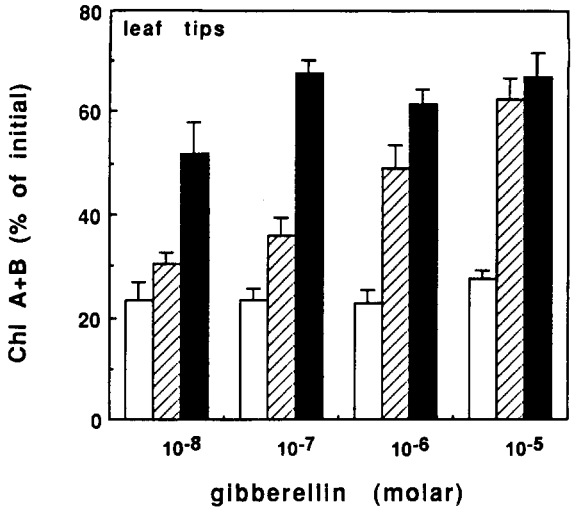

Fig. 4. Effect of various concentrations of GA_4 (*black bars*), GA_3 (*hatched bars*), and GA_{3Me} (*open bars*) on the chlorophyll (Chl) content of detached leaf tips after 8 days in the dark as a percentage of the initial amount. The results are the means of four experiments. *Vertical bars* denote S.E.

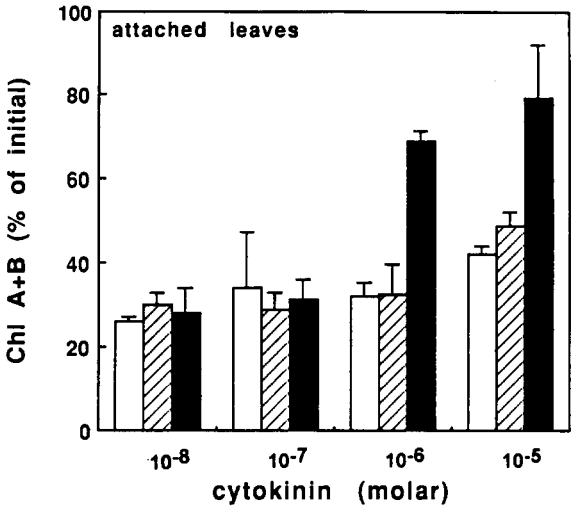

Fig. 5. Effect of various concentrations of zeatin (*black bars*), dihydrozeatin (*hatched bars*), and kinetin (*open bars*) on the chlorophyll (Chl) content of leaves attached to cut flowering stems after 8 days in the dark as a percentage of the initial amount. The results are the means of triplicate experiments. *Vertical bars* denote S.E.

chlorophyll in the concentration range 10^{-8} to 10^{-5} M (Fig. 3). In addition, no difference in the chlorophyll content of the leaves was observed between cut flowering stems treated with the commercial GA_3 and the HPLC-purified GA_3, demonstrating that the impurities present in the commercial product did not affect the efficacy (data not shown). GA_1 and GA_9 also exhibited a clear concentration-dependent retention of chlorophyll and a loss of activity at concentrations $\leq 10^{-8}$ M, but both gibberellins caused a larger retention of chlorophyll in the leaves as compared with GA_3 at 10^{-7} M (data not shown). GA_4 and GA_7 were the only tested gibberellins that showed a delay in chlorophyll loss at exogenous concentrations of 10^{-8} M (Fig. 3). Treatment of the cut flowering stems at a low concentration of 10^{-9} M also resulted in a small but significant increase in the retention of chlorophyll (data not shown). Comparison of the effects of the various gibberellins at the relatively low concentration of 10^{-7} M showed the following ranking order in efficacy of chlorophyll retention: $GA_4 = GA_7 > GA_1 = GA_9 > GA_3 > GA_{20} > GA_{19} = GA_8 = GA_{3Me}$ = no effect.

Figure 4 shows the effect of GA_{3Me}, GA_3, and GA_4 on leaf yellowing in detached leaf tips. The amount of chlorophyll in untreated detached leaf tips decreased to approximately 25% of the initial amount. GA_{3Me} exhibited no effect, GA_3 exhibited a concentration-dependent retention of chlorophyll, and GA_4 was very efficient in delaying chlorophyll loss in leaf tips. Also, the order of efficacy of the various gibberellins is identical to that obtained with the cut flowering stems (data not shown). In conclusion, the results in detached leaf tips and leaves attached to cut flowering stems are similar.

Exogenous Cytokinins

We have examined the effect of various concentrations of five cytokinins (zeatin, dihydrozeatin, isopentenyl adenine, kinetin, and 6-benzylaminopurine) on the retention of chlorophyll both in cut flowering stems and leaf tips. The effects of kinetin, zeatin, and dihydrozeatin are presented in Figure 5. The amount of chlorophyll in untreated cut flowering stems decreased to 25–30% of the initial amount in this experiment. Treatment of *Alstroemeria* cut flowering stems with kinetin at concentrations $\leq 10^{-5}$ M did not result in retention of chlorophyll in the dark, in agreement with observations of Van Doorn et al. (1992). In contrast, treatment with the highest dihydrozeatin concentration resulted in a higher chlorophyll concentration in the leaves compared with untreated cut flowering stems. Zeatin produced the greatest retention of chlorophyll of the cytokinins tested. However, cytokinins generally require higher concentrations for efficient retention of chlorophyll as compared with the gibberellins. The order of efficacy in the retention of chlo-

Table 1. Required concentration of gibberellins and cytokinins for retention of 50% of the initial amount of chlorophyll in the leaves or leaf tips after 8 days incubation in the dark.

Gibberellin	Required concentration (M)	Cytokinin	Required concentration (M)
GA_4	10^{-8}	Zeatin	10^{-7}
GA_7	10^{-8}	6-Benzylaminopurine	10^{-6}
GA_9	10^{-7}	Isopentenyl adenine	10^{-5}
GA_1	10^{-7}	Dihydrozeatin	10^{-5}
GA_3	10^{-6}	Kinetin	$>10^{-5}$
GA_{20}	$>10^{-5}$		
GA_{19}	No significant effect		
GA_8	No significant effect		
GA_{3Me}	No significant effect		

rophyll deduced from chlorophyll concentrations after various cytokinin treatments is zeatin > 6-benzylaminopurine > dihydrozeatin = isopentenyl adenine > kinetin. Analysis of the loss of chlorophyll produced by exogenously added cytokinins using leaf tips yielded similar results (data not shown). In general, a comparison of the effects of 42 different gibberellin and cytokinin treatments in cut flowering stems and leaf tips showed that the effects on leaf yellowing in both systems were similar. Experiments such as those described in Figures 3–5 show the concentration of cytokinins or gibberellins at which 50% of the initial chlorophyll in the leaves or leaf tips is retained after 8 days incubation in the dark can be estimated (Table 1).

Experiments with mixtures of GA_4 and zeatin demonstrated that in the range of GA_4 concentrations ($\leq 10^{-7}$ M) and zeatin concentrations ($\leq 10^{-6}$ M) both plant growth regulators contributed independently to the retention of chlorophyll (data not shown).

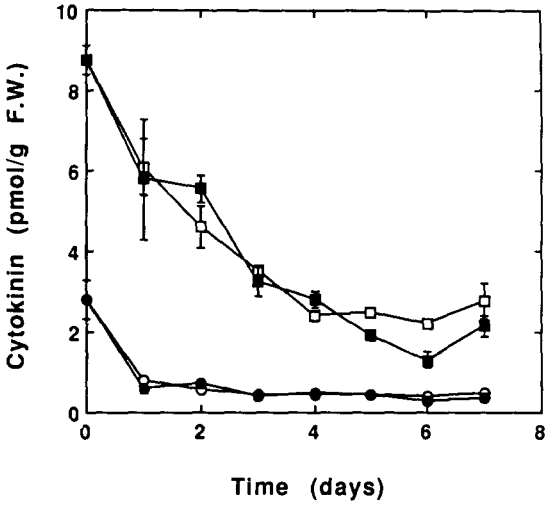

Fig. 6. Endogenous levels of ZR (*circles*) and IPAR equivalents (*squares*) in leaves of control (*open symbols*) and GA_4-treated cut flowering stems (*closed symbols*). *Vertical bars* indicate the range between duplicate experiments. If no bar is shown, the experimental range is smaller than the size of the symbol.

Endogenous Cytokinins

The amount of cytokinins was measured after a phosphatase treatment to convert nucleotide conjugates into free hormones by an enzyme immunoassay (Vonk et al. 1986). In this study the total amount of ZR and IPAR equivalents have been determined (Fig. 6). The leaves contain higher amounts of IPAR equivalents as compared with ZR equivalents comparable, for example, with iris (Vonk et al. 1986). The decrease of ZR equivalents in the leaf was rapid and reached a constant level within the 1st day after the start of the incubation, whereas IPAR equivalents decreased more gradually during the first 5 days. A GA_4 treatment that was effective in delaying leaf senescence in this experiment (data not shown) did *not* result in any significant increase in cytokinin levels of the leaves.

Discussion

Various gibberellins and cytokinins delayed leaf senescence of *Alstroemeria* cut flowering stems. Gibberellins proved to be the most effective. Cytokinins that are used often to delay leaf yellowing were much less effective. The activity of the gibberellins did not involve an increase in the endogenous cytokinin concentration. Auxins and polyamines had no effect on the kinetics of leaf yellowing.

The biologic activity of gibberellins depends among others on vascular transport, metabolic ac-

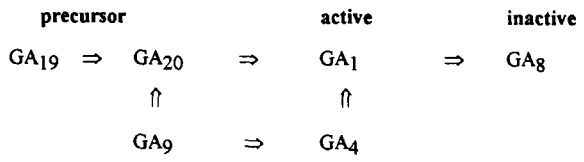

Fig. 7. Some theoretical metabolic interconversions for five gibberellins used in this study.

tivation or inactivation, and transport across the plasmalemma (at least for precursors).

Vascular Transport

Long distance transport in vascular tissue probably does not limit the effect of the plant growth regulators under our experimental conditions since similar results were obtained in leaves attached to cut flowering stems and detached leaf tips.

Metabolic Activation or Inactivation

Some theoretical metabolic interconversions for five gibberellins used in this study are indicated in Figure 7 (Sponsel 1987). GA_8 exhibited no significant biologic activity, illustrating the loss of biologic activity of 2-β-hydroxylated gibberellins (Table 1) (Graebe 1987, Mander 1991 and references cited therein). Additionally, GA_{3Me} was biologically inactive, illustrating that a free carboxyl moiety is generally essential for biologic activity (Mander 1991). The bioactive gibberellins GA_1, GA_4 (but also GA_3 and GA_7, which are not part of the pathway illustrated in Fig. 7) delayed chlorophyll loss efficiently.

GA_9, GA_{19}, and GA_{20} are gibberellins that are not 3β-hydroxylated and are tentatively indicated as precursors in Figure 7. The fact that GA_9 and GA_{20} delayed leaf senescence may indicate that the leaf contains enzymes for conversion into 3β-hydroxylated bioactive gibberellins. GA_{19} exhibits no significant effect on leaf yellowing, indicating that at the tested concentrations the conversion into bioactive gibberellins was not high enough to delay leaf yellowing efficiently. In agreement with a necessity for 3β-hydroxylation, GA_{20} and GA_9 caused a smaller delay of leaf senescence than GA_1 and GA_4, respectively. Future research using tritiated and deuterated GA_9, GA_{20}, and prohexadione (an inhibitor of 3β-hydroxylation) may demonstrate whether GA_9 and GA_{20} are active by conversion into their 3β-hydroxylated analogues GA_4 and GA_1, respectively.

Transport across the Plasmalemma

The importance of efficient transport of gibberellins across the plasmalemma may be questioned since in aleurone cells gibberellin is recognized at the external face of the plasmalemma (Hooley 1994 and references cited therein). For gibberellins that require metabolic conversion for activity by cytosolic enzymes (such as GA_9, GA_{19}, and GA_{20}) the rate of plasmalemma translocation could strongly influence the biologic activity. The extremely low activity of the hydrophilic GA_{20} as compared with the hydrophobic GA_9 could partly be explained by differences in rate of plasmalemma translocation.

Gibberellins and cytokinins both delayed the loss of chlorophyll. In contrast to gibberellins, there is abundant evidence in many different plant species that cytokinins play a role in delaying leaf senescence (for review see Smart 1994). In short, the external application of cytokinins delays leaf senescence. There is a positive correlation between endogenous cytokinin levels and leaf senescence. Transgenic tobacco plants that overproduce the enzyme isopentenyl transferase, which catalyzes cytokinin biosynthesis from its adenylic precursor, exhibit a marked retention of chlorophyll. Therefore, we tested whether the delay of senescence in the presence of gibberellins is caused by an indirect effect on the endogenous cytokinin in the leaves.

During leaf senescence a decrease in both ZR and IPAR equivalents is observed (Fig. 6). The decrease in endogenous cytokinin level in the leaves occurs before any other major change in chlorophyll and protein content. This decrease could be caused by removal of the cut flowering stems from the root system, where the cytokinins are synthesized. The decrease could be involved in triggering the leaf senescence process. However, GA_4 treatment, which was very effective in delaying leaf senescence, did not significantly raise the cytokinin levels as measured by total ZR and total IPAR equivalents in agreement with observations in tobacco leaves using bioassays (Even-Chen et al. 1978). Therefore, we have no indication that gibberellins delay leaf senescence by influencing the endogenous cytokinin levels in the leaves.

Red light, acting through phytochrome, and cytokinins are important for chloroplast development, both in the etioplast to chloroplast transition and during chloroplast senescence. Cytokinins and red light both cause a delay in leaf senescence in many plant species and stimulate the accumulation of the major chlorophyll *a*/*b*-binding protein of light-harvesting complex II. At the moment we are investigating by Northern blotting whether gibberellins (which are very effective in delaying leaf senes-

cence in this system) cause similar enhanced levels of chlorophyll *a*/*b*-binding protein mRNA. These experiments might provide insight into the mechanism by which gibberellins, cytokinins, and phytochrome regulate leaf senescence.

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