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Characterization of Carbohydrate Metabolism during Dark-Induced Senescence

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Abstract To investigate the changes of carbohydrate metabolism in the senescing leaves of *Zea mays* during dark-induced senescence, the changes in the contents of reducing sugar, sucrose and starch as well as the activities of sucrose synthase, three isozymes of invertase, and α -amylase were measured. In the senescing leaves, the content of reducing sugars temporarily increased at 4 d and rapidly decreased thereafter, whereas sucrose contents gradually decreased until 3 d of senescence and significantly decreased thereafter. The activities of intracellular invertases such as soluble acid and alkaline forms gradually enhanced until 4 d of leaf senescence and significantly declined thereafter. The extracellular invertase activity showed no significant changes during leaf senescence. The deactivation of sucrose synthase was observed within 3 d of leaf senescence. On the other hand, the starch contents gradually declined during 2 d of leaf senescence, and showed a temporary increase at 3 d, which is similar to the pattern of sucrose synthase activity. These results imply that sucrose synthase may play an important role in starch accumulation rather than in the breakdown of sucrose in the senescing leaves. The major enzymes which correlated to the breakdown of sucrose during dark-induced senescence were soluble acid and alkaline invertases, not sucrose synthase. Exogenous application of BA negatively affected leaf senescence by delaying the breakdown of sucrose and ABA accelerated leaf senescence by inducing the accumulation of reducing sugar. These results suggest, therefore, that leaf senescence may be mediated by the temporary quantitative changes of reducing sugar induced by the activation of intracellular invertases.

Key words: Carbohydrate metabolism, Dark-induced senescence, Sucrose synthase, Acid and alkaline invertase

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Introduction

Senescence is an important process in plant life cycle and it is thought to (1) alter the metabolism of plants profoundly, (2) disorganize their cellular organelles sequentially, and (3) contribute to their fitness by increasing the number and/or survival of their progeny [1,2]. Usually, leaves senesce by gradually changing their metabolic activity after they are fully developed, and subsequently abscise and die [3].

Chloroplast disassembly occurring in the senescing leaves induces the conversion of leaf function as sink organ of the assimilates, thus there may be the important change of carbohydrate metabolism as senescence progresses. In the previous study, we examined the structural changes of photosynthetic apparatus during dark-induced senescence of maize [4]. Therefore, of particular interest is the changes of carbohydrate metabolism in the senescing leaves occurring chloroplast disassembly. Ono *et al.* (1996) [5] observed that senescence of the leaf, measured as a decrease in leaf nitrogen content, was preceded by the accumulation of carbohydrate. Jordi *et al.* (1993) [6] reported the importance of carbohydrate and nitrogen status of the leaves in the regulation of leaf senescence. During natural senescence the content of sucrose has little changes, whereas that of glucose has slightly increased in the early state of natural senescence. On the contrary, the levels of sucrose and reducing sugar have rapidly decreased in transgenic tobacco leaves during dark-induced senescence [7]. In addition, carbohydrates play a central role for the regulation of senescence-associated genes. the decrease of sugar level gives rise to the expression of *sen1* in the transgenic tobacco leaves [8], and exogenous application of carbohydrates is suggested to cause the suppression of *din1* in the radish cotyledons [3]. However, very little detail is known about the change of carbohydrate metabolism, particularly its relation to enzyme activity, during leaf senescence, even though there has been some research on the carbohydrate metabolism in part of senescing leaves.

Cytokinins has been regarded as inhibitors of senescence in plants and may play a significant role in the regulation

of leaf senescence [9] as observed by a decline in cytokinin level of senescing leaves [10,11]. Recently, Gan and Amasino (1995) [10] reported that in the transgenic tobacco plants that express IPT (isopentenyl transferase) gene there was an overproduction of cytokinin *in vivo*, and a subsequent inhibition of leaf senescence. Also, Jang *et al.* (1997) [12] has reported that cytokinin could repress the sugar's regulation of the senescence-associated gene. On the other hand, an exogenous application of ABA promotes a wide range of senescence-related processes in a variety of organs [13]. Hung and Kao (1997) [14] have reported that ABA strongly promoted senescence of detached *Zea mays* leaves grown in dark conditions. Although numerous studies have been conducted to investigate the effects, actions, and signaling of plant hormones in senescing leaves, there is little data on the role of plant hormone concerning carbohydrate metabolism during leaf senescence. It has been suggested that in many species leaves detached from the plant and placed in darkness will quickly senesce, and such 'artificial senescence' has been used in senescence research [15,16]. Therefore, experimental systems using detached and dark-placed leaves will offer an excellent system in which to investigate not only changes of carbohydrate metabolism but also the effects of plant hormones on carbohydrate metabolism during leaf senescence. The main objective of the present study are (a) to identify the changes in the levels of carbohydrates such as reducing sugar, sucrose, and starch and their relation to carbohydrate cleaving enzymes activity in the detached leaves of *Zea mays*, and (b) to investigate the effect of BA and ABA on the carbohydrate metabolism of the senescing leaves.

Material and methods

Plant material

The seeds of *Zea mays* L. were soaked in running tap water for 5 h and planted in pots, grown at 25℃/18℃ (light/dark) cycle under an 18 h of light and 6 h of dark regime 70 % humidity for 10 days. The leaves of 10 day-old seedlings excised by razor, ,floated in 3 mM MES buffer (pH 5.8) with or without plant hormones under dark condition for 7 days, were used as experimental materials.

The concentration of BA was ascertained to be 5 μM, which was chosen for optimal concentration from the previous report described in Lee *et al.* (1995) [17]. The concentration of ABA was ascertained to be 3 μM, which was chosen for optimal concentration from the results on the chlorophyll contents of detached leaves reported by Moons *et al.* (1997) [18].

Measurements of reducing sugar, sucrose, and starch content

The amount of reducing sugar was determined according to the modified Somogyi-Nelson method (1944) [19]. Sucrose and starch content were determined by the method of Bergmeyer and Bernt (1974) [20]. The total soluble sugar content was determined at A_{340}.

Crude enzyme extraction

Extraction of crude soluble enzymes was performed by the modified Lee *et al.* method (1998 [21]. Detached leaves were homogenized in a Waring blender in an initial plant extraction buffer consisting of 50 mM sodium phosphate, pH 7.0, 1 mM Mg-acetate, 1 mM Na-EDTA, 1 mM DTT and 1 mM PMSF. After centrifugation, the supernatant was used as the crude enzyme. The extraction of extracellular invertase was prepared by the modified Fahrendorf and Beck method (1990) [22]. The final pellet rinsed with distilled water were incubated in 25 mM Tris-HCl buffer (pH 8.0) which contained a high concentration 1.5 M of NaCl, in addition to 1 mM Mg-acetate, 1 mM Na-EDTA, 1 mM DTT and 1 mM PMSF for 12~18 h. The suspension was centrifuged at 18,000 g for 20 min, and the supernatant was designated as the crude extract of extracellular invertase. All purification steps were carried out at 4 ℃

Assays for invertase isozymes activities

Invertase activity was determined by measuring glucose content formed by sucrolysis according to the modified method of Chen and Black (1992) [23]. The amount of glucose formed was measured by the modified glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1974). Activity of alkaline invertase was measure at pH 7.0, whereas that of soluble acid and extracellular invertases was measured at pH 5.0

Assay for sucrose synthase activity

Sucrose synthase activity was determined by measuring fructose content formed by sucrolysis. Enzyme solution (200 μL) was incubated for 1 h at 25 ℃ with 20 mM Na-phosphate (pH 6.5), 100 mM sucrose, 2 mM UDP. The reaction was stopped by adding 0.25 M Tricine-KOH (pH 8.3). The amount of fructose was determined according to the method of Bergmeyer and Bernt (1974) [20].

Assay for α-amylase activity

α-Amylase activity was routinely assayed by measuring the rate of generation of reducing sugars from starch. Appropriate dilutions of the enzyme preparations were made, and 0.2 mL of the diluted preparations was added to 0.5 mL of 100 mM succinate (pH 6.0). The reaction was initiated with 0.5 mL 5 % (w/v) soluble starch (Sigma) and terminated after 10 min by adding 0.2 mL asEaline dinitrosalicyclic acid solution [24] and then placing immediately into a boiling water bath. Color was fully developed after 5 min, and tubes were removed from the bath, allowed to cool, diluted with 10 mL H_2O, and read at A_{540} against air as reference. Activity is defined as the amount of enzyme required to produce 1 μmol of maltose min^{-1}.

Results

The changes in the content of carbohydrate during leaf senescence

The leaves of *Zea mays* were detached and placed in darkness for 7 days to induce 'artificial dark-induced senescence'. Control was defined as detached leaves of maize plants incubated in 3 mM MES solution (pH 5.8) without plant hormones under the same conditions. Fig. 1 shows that changes in the content of carbohydrates such as reducing sugar, sucrose and starch in the detached senescing leaves. The level of ruducing sugar showed little change within 2 d of leaf senescence. After 3 d of leaf senescence, there was s slightly increase in the level of reducing sugar and it reached maximum value at 4 d of leaf senescence. Afterward the level of reducing sugar significantly declined. On the other hand, the amount of sucrose had gradually decreased until 2 d, and then showed remarkable decline during at 3-4 d. The starch content gradually decreased until 3 d, temporarily increased at 4 d, and significantly decreased thereafter.

To investigate the possible relationship between plant hormones and the changes in the content of carbohydrate during leaf senescence, the carbohydrate contents of the leaves treated with plant hormones (BA or ABA) for 7 days were measured. Fig. 2 shows the reducing sugar contents of BA-treated leaves and ABA-treated leaves. The level of reducing sugar in BA-treated leaves gradually decreased until 5 d of leaf senescence, whereas it gradually declined until 2 d, temporarily enhanced at 3 d, and then significantly declined for the last of the senescence period in ABA-treated leaves. In contrast to the changes of reducing sugar contents, the sucrose content of BA-treated leaves showed a gradual decrease rather than a marked decrease (Fig. 3). The sucrose content of BA-treated leaves gradually decreased for 5 d, but its changes in ABA-treated leaves showed a steep declining pattern. At 3 d of senescing leaves treated with ABA, there was a dramatic decrease of sucrose content, which reached the minimum value at 4 d. The change of the starch contents in BA- or ABA-treated leaves significantly differed from the control leaves (Fig. 4). The starch content in BA-treated leaves showed little change for 2 d. Thereafter, it increased gradually until 5 d, reached the maximum value at 6 d, and then significantly declined. In contrast to BA-treated leaves, starch content in the ABA-treated leaves increased at 1 d of leaf senescence and kept on increasing until 3 d and reached the maximum value on the same day. Afterward it decreased significantly throughout 4-6 d and reached minimum value at 7 d.

The changes in the invertase activity during leaf senescence

To demonstrate carbohydrate metabolism in detail, the activities of invertase isozymes were assayed. Invertase could classify into three isozymes according to their optimal pH and subcellular location: soluble acid, alkaline and ex-

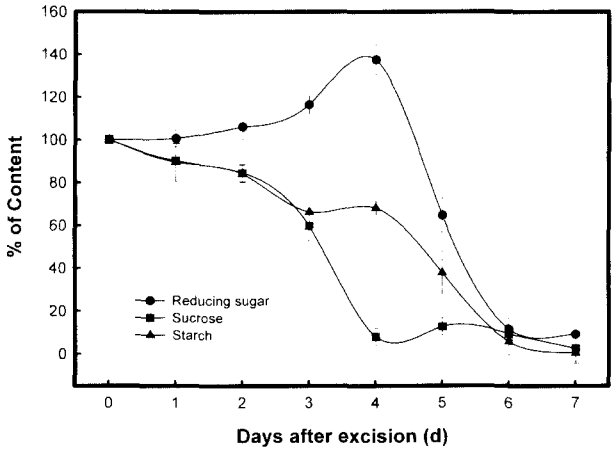

Fig. 1. Changes in the content of carbohydrates from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

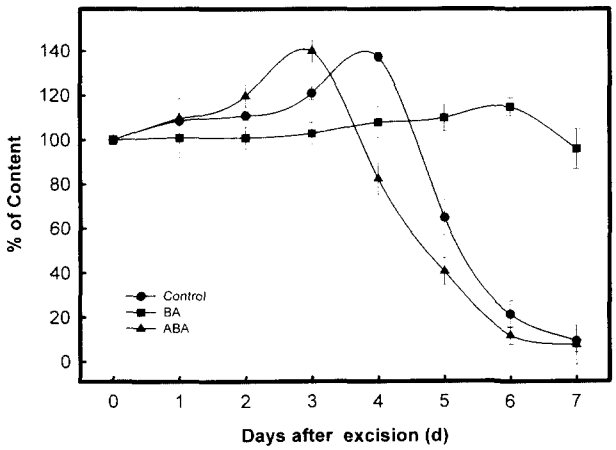

Fig. 2. Changes in the content of reducing sugar from detached leaves of *Zea mays* treated with or without plant hormones under dark conditions for 7 days. Data are means ± SD (n=3).

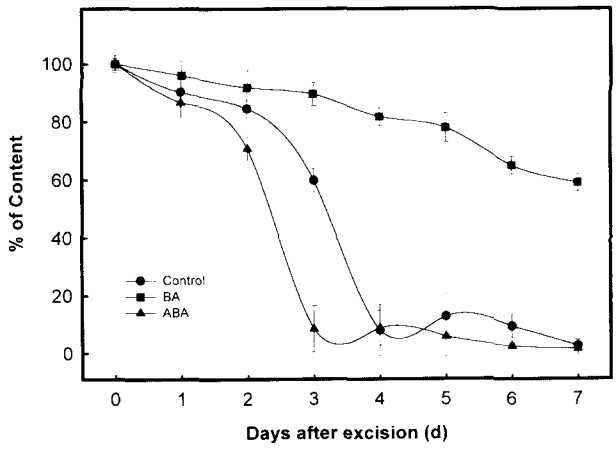

Fig. 3. Changes in the content of sucrose from detached leaves of *Zea mays* treated with or without plant hormones under dark-condition for 7 days. Data are means ± SD (n=3).

tracellular invertases.

The activity of soluble acid invertase increased gradually throughout 3 d of leaf senescence and it reached the maximum value at 4 d. Subsequently, it declined markedly and reached the minimum value at 6 d. The pattern in the change of alkaline invertase activity were similar to that of soluble acid invertase activity during leaf senescence. The only difference was slightly higher alkaline invertase activity than soluble acid invertase activity. However, the activity of extracellular invertase showed no significant changes during leaf senescence. Fig. 6 represents that plant hormones function as a regulatory factors on the soluble acid invertase activity during leaf senescence. The activity of soluble acid invertase in BA-treated leaves gradually increased until 5 d, activated at 6 d, and markedly declined thereafter. On the other hand, soluble acid invertase in ABA-treated leaves showed a faster activation than the control leaves, peaked at 3 d, and drastically decreased thereafter. The activity of alkaline invertase in BA-treated leaves gradually increased, reached the maximum value at 6 d, and then declined. On the other hand, alkaline invertase in ABA-treated leaves showed a significant activation within 2 d, peaked at 3 d, and markedly declined thereafter (Fig. 7). Unlike the intracellular invertase, the extracellular invertase, which is bound to the cell wall, did not show any significant activation in the senescing leaves, and plant hormones such as ABA and BA had no specific effect on the activity of extracellular invertase during leaf senescence (Fig. 8).

The changes in the sucrose synthase activity during leaf senescence

To better understand sucrose metabolism in the senescing

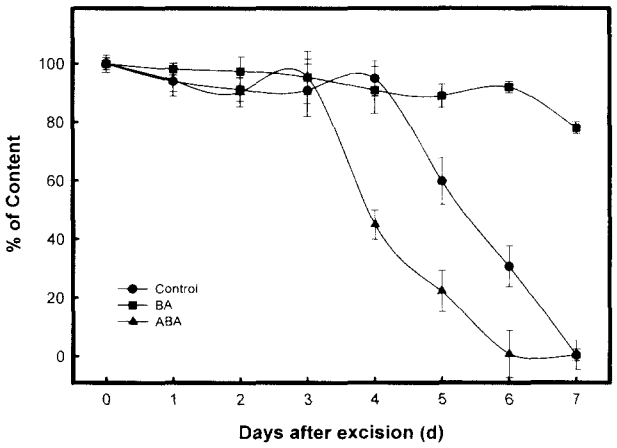

Fig. 4. Changes in the content of starch from detached leaves of *Zea mays* treated with or without plant hormones under dark condition for 7 days. Data are means ± SD (n=3).

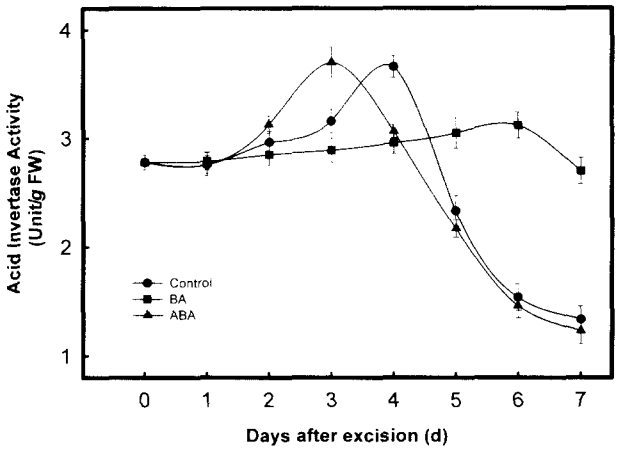

Fig. 6. Changes in the activity of soluble acid invertase from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

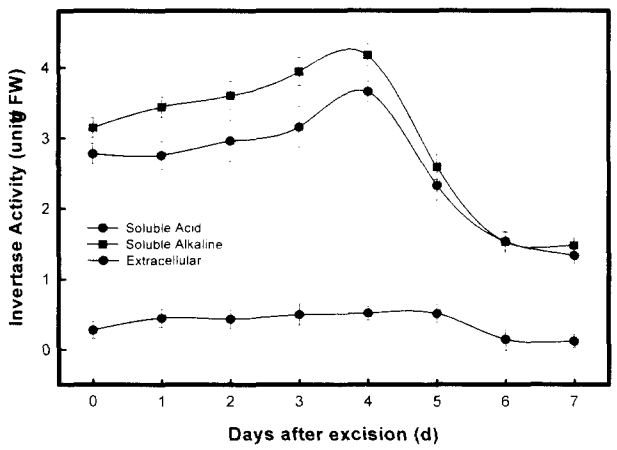

Fig. 5. Changes in the activity of invertase isozymes from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

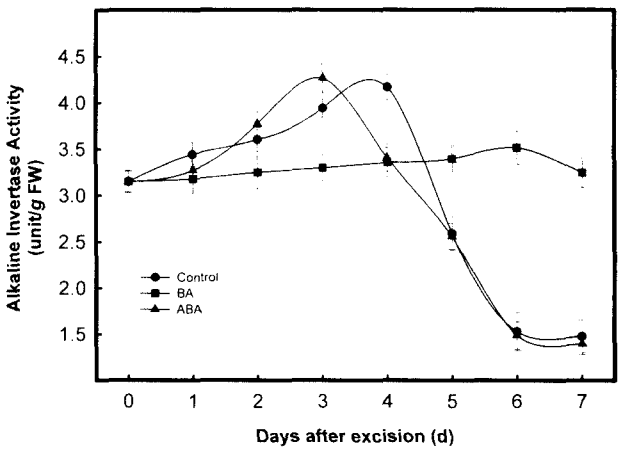

Fig. 7. Changes in the activity of alkaline invertase from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

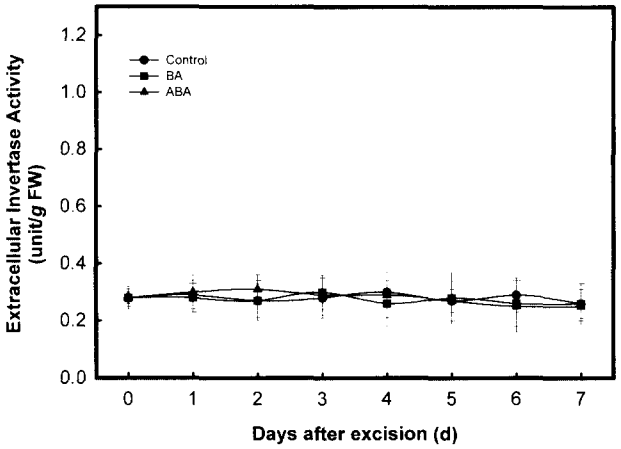

Fig. 8. Changes in the activity of extracellular invertase from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

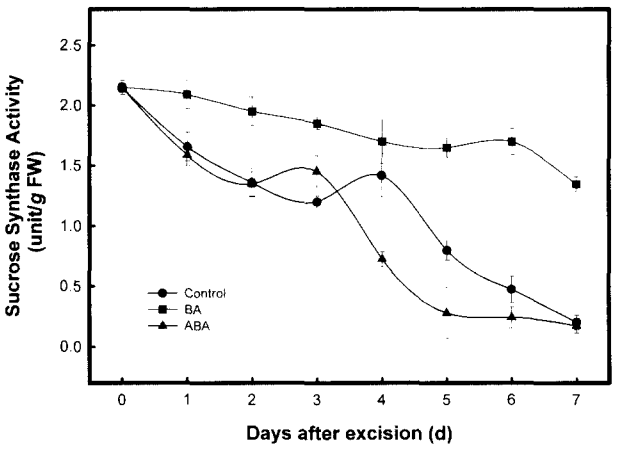

Fig. 9. Changes in the activity of sucrose synthase from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

leaves the activity of sucrose synthase was measured. As shown in Fig. 9, in the control leaves, sucrose synthase was significantly deactivated within 3 d of leaf senescence. After temporary activation of sucrose synthase at 4 d, the activity of sucrose synthase was remarkedly decreased. Exogenous BA application cause the reduction of sucrose synthase deactivation in the senescing leaves, whereas exogenous ABA application promoted the deactivation of sucrose synthase during early stage of leaf senescence.

The changes in the α-amylase activity during leaf senescence

The changes in α-amylase activity during leaf senescence were investigated to better understand the mechanisms of carbohydrate metabolism, especially those involving starch metabolism. α-Amylase showed slightly deactivation for 5 d of leaf senescence in the control plant, and remarkedly declined thereafter (Fig. 10). Its activity in BA-treated leaves was not significantly decreased during leaf senescence, whereas its activity in ABA-treated leaves were similar to that of the control leaves.

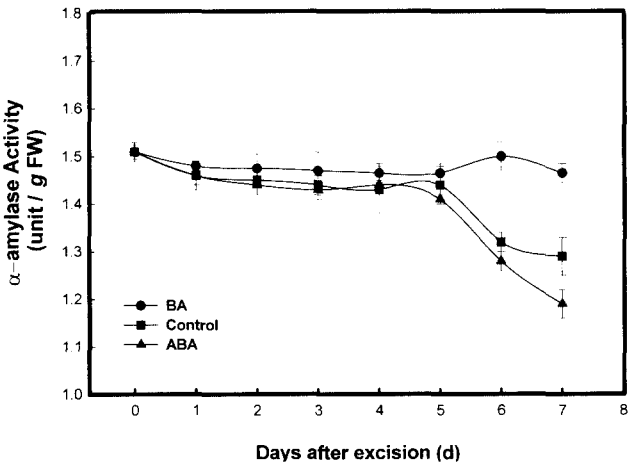

Fig. 10. Changes in the activity of α-amylase from detached leaves of *Zea mays* kept in the dark for 7 days. Data are means ± SD (n=3).

Discussion

Recently, the accumulation of glucose and fructose in old leaves has suggested to be accompanied by lower starch and sucrose content [11]. Also Chung *et al.* (1997) [7] have proposed that the release of sugar represses promoter activity on the *sen1*, senescence-associated gene of *Arabidopsis*. It may be, thus, interested in the study on the relationship between carbohydrates and their relation to enzymes during dark-induced senescence. To elucidate carbohydrate metabolism during the process of leaf senescence, the contents of carbohydrate such as reducing sugar, sucrose, and starch were measured (Fig. 1). The sucrose content decreased gradually during dark-induced senescence. The starch content also gradually decreased until 3 d, whereas the reducing sugar content remarkably increased at 4 d. This result suggests that the accumulation of reducing sugar during dark-induced senescence may be accompanied by the breakdown of cellular starch and sucrose. Consequently, the temporary accumulation of starch and reducing sugar at 4 d of dark-induced senescence may have resulted from a significant dissipation of sucrose. As of leaf senescence processes, there might be a demand for starch accumulation as a sink, not a source, in the leaves.

Plant hormones, such as cytokinin, are known to inhibit the degradation of chlorophyll and photosynthetic protein [9,25,26] and BA has been shown to delay leaf senescence in many plants. However, there is a little information on the effect of plant hormones in the changes of carbohydrate

content during dark-induced senescence. To better understand the effect of plant hormones on carbohydrate status during leaf senescence, detached leaves of maize plants were treated with BA or ABA for 7 days. Exogenous BA application was very effective on delaying the accumulation of reducing sugar when compared with the control kept in the dark (Figs. 2-4). In the contrary, exogenous ABA application accelerated the accumulation of reducing sugar. The decline of sucrose contents was also delayed by exogenous BA application (Fig. 3). Furthermore, a temporal increase of starch content was delayed 2 days compared to that of the control (Fig. 4). Wingler *et al.* (1998) [11] have reported that the accumulation of sugars during leaf senescence resulted from the breakdown of accumulated starch or the preferential export of N_2 from the leaf, which can block the effect of cytokinin, especially in low light. In addition, cytokinin has been known to be able to block some of the responses to sugars [12], which is concordant with these results. On the other hand, exogenous ABA application showed a stronger effect on leading to the accumulation of carbohydrate during dark-induced senescence when compared to the control (Figs. 2-4). These results imply that the exogenous BA application may play an important role on delaying the accumulation of reducing sugar such as glucose, which is known to repress the transcription of photosynthetic genes and result in the delay of senescence. Whereas, exogenous ABA application may induce the accumulation of glucose and accelerate the leaf senescence in *Zea mays*.

These senescence-associated changes in carbohydrate contents can be considered to be a physiological event by which senescing leaves acquire an alternative carbon source and energy to sustain metabolic integrity. In addition to carbohydrate effect on leaf senescence, numerous enzymatic alterations associated with carbohydrate metabolism have been reported. Lee *et al.* (1998) [21] have suggested that the changes of carbohydrate content during dark-induced senescence are regulated by enzymes which hydrolyze sucrose into glucose and fructose. Therefore, enzymes related in sucrose cleavage such as invertase and sucrose synthase were also measured (Figs. 5-9). Invertase is known to play an important role in the hydrolysis of sucrose into its constituent monosaccharides, glucose and fructose. Invertase is present in multiple forms in many plants. Two major forms were distinguished on the basis of their optimal pH: the acid (pH 4.0-5.0) and alkaline (pH 7.0-8.0) forms [27]. The former could be subdivided into soluble (intracellular) and extracellular forms according to its subcellular localization. In many plants, soluble acid invertase activity normally decreases during leaf maturation, probably as a result of the onset of sucrose export capacity, i.e. the sink-to-source transition. Dark-induced senescence engendered a marked increase of soluble acid and alkaline invertase activity that was correlated with higher reducing sugar accumulation (Fig. 5). In the senescing leaves of mung bean, only the soluble acid invertase activity was increased [17]. However, the activity of alkaline invertase showed little changes during dark-induced senescence, which contradicts the results of present study. Vorster and Botha (1998) [28] have reported that the activity of alkaline invertase may also play a key role in the control of hexose concentrations in the cytosol of C_4 plants such as sugarcane. It is consistent with the present results, which showed high activity of alkaline invertase on hexose accumulation during dark-induced senescence in *Zea mays* as C_4 plant. The activity of extracellular invertase did not show any significant changes during dark-induced senescence (Fig. 5) though Strum (1996) [29] has reported that extracellular invertase, among the three different isoforms, is expressed at very different times and places during plant development and the extracellular invertase seems to only have a function during vegetative plant growth [30]. These results imply that soluble acid and alkaline invertases among invertase isozymes are strongly related with the accumulation of reducing sugar during dark-induced senescence.

Both soluble acid invertase and alkaline invertase seemed to be more affected by plant hormones such as BA and ABA than extracellular invertase (Figs. 6 and 7). When BA was applied to the senescing leaves, the activation of both soluble acid and alkaline invertases maintained constitutively all over leaf senescence measured compared to the control leaves, thus suggesting that BA may regulate the levels of reducing sugar through offering the stability of intracellular invertase during leaf senescence. On the other hand, exogenous ABA application was effective in activation of soluble acid and alkaline invertases at the early stage of leaf senescence compared with control leaves.

Sucrose synthase hydrolyze sucrose into UDP-glucose and fructose as well as catalyzes irreversibly the convertion of UDP-glucose and fructose into sucrose. In many plants, sucrose synthase is a dominant sucrolysis enzymes in certain plant sinks as observed in high sucrose synthase activity in some actively growing and filling sinks, and lower sucrose synthase activities in the same sinks at maturity and when quiescent [31]. Thus, the activity of sucrose synthase is a lower level in leaves at maturity or senescing leaves. This report coincided with the results in the present study. In other words, during the dark-induced senescence, a leaf no longer synthesizes much sucrose, thus sucrose inhibits the sucrose synthase activity, which largely depends on the amount of available sucrose. These observations suggest that sucrose synthase plays more important role for sucrose breakdown than sucrose synthesis in the senescing leaves. During the dark-induced senescence, sucrose synthase activity significantly decreased until 3 d and a temporally increased at 4 d (Fig. 9). Consequently, the decreased activity of sucrose synthase may be result of the breakdown of sucrose. Wang *et al.* (1993) [32] have suggested that sucrose synthase was found to be positiviely correlated with relative growth rate of tomato fruit and with starch content in the pericarp tissue. Moreover, a positive correlation between the levels of sucrose synthase and starch content in tomato fruit [33].

The results indicate that the changes of sucrose synthase activity during dark-induced senescence may affects the changes of starch content in the *Zea mays*. Starch content gradually decreased within 3 d of dark-induced senescence and then temporally increased at 4 d, which is similar to the result in the sucrose synthase activity (Figs. 1,9). Therefore, sucrose synthase-catalyzed sucrose breakdown may be one of the control steps in starch accumulation.

Acknowledgements

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