Changes in the Synthesis of Rubisco in Rice Leaves in Relation to Senescence and N Influx

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• Background and Aims The amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1-39) synthesized in a leaf is closely correlated with N influx into the leaf throughout its lifetime. Rubisco synthesis and N influx are most active in the young leaf during expansion, but are very limited in the senescent leaf. However, it is not established whether Rubisco synthesis can be observed if N influx is increased, even in a very senescent leaf. This study first investigated changes in the relationships between \( rbcS \) and \( rbcL \) mRNAs contents and Rubisco synthesis per unit of leaf mass with leaf senescence. Next, leaves were removed during late senescence, to examine whether Rubisco synthesis is re-stimulated in very senescent leaves by an increase in N influx.

• Methods Different N concentrations (1 and 4 mM) were supplied to \( Oryza sativa \) plants at the early (full expansion), middle and late stages (respectively 8 and 16 d after full expansion) of senescence of the eighth leaf. To enhance N influx into the eighth leaf 16 d after full expansion, all leaf blades on the main stem, except for the eighth leaf, and all tillers were removed and plants received 4 mM N (removal treatment).

• Key Results Rubisco synthesis, \( rbcS \) and \( rbcL \) mRNAs and the translational efficiencies of \( rbcS \) and \( rbcL \) mRNAs decreased with leaf senescence irrespective of N treatments. However, in the removal treatment at the late stage, they increased more strongly with an increase in N influx than in intact plants.

• Conclusions Although Rubisco synthesis and \( rbcS \) and \( rbcL \) mRNAs decrease with leaf senescence, leaves at the late stage of senescence have the potential actively to synthesize Rubisco with an increase in N influx.

Key words: Senescence, Rubisco, N influx, \( rbcS \) mRNA, \( rbcL \) mRNA, translational efficiency, \( rbcL \) DNA, \( Oryza sativa \).

INTRODUCTION

Nitrogen (N) is an essential macronutrient for plant growth, and crop production is often greatly affected by N nutrition. In rice seedlings, about 70 % of N in the above-ground part is allocated to leaf blades and supports their photosynthetic function (Mae and Ohira, 1982). Approximately 80 % of total leaf N is invested in chloroplasts (Makino and Osmond, 1991). A number of proteins participate in photosynthetic reactions in chloroplasts, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) being the most abundant. Rubisco is both an enzyme of photosynthesis and the most abundant leaf protein. It accounts for 12–35 % of total leaf N in \( C_3 \) plants (Evans and Seeman, 1989; Kumar \textit{et al.}, 2002; Makino, 2003; Makino \textit{et al.}, 2003). It comprises eight small subunits (SSUs) and eight large subunits (LSUs), which are products of the nuclear \( rbcS \) genes and the chloroplast \( rbcL \) gene, respectively.

It is well known that the expression of many photosynthesis-related genes is very high in young, expanding leaves in contrast to senescent leaves (Mullet, 1993), and that an increase in the amount of N supplied to young, expanding leaves increases the amounts of photosynthesis-related proteins and promotes photosynthesis (Terashima and Evans, 1988; Makino \textit{et al.}, 1994a, \textit{b}, 1997; Nakano \textit{et al.}, 1997). When a leaf is senescent, photosynthesis-related proteins are actively degraded and are hardly synthesized again (Thomas and Stoddart, 1980; Matile \textit{et al.}, 1999; Lim \textit{et al.}, 2003; Yoshida, 2003; Jones, 2004; Krupinska and Humbeck, 2004). The amount of soluble protein, chlorophyll (Chl) and chloroplast DNA, and the number and volume of chloroplasts decrease substantially during senescence of rice, in both coleoptiles (Inada \textit{et al.}, 1998a, \textit{b}) and leaf blades (Inada \textit{et al.}, 1999). Similar results have been shown in leaves of wheat (Wittenbach \textit{et al.}, 1982; Mae \textit{et al.}, 1984; Ono \textit{et al.}, 1995). One of the most obvious enzymatic events during senescence of leaves is proteolysis, and genes encoding several different types of protease and RNase have been identified (Buchanan-Wollaston, 1997; Lim \textit{et al.}, 2003; Yoshida, 2003; Jones, 2004; Donnison \textit{et al.}, 2007). Thus, a senescent leaf appears not to have the ability to synthesize Rubisco protein. However, it has not yet been clarified if an increase in N nutrition affects the potential ability of such a leaf to synthesize Rubisco.

The amount of Rubisco in a leaf is determined by the balance between its synthesis and degradation: these processes were studied in rice leaves using \( ^{15} \text{N} \) as a tracer (Mae \textit{et al.}, 1983; Makino \textit{et al.}, 1984). They indicate that Rubisco synthesis is most active during leaf expansion but very limited after full leaf expansion, and that Rubisco is actively degraded during leaf senescence. In addition, N influx into the leaf seems to be closely related to Rubisco synthesis, and N influx declines in the senescent leaf. This suggests that Rubisco synthesis may be re-stimulated if N influx is increased in a very senescent leaf. Imai \textit{et al.},...
(2005) examined the effect of N nutrition on the relationships between \( rbcS \) and \( rbcL \) mRNAs contents and Rubisco synthesis in the leaves of rice from emergence to early senescence. Although the changes in \( rbcS \) and \( rbcL \) mRNAs are similar to those in Rubisco synthesis in young, expanding leaves, it has been suggested that the decrease in Rubisco synthesis is much faster than the decrease in \( rbcS \) and \( rbcL \) mRNAs after leaf expansion (Imai et al., 2005). These results indicate that Rubisco synthesis after leaf expansion is more correlated with N influx into the leaf than with \( rbcS \) and \( rbcL \) mRNAs. Thus, it has been suggested that the relationships between the transcription of \( rbcS \) and \( rbcL \) and the translational activity of their mRNAs may change depending on leaf age and N influx into the leaf.

This study first investigated the changes in the relationships between \( rbcS \) and \( rbcL \) mRNAs and Rubisco synthesis with the progress of leaf senescence. Secondly, to examine whether Rubisco synthesis is re-stimulated in very senescent leaves by an increase in N influx, a treatment (called removal treatment) was applied, at the late stage of leaf senescence, by removing all leaves except the eighth leaf on the main stem and all tillers.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Rice (Oryza sativa L. cv. Notohikari) plants were grown hydroponically in an air-conditioned greenhouse (Koitotoron S-180A; Koito, Tokyo, Japan) with a day/night temperature regime of 25/20°C and 60 % relative humidity under natural sunlight supplemented with 400 W metal halide lamps. Twelve seedlings were transplanted into each of twenty-five 3.5 L plastic pots containing nutrient solution. The basal nutrient solution was described by Makino et al. (1983), with some modifications. Leaves were homogenized from rice leaves. The Chl content and the LHCII content were determined by formamide extraction of Coomassie brilliant blue R-250-stained subunit bands from gels (Makino et al., 1986) using calibration curves for Rubisco purified from rice leaves. The Chl content and the LHCII content were determined by the method of Arnon (1949) and Imai et al. (2005), respectively.

**Determination of total leaf N, Chl, Rubisco and light-harvesting chlorophyll \( a/b \)-binding protein of photosystem II**

Frozen leaf blades were homogenized using a chilled pestle and mortar in 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM iodoacetic acid, 120 mM 2-mercaptoethanol and 5 % (v/v) glycerol (Makino et al., 1984). Total leaf N was determined from a part of the homogenate (Makino et al., 1988). The remaining homogenate was used for the determination of Rubisco, Chl, light-harvesting chlorophyll \( a/b \)-binding protein of photosystem II (LHCII), and for isolation of Rubisco for \( ^{15} \)N analysis. The Rubisco content was determined spectrophotometrically by formamide extraction of Coomassie brilliant blue R-250-stained subunit bands from gels (Makino et al., 1986) using calibration curves for Rubisco purified from rice leaves. The Chl content and the LHCII content were determined by the method of Arnon (1949) and Imai et al. (2005), respectively.

**Isolation of Rubisco for \( ^{15} \)N analysis**

Isolation of Rubisco was as described in Mae et al. (1983), with some modifications. Leaves were homogenized in the buffer for the determination of Chl, Rubisco, LHCII and total leaf N contents, and Triton X-100 (final concentration 0.4 %; Bio-Rad, Hercules, CA, USA) was added to the homogenate. The mixture was centrifuged at 12 000 g for 15 min at 4 °C and the supernatant was mixed with an equal volume of glycerol and stored at –30 °C. This mixture with glycerol was applied to a polyacrylamide minislab gel without SDS (3.0 % stacking gel, 5.0 % separation gel). The part of the gel corresponding to Rubisco, which could be detected by light refraction because of its high concentration, was cut out and macerated in 50 mM sodium phosphate buffer (pH 7.0), and then left to stand overnight at 4 °C. After the mixture was centrifuged at 12 000 g at 4 °C for 3 min, protein in the supernatant was precipitated by the addition of 40 % (w/v) trichloroacetic acid (final concentration of 4.5 %). The precipitate collected by centrifugation at 12 000 g for 15 min at 4 °C was washed with 80 % (v/v) ethanol. The precipitate, which was practically pure Rubisco according to SDS–PAGE, was dissolved in 0.1 M NaOH. Aliquots of this solution were placed in microtubes. These microtubes were dried, and the abundance of \( ^{15} \)N in the tubes was determined by emission spectrography (Kano et al., 1974) with a \( ^{15} \)N-analyser (N-151; JASCO, Tokyo, Japan).
Estimation of N influx in the eighth leaf blade

N influx during a 2 d period was calculated with the following equation (Mae et al. 1983). Here, the N efflux was assumed to be zero while labelled N was increasing.

\[
\text{N efflux}_{t-t'} = \frac{(^{15}N_t - ^{15}N_{t'})}{^{15}N_t \times N_t} \\
\text{N influx}_{t-t'} = N_{t'} - (N_t - \text{N efflux}_{t-t'})
\]

where \(N_t\) and \(N_{t'}\) are the total leaf N at \(t\) or \(t'\); \(t\) is the first day of each 2 d period, \(t'\) is the second day after \(t\). N efflux \(t-t'\) and N influx \(t-t'\) are N efflux from or influx into the leaf between \(t\) and \(t'\). \(^{15}N_t\) or \(^{15}N_{t'}\) is the labelled N content in the leaf at \(t\) or \(t'\), calculated from the following equation:

Labelled N = \(^{15}\text{N atom \% excess}\) of a leaf\(^{15}\text{N atom \% excess}\) of \((^{15}\text{NH}_4)_2\text{SO}_4\) fed to the plants × total leaf N.

Estimation of Rubisco synthesis in the eighth leaf blade

The amount of Rubisco synthesized during a 2 d period was calculated with the following equation (Mae et al., 1983). Here, the amount of degraded Rubisco was assumed to be zero while labelled Rubisco was increasing.

\[
\text{Rubisco degradation}_{t-t'} = \frac{(^{15}N_{t'} - ^{15}N_t)}{^{15}N_t \times N_t} \\
\text{Rubisco synthesis}_{t-t'} = N_{t'} - (N_t - \text{Rubisco degradation}_{t-t'})
\]

where \(N_t\) and \(N_{t'}\) are the amount of Rubisco at \(t\) or \(t'\); \(t\) is the first day of each 2 d period, \(t'\) is the second day after \(t\). Rubisco degradation \(t-t'\) and Rubisco synthesis \(t-t'\) are respectively Rubisco degradation or synthesis in the leaf between \(t\) and \(t'\). \(^{15}N_t\) or \(^{15}N_{t'}\) is the labelled N content in the leaf at \(t\) or \(t'\), as calculated from:

Labelled Rubisco = \(^{15}\text{N atom \% excess}\) of Rubisco of a leaf\(^{15}\text{N atom \% excess}\) of \((^{15}\text{NH}_4)_2\text{SO}_4\) fed to the plants × Rubisco content.

Northern and Southern blot analyses

RNA an DNA were extracted by the methods of Suzuki et al. (2001a, b, respectively). More than 80% of RNA and DNA in the leaf blades of rice could be extracted irrespective of leaf age. Northern and Southern blot analyses and the quantification of \(rbcS\) and \(rbcL\) mRNAs and \(rbcS\) DNA were described by Imai et al. (2005). Single bands were observed for \(rbcS\), \(rbcL\) and NADH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, and for \(rbcL\) and \(rbcS\) DNAs, by northern and Southern gel blot analyses throughout the experimental period. GAPDH mRNA and \(rbcS\) DNA were the internal standards. \(rbcS\) and \(rbcL\) mRNAs and \(rbcL\) DNA were quantified by dot-blot analysis and their signal intensities were respectively multiplied by the amounts of total RNA and total DNA shown in Fig. 6. The amounts of \(rbcS\) and \(rbcL\) mRNAs and \(rbcL\) DNAs were expressed relative to the amounts at the full expansion.

RESULTS

Length and fresh weight of leaf blades increased rapidly after emergence and reached their maxima on the sixth day after emergence (full expansion). Thereafter, leaf length and fresh weight remained constant regardless of the N treatments. Leaf length and fresh weight were not significantly different among the N treatments throughout the experimental period (\(P < 0.10\), Student’s t-test). Averages of the fresh weights were used to calculate the per leaf blade data.

Changes in the amounts of total leaf N and N influx

The amount of total N per leaf lamina (total leaf N) rapidly increased during leaf expansion and then gradually declined in the 1 mM N treatment (Fig. 1A). The amount of
total leaf N was considerably increased in the 4 mM N treatment on days 0, 8 and 16 after full expansion, and in the removal treatment. The maximum amount of total leaf N was significantly greater in the 4 mM N treatment on days 0, 8 and 16 after full expansion and in the removal treatment than in the 1 mM N treatment at the same stage (Student’s t-test, \( P < 0.05 \)). The abundance of \(^{15}\)N in total leaf N was greatest at the end of \(^{15}\)N labelling and then declined rapidly until full expansion (Fig. 1B). Thereafter, it gradually declined in the 1 mM N treatment. The abundance of \(^{15}\)N in total leaf N in the 4 mM N treatment and in the removal treatment declined more than in the 1 mM N treatment after each treatment.

N influx for a 2 d period was estimated from the changes in the amount of total leaf N and its \(^{15}\)N content (Fig. 2). The N influx was active during leaf expansion, but very limited after the completion of leaf expansion in the 1 mM N treatment. The N influx was greatly increased in the 4 mM N treatment on days 0, 8 and 16 after full expansion, and was enhanced in the removal treatment. However, the extent of the increase in the N influx in the 4 mM N treatment decreased with the progress of leaf senescence.

Changes in the amount of Rubisco and synthesized Rubisco

The amount of Rubisco rapidly increased during leaf expansion and then gradually declined in the 1 mM N treatment (Fig. 3A). The amount of Rubisco was also increased in the 4 mM N treatment at all stages and in the removal treatment. The maximum amount of Rubisco was significantly greater in the 4 mM N treatment on days 0, 8 and 16 after full expansion and in the removal treatment than in the 1 mM N treatment at the same stage (Student’s t-test, \( P < 0.05 \)). The \(^{15}\)N abundance in Rubisco was the highest at the end of \(^{15}\)N labelling and then declined rapidly until full expansion (Fig. 3B). Thereafter, it gradually declined in the 1 mM N treatment. The \(^{15}\)N abundance in Rubisco in the 4 mM N treatment and the removal treatment declined more than that in the 1 mM N treatment after each treatment.

The amount of Rubisco synthesized for each 2 d period was estimated from the changes in the amount of Rubisco.
and its $^{15}$N content (Fig. 4). Rubisco synthesis was also increased in the 4 mM N treatment at all stages of leaf senescence and in the removal treatment. The amount of synthesized Rubisco was greater in the removal treatment than in the 4 mM N treatment on the 16th day after full expansion.

**Ratio of Rubisco synthesized to N influx in senescent leaves**

Table 1 shows the percentages of synthesized Rubisco to N influx in the 1 and 4 mM N treatments during 0–4, 8–12 and 16–20 d after full expansion and in the removal treatment. The ratio (percentages) in the 1 and 4 mM treatments decreased with leaf senescence, but this decrease was faster in the 1 mM N treatment than in the 4 mM N treatment. The percentages at all periods of leaf senescence tended to be greater in the 4 mM treatment than in the 1 mM N treatment. In the removal treatment, the percentages also tended to be higher than that in the 4 mM N treatment at the same stage.

**Changes in the amounts of LHCII and Chl**

The amounts of LHCII and Chl of thylakoid membranes in chloroplasts rapidly increased during leaf expansion (Fig. 5A and B). These gradually declined in the 1 mM N treatment 37.1 ± 7.9b.

**Table 1. Amount of Rubisco synthesized as a percentages of the N influx in the senescent leaves during the indicated period**

<table>
<thead>
<tr>
<th></th>
<th>Days 0–4 after full expansion</th>
<th>Days 8–12 after full expansion</th>
<th>Days 16–20 after full expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM N</td>
<td>37.2 ± 6.2a</td>
<td>23.1 ± 5.1a</td>
<td>13.1 ± 3.1a</td>
</tr>
<tr>
<td>4 mM N</td>
<td>44.0 ± 7.9b</td>
<td>41.2 ± 8.5b</td>
<td>37.1 ± 7.9b</td>
</tr>
<tr>
<td>Removal</td>
<td></td>
<td></td>
<td>37.1 ± 7.9b</td>
</tr>
</tbody>
</table>

The percentages were calculated as the average amount of Rubisco synthesized to the average amount of N influx for each 4 d period after the 1 and 4 mM N treatments and the removal treatment from the data in Figs 2 and 4. The different letters indicate that the means are significantly different between the treatments during the same period using Student’s $t$-test ($P < 0.05$).
treatment after full expansion. The amounts of LHCII and Chl as well as the amount of Rubisco tended to increase in the 4 mM N treatment on days 0, 8 and 16 after full expansion, and in the removal treatment. There were no significant differences in the amounts of Chl and LHCII between the treatments throughout the experimental period (Student’s t-test, \( P < 0.05 \)).

Changes in the amounts of total RNA and total DNA

The amount of total RNA increased just after each N supply, but then declined (Fig. 6A). This response was greater in the 4 mM N than in the 1 mM N treatment. The maximum amount of total RNA was significantly greater in the 4 mM N treatment on days 0, 8 and 16 after full expansion, and in the removal treatment than in the 1 mM N treatment at the same stage (Student’s t-test, \( P < 0.05 \)). In contrast, the amount of total DNA was not affected by the N supply throughout senescence (Fig. 6B). In the removal treatment, the amount of total DNA tended to increase, but the difference was not significant. There was no significant difference in the amount of total DNA between the treatments throughout the experimental period (Student’s t-test, \( P < 0.05 \)).

Changes in \( rbcS \) and \( rbcL \) mRNAs and \( rbcL \) DNA

Although Rubisco content did not increase just after each N supply in the 1 mM N treatment, \( rbcS \) and \( rbcL \) mRNAs increased (Fig. 7A and B). These responses were greater in both the 4 mM N treatment and the removal treatment, but in the 4 mM N treatment they decreased quickly. The extent of the increase was greater for \( rbcS \) mRNA than for \( rbcL \) mRNA irrespective of N supply. The maximum amounts of \( rbcS \) and \( rbcL \) mRNAs were significantly higher in the 4 mM N treatment on days 0, 8 and 16 after full expansion, and in the removal treatment than in the 1 mM N treatment at the same stage (Student’s t-test, \( P < 0.05 \)). \( rbcL \) DNA in the 1 mM N treatment reached a maximum at the time of full expansion, whereas \( rbcS \) and \( rbcL \) mRNAs had already decreased (Fig. 7C). The effect of N supply on \( rbcL \) DNA was smaller than that on \( rbcS \) and \( rbcL \) mRNAs. There was no significant difference in \( rbcL \) DNA between the treatments throughout the experimental period (Student’s t-test, \( P < 0.05 \)).

Ratios of Rubisco synthesis to \( rbcS \) and \( rbcL \) mRNAs

Table 2 shows the ratios of Rubisco synthesis to \( rbcS \) and \( rbcL \) mRNAs in the 1 and 4 mM N treatments at all stages of leaf senescence and in the removal treatment. Although Rubisco synthesis and \( rbcS \) and \( rbcL \) mRNA amounts decreased with leaf senescence, the extent of the decrease was larger for the former than the latter. Thus, the ratios in the 1 and 4 mM N treatments decreased with leaf senescence, indicating that changes in \( rbcS \) and \( rbcL \) mRNAs do not always correspond to changes in Rubisco synthesis, and that the translational efficiency of Rubisco is increasingly downregulated as leaf senescence progresses. However, this ratio was always higher in the 4 mM N treatment and the removal treatment than in the 1 mM N treatment, indicating that an increase in N supply promotes Rubisco synthesis at the translational stage as well as at the transcriptional stage. Figure 8 shows the relationship between Rubisco synthesis and N influx for all treatments on the 16th day after full expansion. Both parameters were highly positively correlated with each other, irrespective of treatments. Thus, Rubisco synthesis and the translational efficiencies of \( rbcS \) and \( rbcL \) mRNAs were greatly enhanced even at the late stage of leaf senescence when N influx into the leaf was abundant.

DISCUSSION

It is generally accepted that a senescent leaf plays a role as a tissue providing nutrients to other parts of the plant (source tissue). The purpose of the removal treatment on
the 16th day after full expansion was to examine whether a senescent leaf can again play a role as a ‘sink’ tissue. Although a senescent leaf has the potential ability to increase N influx and Rubisco synthesis, senescent leaves did not synthesize Rubisco as actively as young expanding ones in the 1 mM N treatment (Fig. 4). While photosynthetic capacity was not measured, that on the 16th day after full expansion probably did not increase as greatly as a young expanding leaf would, in accord with Rubisco

### TABLE 2. The ratios of the amount of Rubisco synthesized to the amounts of rbcS and rbcL mRNAs in the senescent leave during the indicated period

<table>
<thead>
<tr>
<th></th>
<th>Days 0–4 after full expansion</th>
<th>Days 8–12 after full expansion</th>
<th>Days 16–20 after full expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesized Rubisco/rbcS mRNA (µmol/relative amount)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM N</td>
<td>1.26 ± 0.31a</td>
<td>0.80 ± 0.19a</td>
<td>0.69 ± 0.18a</td>
</tr>
<tr>
<td>4 mM N</td>
<td>4.11 ± 0.83b</td>
<td>3.20 ± 0.77b</td>
<td>2.51 ± 0.55b</td>
</tr>
<tr>
<td>Removal</td>
<td></td>
<td></td>
<td>3.88 ± 0.77c</td>
</tr>
<tr>
<td>Synthesized Rubisco/rbcL mRNA (µmol/relative amount)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM N</td>
<td>1.48 ± 0.38a</td>
<td>0.57 ± 0.13a</td>
<td>0.46 ± 0.10c</td>
</tr>
<tr>
<td>4 mM N</td>
<td>5.82 ± 0.91b</td>
<td>4.23 ± 0.93b</td>
<td>3.65 ± 0.77b</td>
</tr>
<tr>
<td>Removal</td>
<td></td>
<td></td>
<td>8.00 ± 1.59c</td>
</tr>
</tbody>
</table>

The ratio was calculated as the average amount of Rubisco synthesized to the average amounts of rbcS and rbcL mRNAs for each 4 d period after the 1 and 4 mM N treatments and the removal treatment from the data in Figs 4 and 7. The different letters indicate that the means are significantly different between the treatments during the same period using Student’s t-test (P < 0.05).

The 16th day after full expansion was to examine whether a senescent leaf can again play a role as a ‘sink’ tissue. Although a senescent leaf has the potential ability to increase N influx and Rubisco synthesis, senescent leaves did not synthesize Rubisco as actively as young expanding ones in the 1 mM N treatment (Fig. 4). While photosynthetic capacity was not measured, that on the 16th day after full expansion probably did not increase as greatly as a young expanding leaf would, in accord with Rubisco.
and Chl contents (Figs 3A and 5A). Additionally, N supply hardly affects the specific activity of Rubisco (Makino et al., 1983). It is unclear if this is because N largely moved into the young tissues in which sink activity was much higher than in the eighth senescent leaf, or if the eighth senescent leaf on the 16th day after full expansion had already reached the limit of its ability to synthesize Rubisco. Substances such as cytokinin, which is known to activate the bioactivity of plant cells (Kiba et al., 2004, 2005; Brenner et al., 2005; Sakakibara et al., 2006), might be needed to synthesize de novo Rubisco in a senescent leaf. Table 1 shows that the preferential investment of N in Rubisco is increased with an increase in the N influx. This result is consistent with the reports of Makino et al. (1992, 1997). Thus, the mechanism for the synthesis of Rubisco in a senescent leaf seems to be essentially the same as that in a young expanding leaf. If N influx into a senescent leaf can be more strongly enhanced, de novo Rubisco synthesis and the translational efficiencies of rbcS and rbcL mRNAs may be further raised. Since N influx probably involves various substances such as amino acids, nitrate ions and phytohormone (Hayashi and Chino, 1990), it is intended to investigate in the future which of these substances are the main ones involved in Rubisco synthesis in leaf cells.

In general, in field cultivation of rice plants in most areas, unlimited N nutrition is supplied. However, in hydroponic laboratory experiments, the N supply is limited. In the 3-5 L pots used in this research, preliminary experiments showed that the amounts of N in the 1 and 4 mM N solutions were absorbed in only a few days (data not shown). Thus, the environment related to N supply between field cultivation and hydroponics is very different. However, the reason why hydroponics was used was the ease and accuracy of changing N concentrations. Moreover, this experiment was done in an air-conditioned greenhouse, i.e. a small-scale environment in which temperature and light intensity were essentially controllable. Thus, Rubisco synthesis and its relationship to N influx in rice grown in the paddy field remain to be examined.

Unfortunately, reusing 15N produced from Rubisco degradation was not taken into consideration in the equations for the calculation of Rubisco synthesis (see Materials and Methods). It is reported that Rubisco synthesis and degradation occur in several hours (Sasaki et al., 1985; Berry et al., 1990; Ishida et al., 1997, 1998). Thus, Fig. 4 is thought to show the total results of the turnover of Rubisco during 2 d. The purpose was to examine the changes in the relationship between Rubisco synthesis and N influx with leaf senescence, so cultivation of rice plants during a long period was needed, and related equations were applied to the calculation of Rubisco synthesis. It will be necessary to develop other methods and formulae that can be used for estimations of reused N to understand more accurately the regulatory mechanism of Rubisco turnover.

N influx into leaves decreased with leaf senescence (Fig. 4), and the ratio of N influx invested to Rubisco synthesis also decreased in both the 1 and 4 mM N treatments (Table 1). However, the decrease in the ratio was slower in the 4 than the 1 mM N treatment (Table 1). These results suggest that when considerable N flows into a senescent leaf, N is preferentially invested in Rubisco synthesis, as in young expanding leaves (Makino et al., 1992). This might be a valid result because a senescent leaf works as a source tissue and Rubisco has an important role as a material for the storage of N. It is reported that about 60% of total N in an ear of rice grown in a field originates from senescent leaves (Mae and Ohira, 1981). N influx into a senescent leaf for Rubisco synthesis may mean not only re-activating photosynthesis but also might secure the necessary N for the ear to mature.

The rbcS and rbcL mRNA contents decline during leaf senescence (Nie et al., 1995a, b; Crafts-Brandner et al., 1996, 1998; Ono and Watanabe, 1997; Suzuki et al., 2001b; Imai et al., 2005). However, the increase in rbcS and rbcL mRNAs with 4 mM N supply hardly changed, irrespective of leaf age, whereas N influx and Rubisco synthesis decreased with leaf senescence (Figs 2, 4, 7A and B). These results mean that the translational efficiencies of rbcS and rbcL mRNAs gradually declined as leaf senescence progressed. Since translational efficiencies are just indexes calculated as shown in Table 2, they scarcely take into consideration post-transcriptional and translational steps such as rbcS and rbcL mRNA processing and the translational rate of ribosomes. However, in vitro transla-
tional activity of rbcS and rbcL mRNAs declines with leaf senescence in Amananthus hypochondriacus (Nikolau and Klessig, 1987) and Phaseolus vulgaris (Bate et al., 1991), so that the indexes calculated are useful for evaluating the efficiencies of rbcS and rbcL mRNA translation to mature Rubisco. Table 2 shows that the translational efficiencies of both rbcS and rbcL mRNAs in the removal treatment were higher than those in the 4 mM N treatment during the same period. In the removal treatment, not only increases in rbcS and rbcL mRNA translation but also enhancement of the translational efficiencies would have greatly affected upregulation of de novo Rubisco synthesis. It has not been clarified which translational steps, such as those of rbcS and rbcL mRNAs by activated ribosomes or the assembly of SSU with LSU in chloroplasts, were enhanced by an increase in N influx. Further molecular investigations are needed to clarify the relationships between N influx and post-transcriptional and translational regulation of rbcS and rbcL mRNAs.

The rbcL DNA is considered an index of the copy number of the chloroplast genome. It was previously reported that N supply to young expanding leaves should increase the copy number of the chloroplast genome (Imai et al., 2005). Moreover, the present results suggest the possibility that even during leaf senescence the copy number of the chloroplast genome may be increased by N supply (Fig. 7C). It would therefore be interesting to ascertain if synthesis of the chloroplast genome with N supply can be observed during leaf senescence. Though the possibility might be extremely low, the chloroplast number in a leaf cell might change with N supply. Generally, de novo synthesis of the chloroplast genome has been thought to take place only at the beginning of leaf development; however, the present and earlier results indicate that a
chloroplast genome is likely to be re-synthesized with N supply throughout the lifetime of a leaf.

Conclusions

Supply to a senescent leaf increased N influx, Rubisco synthesis, rbcS and rbcL mRNAs and the translational efficiencies of rbcS and rbcL mRNAs. Rubisco synthesis declined with leaf senescence, as shown by the decreased translational efficiencies of rbcS and rbcL mRNAs. However, when N influx was abundant, Rubisco was again actively synthesized, even in the late stage of leaf senescence, while translational efficiencies were also increased in the same way. Thus, senescent leaves have the potential to synthesize Rubisco in response to N influx. Thus, N influx into a leaf is likely to be one of the most important factors determining the extent of leaf senescence. It still remains to investigate which of the steps in post-transcription and translation of rbcS and rbcL mRNAs are affected with N influx and what substances contained in the N influx contribute to the upregulation of Rubisco synthesis.

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