Cold-induced Accumulation of RNAs and Cloning of cDNAs Related to Chilling Injury in Rice

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Summary

Rice seedlings were exposed to cold (5 °C) at a relative humidity of 100 % for 24 h and a subtracted cDNA library was constructed. Six clones isolated by differential screening of the subtracted cDNA library were designated as RC (responsive to cold) clones. Increase of RNA level detected with these cDNA probes was observed within 3 h under cold treatment. Thereafter the RNA level either remained high or decreased during the initial two days of cold treatment. Reincubation of plants at 28 °C following the 5 °C treatment caused a rapid decrease of RNA levels detected with all the clones. RNAs detected with four of the RC clones (RC42, RC104, RC114, RC235) were also accumulated when seedlings were subjected to drought, abscisic acid (ABA) and NaCl treatments. However, accumulation of RNAs detected with two of the RC clones (RC167 and RC179) under these stress treatments was not observed, suggesting the existence of a specific pathway that is generated only by cold treatment, but not by other stresses. Examination of chilling injury and cold-induced accumulation of these RNAs in 22 rice varieties using four cDNA probes suggests that the accumulation of RC167 and RC235 mRNAs was associated with chilling sensitivity.

Key Words : *Oryza sativa*, chilling injury, cold tolerance, cDNA cloning, cold-responsive genes, subtractive cloning.

Introduction

Plants from tropical and subtropical regions show injury symptoms when exposed to non-freezing, low temperatures below 10 - 15 °C (Graham and Patterson 1982). The extent of the chilling injury depends on the duration and severity of treatment and other growth conditions as well as genotypes (Morishima and Oka 1981, Rikin 1992). The genotypes of cultivated rice, *Oryza sativa* L. can be classified into two major groups, japonica and indica, depending on their biochemical characters or RFLPs of genomic DNA (Tanaka *et al.* 1989). Chilling sensitivity is one of the basic characters used for discriminating these two groups. Most of the japonica types are more cold-tolerant than the indica types (Morishima and Oka 1981).

It is generally recognized that patterns of protein synthesis and mRNA level change when plants are ex-

posed to cold (Graham and Patterson 1982, Guy *et al.* 1985, Hahn and Walbot 1989, Schaffer and Fischer 1989, Thomashow 1990). In overwintering plants from temperate regions, growth under nonfreezing- low-temperature causes hardening, during which the freezing tolerance increases. Cold-regulated genes or their cDNAs have been isolated, and their structures and functions were analyzed (reviewed by Thomashow 1990). Studies on the responses of chilling sensitive plants to cold at the gene level have been reported recently in tomato (Schaffer and Fischer 1989) and rice (Aguan *et al.* 1991, Binh and Oono 1992, Christie *et al.* 1991, Hahn and Walbot 1989). However the function or importance of the cold-regulated genes in chilling-sensitive plants remains largely unknown.

In the present study, we isolated several cDNA clones of cold-accumulated RNAs and examined their expression in response to cold, drought, abscisic acid (ABA) and NaCl treatments. Analysis of mRNA accumulation in 22 rice varieties enabled to identify two RNAs associated with chilling sensitivity.

Materials and Methods

1. Plant Materials

A japonica rice variety, Hayayuki, was used to construct cDNA libraries and to examine the changes of the RNA level in leaves under stress conditions. For the examination of cold injury and RNA accumulation under cold conditions, a total of 22 rice varieties consisting of japonica, javanica and indica varieties were used (Table 2). Seeds were surface-sterilized and germinated on agar (0.7 %) or vermiculite containing a liquid medium (Satake *et al.* 1978). Seedlings were grown for two weeks under fluorescent light (about 600 $\mu\text{E}/\text{m}^2\cdot\text{sec}$, 16 h light period every day) at 26 - 28 °C and 60 - 90 % of relative humidity (RH). For the cold treatments, which were always started at the beginning of the light period of a day, plants were transferred to a growth chamber set at 5 °C and incubated for various periods of time. Light intensity during cold treatment ranged from 300 to 600 $\mu\text{E}/\text{m}^2\cdot\text{sec}$ and RH in the box was nearly 100 %. Drought treatment was applied by removing the liquid medium and exposing plants to open air (60 - 90 % RH) for 24 h. In the abscisic acid treatment, a 100 μM solution of ABA was sprayed on the seedlings once every 6 h during the 24 h period. In the NaCl treatment, plants were transferred to the liquid medium containing 2 % of NaCl and incubated for 24 h.

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2. RNA extraction and cDNA cloning

Total RNA for cDNA cloning was extracted from 10 g of leaves of rice seedlings by the GTC/CsCl method (Sambrook *et al.* 1989), and poly(A)⁺ RNA was isolated using an oligo(dT) cellulose column (Sambrook *et al.* 1989). cDNA was synthesized using a commercial kit (Amersham) and a subtracted library was constructed in the pUC19 vector after subtracting single-stranded cDNAs of cold-treated plants one time to a 20-fold excess of poly(A)⁺ RNA of control plants (Davis *et al.* 1984). Total RNA for Northern blotting was extracted from 1 g of leaves by the phenol/SDS method (de Vries *et al.* 1988).

3. Colony hybridization and Northern hybridization

Preparation of plasmid DNA and radioactive probes of cDNAs, screening of cDNA library by differential colony hybridization, Northern hybridization, were carried out as previously described (Sambrook *et al.* 1989). In the Northern hybridization, filters were finally washed with 0.1 × SSC - 0.1 % SDS solution at 68 °C for 30 min.

4. Evaluation of chilling sensitivity

Seedlings of 22 rice varieties were grown for 2 weeks, then exposed to cold at 5 °C for 4 days and reincubated for one week at 28 °C. Light intensity was 300 - 600 μE/m²·sec during the cold treatment and 600 μE/m²·sec during the 28 °C reincubation, respectively. RH during and after the cold treatment was nearly 100 %. Chilling sensitivity of all the varieties was scored on a 0 - 5 scale by counting the number of dead plants (0; no injury, 1; 1-20 % death, 2; 21-40 % death, 3; 41-60 % death, 4; 61- 80 % death, 5; death of 81-100 % plants).

Results

1. Cloning of cDNAs of cold-responsive genes

In order to isolate cDNAs of cold-responsive genes, a subtracted cDNA library was constructed and 550 clones were screened by differential colony hybridization to pick up cDNAs of relatively abundant cold-accumulated RNAs. Fifty-eight clones were isolated as cold-responsive clones, and were designated as RC (responsive to cold) clones. Six of the 58 clones, which showed the most intense signal in colony hybridization, were used to analyze the changes in the level of RNAs under cold conditions and the effect of other stress treatments (Fig. 1, Fig. 2). These six clones were different from one another based on the sizes of their

Fig. 1. Northern analysis of cold-accumulated RNAs. Twenty micrograms of total leaf RNA isolated before (left lanes) and after (right lanes) cold treatment (at 5 °C for 24 h) was applied per lane. The sizes of the bands are indicated in kilobases on the right handside.

transcripts and the expression patterns under cold or stress conditions. The characteristics of these clones are shown in Table 1. The seventh clone in Fig. 1, IRC202 (irresponsive to cold), is an "internal control" clone isolated from the non-subtracted cDNA library. The RNA level which did not increase by the cold treatment was similar to that of the majority of the cDNA clones in the non-subtracted cDNA library.

2. Changes in the level of RNAs under cold conditions and effect of other stress treatments

Examination of the level of six cold-accumulated RNAs during the initial two days of cold treatment indicated that the RNA level increased within 3 h, then either peaked during 3 to 24 h and started to decrease (in RC42, RC167 and RC179), or remained high during the two-day period (in RC104, RC114 and RC235) (Fig. 2 (a)). When plants were returned to 28 °C following one day of cold treatment, the RNA level of these clones decreased to the original level within 6 to 12 h (Fig. 2 (a)). The RNA level of three clones, RC167, RC179 and RC235, was hardly detected before and after cold treatment.

Some of the cold-accumulated RNAs (RC42, RC104,

Table 1. Characterization of RC clones

	RC42	RC104	RC114	RC167	RC179	RC235	IRC202
cDNA (kb)	0.17	0.19	0.36	0.34	0.27	0.10	0.22
RNA (kb)	3.4	1.1*	3.4*	3.8	5.0	2.4	3.6*
	1.5*	0.72	1.0	2.7*	3.3	1.8	
				1.7	2.4*	0.85*	
				1.2	1.5		

*; The band detected most strongly in the Northern experiment.

RC114, RC235) were also responsive to some extent to drought, ABA or NaCl treatments, while two (RC167, RC179) hardly responded to these treatments (Fig. 2 (b)).

3. Chilling injury and cold-induced accumulation of RNAs in 22 rice varieties

The relationship between chilling injury and cold-induced accumulation of RNAs was examined using 22 rice varieties. Preliminary experiments showed that when plants were reincubated at 28 °C after 1 or 2 days of cold treatment at 5 °C, no obvious chilling injury was observed even in the most chilling-sensitive varieties. On the other hand, after 10 days of cold treatment,

plants in the most cold-tolerant varieties died. Therefore, cold treatment for four days was adopted to evaluate the chilling sensitivity. Fig. 3 and Table 2 show the level of RNAs detected by cDNA probes under 24 h of cold treatment and the sensitivity in 22 rice varieties, respectively. RC167 clone showed RNA accumulation specifically in the chilling-tolerant varieties (varieties No. 1 - 11 and 14 in Fig. 3). We also detected exceptional RNA accumulation in the two chilling-sensitive varieties (Local Sardri (No. 12) and Jamuna (No. 20)). In contrast, RC235 showed a higher accumulation in the chilling-sensitive varieties (varieties No. 12 - 13 and 15 - 22 in Fig. 3) than in the tolerant varieties. These results suggest that the accumulation of these two RNAs was associated with the chilling sensitivity.

Discussion

We were able to isolate cDNA clones of cold-responsive genes effectively from a subtracted library. Cold-responsive genes or their cDNAs have been isolated in many plants (reviewed by Thomashow 1990). However, all the RC clones we isolated in this report were new cDNAs based on the partial nucleotide sequences and the deduced amino acid sequences (data not shown). No significant sequence homology was detected in the computer-homology search of the nucleotide and amino acid sequences in relation to GenBank, EMBL, PIR or SWISS-PROT database. Some of the cold-responsive genes we isolated were also responsive to other stresses. It has been suggested that ABA was involved in cold or drought tolerance (Thomashow 1990). Salt

Fig. 2. Dot blot analysis of RNA levels in (a) cold treatment at 5 °C, and (b) drought, abscisic acid (ABA) and NaCl treatments. Seedlings were exposed to low temperature for different periods of time (3, 6, 12, 24, and 48 h) or subjected for 24 h to drought, ABA or NaCl treatments as indicated on the top. Twenty micrograms of total RNA isolated from leaves was blotted on nylon membranes and hybridized with cDNA probes of the cold-responsive genes indicated on the left handside.

Table 2. Chilling sensitivity at seedling stage in 22 rice varieties

No.	Variety	Chilling sensitivity score		
		Exp. I	Exp. II	Average
1	Senshou	2	2	2.0
2	USSR22	1	0	0.5
3	Somewake	2	1	1.5
4	Hayayuki	2	1	1.5
5	Koshihikari	2	1	1.5
6	Lijiang Xintuan Heigu	1	0	0.5
7	Silewah	2	1	1.5
8	Bomba 1	2	1	1.5
9	Nipponbare	3	2	2.5
10	Norin33	2	2	2.0
11	Toyohatamochi	3	2	2.5
12	Local Sardri	4	3	3.5
13	T246	3	5	4.0
14	Ketan Nangka	3	2	2.5
15	Taichung Native 1	4	5	4.5
16	Er Jiu Qing	4	5	4.5
17	Nepal 556	5	4	4.5
18	Kele	4	3	3.5
19	Dular	4	3	3.5
20	Jamuna	5	4	4.5
21	IR8	5	4	4.5
22	IR36	5	5	5.0

Fig. 3. Cold accumulation of RNAs in 22 rice varieties. Seedlings of 22 rice varieties (shown in Table 2) were exposed to cold (5 °C) and at 100 % relative humidity for 24 h. Twenty micrograms of leaf RNA was blotted on nylon membranes and hybridized with cDNA probes of the cold-responsive genes indicated on the left handside.

stress has also been reported to activate ABA- and drought-inducible genes (Mundy and Chua 1988). All of these stresses share a common aspect in that they lower the water potential. The fact that cold-responsive genes, RC42, RC104, RC114 and RC235 were induced by ABA, drought or NaCl treatments also suggests the existence of a common pathway that is generated by these physiological factors. However, the fact that some of the cold-responsive genes were induced only by cold treatment suggests that a specific pathway was generated by cold treatment, and not mediated by ABA. RC167 and RC179 are interesting in this regard.

It is anticipated that some of the cold-accumulated RNAs play a role in the chilling injury or cold tolerance of seedlings. To analyze the relationship between the accumulation of the specific RNAs and cold tolerance, it is important to compare plants closely related genetically (Guy *et al.* 1985). Among the genetic studies on chilling sensitivity at seedling stage in rice, Nagamine and Nakagahra (1990) reported that the viability of seedlings after 4 days of cold treatment was controlled mainly by a single dominant gene. We could detect some association between the accumulation of the specific RNAs and cold tolerance. However, there were also exceptional data. RFLP analysis or transgenic experiments relating to the genes of RC167 and RC235 should be conducted for further examination of the relationship between the RNA accumulation and chilling sensitivity. These RNAs were characteristic in that they were hardly detectable before cold treatment and their levels remarkably increased in response to cold.

Binh and Oono (1992) reported that two RNA components showed a substantial accumulation during several days of cold treatment in a chilling-tolerant japonica rice. mRNA levels of the enzymes of the glycolytic pathway also increased after several days in response to cold (Christie *et al.* 1991). Such a gradual increase of the level of specific mRNA may be related to adaptive changes of the cellular metabolism (Guy *et al.* 1985). In contrast, we isolated cDNA clones of rapidly-accumulated RNAs. Especially, mRNA levels of RC167 and RC179 started to decrease in one or two days of cold treatment. Such genes may be involved in early response to cold. It is necessary to study both rapidly- and slowly-accumulated RNAs to identify the genes involved in chilling injury or cold tolerance.

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