Accumulation of Maize Response Regulator Proteins in Mesophyll Cells after Cytokinin Treatment

Atsushi DEJI,1 Hitoshi SAKAKIBARA,2,† Shinya OKUMURA,1 Tsukasa MATSUDA,1
Yuji ISHIDA,3 Shigehiro YAMADA,3 Toshihiko KOMARI,3 Tomoaki KUBO,3
Tomoyuki YAMAYA,2 and Tatsuo SUGIYAMA1,2

1Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
2RIKEN (The Institute of Physical and Chemical Research), Plant Science Center, 1-7-22, Suehiro, Tsurumi, Yokohama 230-0045, Japan
3ORYNOVA K.K., 700 Higashibara, Iwata, Shizuoka 428-0802, Japan

Received February 25, 2002; Accepted April 5, 2002

The maize response regulator genes ZmRR1 and ZmRR2 respond to cytokinin, and the translated products seem to be involved in nitrogen signal transduction mediated by cytokinin through the His-Asp phosphorelay. To elucidate the physiological function of the proteins, we examined the temporal and spatial distribution in maize leaves by immunochemical analysis and use of transgenic plants. ZmRR1 and ZmRR2 polypeptides could be distinctively detected by western blotting. The polypeptides accumulated in leaves within 5 h of the supply of nitrate to nitrogen-depleted maize, and the accumulation was transient. The extent of induction was larger in the leaf tip, which is rich in photosynthetically matured cells, than elsewhere. In leaves, the polypeptides accumulated mostly in mesophyll cells. Histochemical analyses of transgenic maize harboring a ZmRR1 promoter-β-glucuronidase fusion gene also showed most of the expression to be in these cells. These results suggest that ZmRR1 and ZmRR2 are induced in mesophyll cells and function in nitrogen signal transduction mediated by cytokinin.

Key words: cytokinin; His-Asp phosphorelay; mesophyll cell; response regulator; Zea mays

Cytokinin is involved in various processes of growth and development of plants, such as cell division, chloroplast differentiation, inhibition of leaf senescence, regulation of photosynthesis, and nutrient metabolism. The expression of various genes probably is modulated by the action of this phytohormone, and in fact, many cytokinin-regulated genes have been reported. In terms of the signaling pathway, the His-Asp phosphorelay system seems to be involved in perception and signal transduction in higher plants. Three genes for the sensory His-protein kinase homolog, AHK2, AHK3, and AHK4 (CRE1), have been identified as genes encoding cytokinin receptors in Arabidopsis thaliana. We earlier isolated cDNAs, designated ZmRR1 and ZmRR2, that encode the response regulator domain in maize. The accumulation of the transcripts starts within 30 min of the start of treatment of detached leaves with trans-zeatin (t-zeatin), a naturally occurring cytokinin, and in whole maize plants, accumulation of the transcripts begins when inorganic nitrogen sources are supplied. We analyzed stable maize transformants harboring the ZmRR1 promoter-β-glucuronidase (GUS) fusion gene, and attributed the accumulation to transcriptional regulation. Similar changes have been found in response regulator genes in A. thaliana. On the other hand, in terms of the metabolic response of cytokinin to nitrogen availability, significant correlation between the cytokinin level and the nitrogen nutritional status has been found in several plant species: Urtica dioica, barley, and maize. In maize, isopentenyladenosine-5-monophosphate, a cytokinin, rapidly accumulates in roots, and later t-zeatin riboside and t-zeatin accumulate in the xylem sap and leaf, respectively. These findings suggest that cytokinin may be a mediator conducting the nutritional signal from root to shoot, and that the His-Asp phosphorelay system may be involved in the signaling pathway in the leaves.

One characteristic of the expression of response regulator genes in both maize and A. thaliana is that the cytokinin-responsive accumulation of the transcript is transient. The level of transcripts decreases to the initial level within several hours even during continuous treatment with nitrogen, cytokinin, or

† To whom correspondence should be addressed. Fax: +81-45-503-9609; E-mail: sakaki@postman.riken.go.jp

Abbreviations: GUS, β-glucuronidase; t-zeatin, trans-zeatin; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
both. Characterization of the accumulation patterns at the protein level is needed before discussion of the physiological significance of this profile.

Maize, a C₃ plant, has two kinds of photosynthetic cells, bundle sheath cells and mesophyll cells. The enzymes involved in nitrogen and carbon metabolism are distributed differently in these two kinds of cells, so their nutritional and hormonal signaling systems may differ. At present, there is no information about the cellular distribution of His-Asp phosphotransfer factors in maize leaf. We wanted to examine the spatial distribution of the signaling pathway including the response regulators to better understand their physiological functions. In this study, we found a difference in the response of the genes in maize to the cytokinin-mediated nitrogen signal between the two kinds of cells, and these differences suggest functional differences of the cells in the signaling pathway.

Materials and Methods

Plant materials and growth conditions. Maize (Zea mays L. cv. Golden Cross Bantam T51) plants were grown for about 15 days in vermiculite with Hoagland’s nutrients with a limited (0.8 mM NaNO₃) supply of nitrogen under fluorescent light, at an intensity of about 700 μE m⁻² s⁻¹, in a growth chamber (Koitotron KG-201SHL-D, Koito Industries, Tokyo). The photoperiod was 14 h of light and 10 h of dark and the temperature was 28°C in the light and 20°C in the dark. Leaves were harvested after 16 mM nitrate was supplied at the indicated times in the figures, or leaves were detached and treated with 5 μM t-zeatin as described previously.

Stable transformants of the maize line T1.6.8, has a GUS reporter gene driven by a 3.4-kb ZmRR1 promoter region, were grown in the same growth chamber.

Preparation of monoclonal antibodies against ZmRR1. Monoclonal antibodies were prepared essentially as described by Matsuda and Kabat. BALB/c mice were immunized with recombinant His-ZmRR1 emulsified in Freund’s adjuvant. Spleenocytes were extracted and fused with P3 myeloma cells by the polyethylene glycol method. Hybridomas were screened for production of antibodies with the desired properties by an enzyme-linked immunosorbent assay, and subcloned by limiting dilution. For large-scale preparation of the antibodies, the hybridoma was injected intraperitoneally into CDF mice that had been primed 1–4 days earlier with pristane (2,6,10,14-tetramethylpentadecane), and the ascites fluid was harvested.

Expression plasmids. Complementary DNAs containing the reading frame of ZmRR1 and ZmRR2 were excised from pQEZmRR1 and pQEZmRR2, respectively, with NcoI and HindIII digestion. Each fragment was inserted at the NcoI/HindIII site of pTrc99A vector (Amersham Pharmacia Biotech). The JM109 strain of Escherichia coli harboring pREP4 (Qiagen) was used as the host for protein expression.

Extraction of proteins and western blotting. About 1 g of leaves was ground in a mortar with a pestle with a small amount of quartz sand in one volume of ice-cold extraction buffer A (50 mM Na-phosphate (pH 8.0), 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 10 mM 2-mercaptoethanol). The homogenate was centrifuged at 12,000 × g for 5 min at 4°C. The resulting supernatant was analyzed by western blotting. The antigen-antibody complexes on the polyvinylidene difluoride membrane (Immobilon; Millipore) was made visible by reaction with alkaline phosphatase conjugated to goat antibodies raised against mouse IgG. The protein concentration in the supernatant was assayed by the method of Bradford with bovine serum albumin as the standard.

Isolation of mesophyll cells and bundle sheath cells. Mesophyll cells and bundle sheath cells were isolated by the method of Ohnishi and Kanai from about 15 g of tissue. The resultant populations of cells were washed twice or more with a solution of 0.5 M sorbitol, 10 mM HEPES-KOH (pH 7.0), 1 mM MgCl₂, and 1 mM CaCl₂ to remove as much bovine serum albumin as possible. The total extracts from both populations of cells were obtained by homogenization in glass homogenizers with extraction buffer A and centrifugation to remove insoluble materials.

Histochemical reporter analysis. A mature leaf of a transformant was cut off and divided into two parts at the main vein. Five μM t-zeatin was sprayed on one segment and water was sprayed on the other. The parts were incubated on moistened filter paper with distilled water for 7 h at 30°C. The tissues were cut into small pieces approximately 5 mm square and further incubated in staining buffer [100 mM Na-phosphate (pH 7.0), 1.9 mM 5-bromo-4-chloro-3-indoly-β-d-glucuronic acid (X-Glu), 20% methanol, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 0.3% Triton X-100] for 50 h at 37°C. After incubation, the leaf parts were soaked in 80% ethanol for 50 h to decrease chlorophyll color, and then were embedded in 5% agar. The blocks were sectioned with a microtome (DTK-100, Dosaka EM, Kyoto, Japan) and examined with a microscope (Olympus BX60).

Results

Separation and immunological detection of
Expression of Maize Response Regulators

ZmRR1 and ZmRR2 proteins

For the immunological study, monoclonal antibodies against recombinant ZmRR1 were prepared because the polyclonal antibodies previously raised cross-reacted with other polypeptides. We selected a hybridoma line R12 that excreted IgG that reacted with both ZmRR1 and ZmRR2. Figure 1 shows the results of the immunological detection of ZmRR1 and ZmRR2 proteins extracted from E. coli transformants and from maize leaves. Although they had similar calculated molecular masses (ZmRR1, 16.7 kDa; ZmRR2, 17.0 kDa), the migration of ZmRR2 on the SDS-polyacrylamide gel was slower than that of ZmRR1 (lanes 2 and 3). The two recombinant proteins had almost the same band intensity. In the leaf extracts, two polypeptides with mobilities corresponding to those of the recombinant proteins accumulated after cytokinin treatment (lanes 4 and 5). The signal intensity of ZmRR2 in maize leaves was about one-third that of ZmRR1. This result corresponded well with the results about transcript levels. Therefore, we used this antibody as a probe for simultaneous detection of both ZmRR1 and ZmRR2 polypeptides.

Changes in amounts of ZmRR polypeptides during replenishment

A recent study showed that t-zeatin in leaves increases within 4 h after nitrate is supplied to nitrogen-depleted maize, and that the elevated level is maintained even after 24 h. The accumulation of transcripts of ZmRR1 and ZmRR2 was simultaneous, and decreased to below the detectable level after 24 h. Polypeptide(s) detectable with the polyclonal antibodies accumulate in 4 h in response to nitrate replenishment, but changes thereafter were not reported. In an examination of how the effects of nitrogen supplement on proteins, changes during nitrate resupply were monitored by western blotting.

Changes in time in accumulation of ZmRR polypeptides in leaves of maize supplied with nitrogen. Total proteins were extracted from leaves at the indicated times after the supply of nitrate began (Arnon-Hoagland medium with 16 mM nitrate; +Nitrate). In the control treatment, the medium was the same, without nitrate (−Nitrate). The amount of protein put in each lane of the gels was 80 μg.

Spatial accumulation of ZmRR polypeptides in leaves during recovery from Nitrogen Deficiency

Leaves of nitrate-replenished (+) or control (−) maize were divided into three parts, basal (Base), middle (Mid), and tip (Tip). Total extracts (80 μg of protein) of the parts were subjected to SDS-PAGE and ZmRR polypeptides were made visible by western blotting.

Spatial accumulation of ZmRR polypeptides in leaves during nitrate replenishment

In monocotyledonous leaves, cells mature photosynthetically as a function of the distance from the base. To elucidate the spatial difference in ZmRRs accumulation in the nitrogen recovery process, the youngest fully developed leaves, which were the third leaves of nitrate-replenished maize plants, were divided into three parts, base, middle, and tip, and the amounts of the ZmRR polypeptides were examined (Fig. 3). Total proteins were extracted from the tissues and the polypeptides were detected by western
Histochemical Location of GUS Activity in Leaves of Transgenic Maize Plants.
(A, B) Cytokinin-dependent induction of GUS activity in mesophyll cells. Detached leaf blades of ZmRR1-GUS plants were treated with 1 μM t-zeatin (A) or water (B) for 7 h. Cross-sections were stained for GUS activity. MC, mesophyll cells; BSC, bundle sheath cells.

Accumulation of the ZmRR polypeptides in mesophyll cells in response to cytokinin

In a further check of the cellular location of the ZmRRs at the protein level, we prepared mesophyll cells and bundle sheath cells separately from cytokinin-treated and untreated leaves of wild-type maize, and detected the polypeptides by western blotting. The cross-contamination between the two kinds of cells was low, as judged by the distribution of the marker enzymes Rubisco and phosphoenolpyruvate carboxylase for bundle sheath cells and mesophyll cells, respectively. In cytokinin-treated leaves, ZmRR1 and ZmRR2 accumulated mostly in mesophyll cells; little accumulation was found in bundle sheath cells. When we analyzed the leaves of nitrate-replenished maize, results were similar (data not shown). These results indicate that the response regulator polypeptides ZmRR1 and ZmRR2 of maize leaves accumulate mostly in mesophyll cells.

Discussion

ZmRRs were transiently expressed in mature mesophyll cells in response to a cytokinin-mediated nitrogen signal. The different mobilities of ZmRR1 and ZmRR2 polypeptides on SDS-PAGE enabled us to monitor the accumulation of each protein separately. The proteins had the same accumulation pattern, suggesting that they have similar physiological roles in the same compartment.

The transient accumulation of the ZmRRs in response to nitrate replenishment implies that they function at an early stage of nitrogen recovery in maize leaves by increasing in amount. Both ZmRR1
and ZmRR2 have phosphotransfer activity, so the such increases would be reflected in the amplification of the cytokinin-mediated nitrogen signal in mesophyll cells through the His-Asp phosphorelay. As shown previously, treatment with cycloheximide, an inhibitor of cytosolic protein synthesis, causes greater accumulation of the transcripts for ZmRR1 and ZmRR2 and A. thaliana response regulators in response to cytokinin. These findings suggest that a repressor protein(s), the biosynthesis of which is up-regulated by cytokinin, may be involved in the transient expression.

Recently, the Arabidopsis response regulator gene ARR5 has been investigated in detail, and found to be expressed mostly in root and shoot apical meristems, at the junction of the pedicel and the silique, and in the central portion of mature roots, and expressed weakly in older leaves and cotyledons. We found that transcripts of ZmRR1 and ZmRR2 are abundant in tassels, ear, and leaf sheath of mature maize plants, and increases in leaf mesophyll cells in response to cytokinin. Neither the transcripts nor GUS activity were detected in any parts of the root (data not shown). The expression site of ZmRRs did not coincide with that of ARR5. We have found other maize response regulator genes, naming them ZmRR3 to ZmRR10 (Sakakibara et al., unpublished).

Expression of the ZmRRs mostly in mesophyll cells tempts us to speculate about the target factor(s) of the response regulators. Several genes have mesophyll-specific expression, and they are involved in nitrate reduction, C4 photosynthesis, photosystem II-related proteins, and so on. Some of them are regulated by nitrogen availability and cytokinin. In addition to metabolic differentiation, developmental differences in organelles, such as granum formation exclusive to mesophyll chloroplasts, also have been found. The signaling pathway including ZmRR1 and ZmRR2 may be involved in those phenomena.

Acknowledgments

This work was supported by Grants-in-aid for Scientific Research on Priority Areas (numbers 09274101 and 09274102 to TS, 12142202 to HS) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan and by Japan Tobacco Inc., Plant Breeding and Genetics Laboratory. A. Deji is a Research Fellow of the Japan Society for the Promotion of Science.

References


16) Takei, K., Sakakibara, H., Taniguchi, M., and


