Co-Expression of Antifungal Protein Genes in Transgenic Rice

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Introduction

Fungal diseases are the most serious problems in rice production throughout the world. The loss of rice yield due to fungal pathogens is estimated to be several billion dollars (Thurston, 1984). The most important fungal pathogens of rice include *Magnoportha grisie* and *Rhizoctonia solani* which cause rice blast and sheath blight, respectively. In particular, rice blast is generally considered as the principal disease of rice because of its worldwide distribution and its destructiveness under favorable conditions.

The control of fungal disease in modern rice cultivation is mostly achieved by the integrated pest management. Alternative strategies are being tested which aim to develop plants with a broad range resistance to fungal pathogens by genetic engineering (for review, see Cornelissen and Melchers, 1993; Strittmatter and Wegener, 1993).

Plants naturally respond to fungal attack by a complex network of defence mechanisms. These include the synthesis of polymers forming physical barriers (cutin, lignin, callose), of antimicrobial metabolites (phytoalexins), and of pathogenisis-related proteins (PR proteins). Hydrolytic enzymes, such as chitinases and b-1,3-glucanases which degrade fungal cell wall structural polysaccharides are well known PR proteins (Kombrink et al., 1988; Legrand et al., 1987). Three classes of plant b-1,3-glucanases and five classes of plant endochitinases have been described (Collinge et al., 1993; Melchers et al., 1994). Several of these enzymes have been shown to inhibit fungal growth in vitro, and their respective genes are therefore good candidates as antifungal genes. In fact, recent studies have shown that expression of the chitinases in transgenic plants can mediate increased protection against phytopathogenic fungi (Broglie et al., 1991; Lin et al., 1995).

Genes encoding ribosome-inactivating proteins (RIPs) are also candidates as defence transgenes. RIPs inactivate eucaryotic ribosomes by enzymatically cleaving the N-glycosidic bond of a specific adenine residue in the ribosomal 28 S RNA (Endo and Tsurugi, 1987; Endo et al., 1988). This irreversible modification renders the ribosome unable to bind elongation factor 1 a, thereby blocking translation. Cytosolic Type I RIPs from cereals are not significantly active on plant ribosomes in vitro

(Taylor et al., 1994), but readily modify foreign ribosomes, including those of fungi. Logemann et al. (1992) have shown that expression of a barley RIP resulted in an increased protection of transgenic tobacco plants against *Rhizoctonia solani* without affecting plant growth.

A stiking feature of response of plants to pathogen attack is the induction of a large number of genes encoding diverse proteins, many of which are believed to have a role in defence, suggesting that different protective mechanisms may have complementary roles in the overall expression of resistance. Leah et al. (1991) demonstrated that chitinases and b-1,3-glucanases, when they were combined, showed synergistic antifungal properties, leading to enhanced mycelial cell wall destructions in vitro. Evidence for synergistically enhanced protection in planta by co-expression of a rice basic chitinase and an acidic b-1,3-glucanase was shown by Zhu et al. (1994). It was further evidenced that combinatorial expression of a barley class II chitinase, a b-1,3-glucanase and a Type I RIP led to a significantly enhanced protection against fungi, indicating synergistic protective interactions of the co-expressed antifungal proteins in vivo (Jach et al., 1995).

To evaluate synergistic interactions between antifungal proteins in vivo and also to make transgenic rice plants hightened in antifungal activity, we chose to co-express of a rice basic chitinase gene plus RIP genes from other cereals in transgenic rice. To this end, we constructed two expression vectors containing either a rice basic chitinase plus a maize processed RIP genes or the rice basic chitinase plus a barley RIP genes on a single plasmid for each pair of combination. The two constructs were then cotransformed with a plasmid containing a herbicide resistance gene into rice by particle bombardment. Analysis of the first (R_0) and second (R_1) generation of transgenic rice plants revealed the integration of the transgenes, stable inheritance to their progenies in a Mendelian fashion and the high levels of expression of the transgenes in transformed rice plants.

Antifungal protein genes used for rice transformation

Purified barley RIP30 was shown to inhibit the growth of fungi assayed in vitro, this inhibition is synergistically enhanced in the presence of chitinases or (1-3)-b-glucanases which are known to degrade fungal cell wall polysaccharides (Leah et al., 1991). This synergistic inhibition suggests that inhibition by RIP is enhanced when hyphal cell walls are permeabilized by the action of the chitinases. Because of the synergistic effect of the RIP and chitinase on fungal growth inhibition, we chose to express the rice chitinase gene RCH10 (Zhu and Lamb, 1991) along with the maize MOD5 (Bass et al., 1992; Walsh et al., 1991) or the barley cRIP30 (Leah et al., 1991) genes in transgenic rice plants. RCH10 is a rice (*Oryza sativa*) gene encoding a basic chitinase whose transcripts normally accumulate to a high level in roots, but only low levels are found in stem and leaf tissue (Zhu and Lamb, 1991; Zhu et al., 1993). The rice chitinase gene together with an acidic b-1,3-glucanase gene from alfalfa was co-expressed in transgenic tobacco plants, generating an enhanced

protection against fungal attack (Zhu et al., 1994). A maize RIP, which is also called b-32 protein, is known to be synthesized and stored in the kernel as a 34-kDa inactive precursor. During germination, this neutral precursor is converted into a basic, active form by limited proteolysis, which removes 25 amino acids (2.8 kDa) from the center of the polypeptide chain (Walsh et al., 1991). Additional processing also occurs at the amino and carboxy termini of the polypeptide (Walsh et al., 1991). Dr. Boston's group in North Carolina State University modifed the b-32 cDNA (Pro-ZmcRIP3a) into a processed form of it, naming it MOD5 as shown in Fig. 1. They removed 135 bp

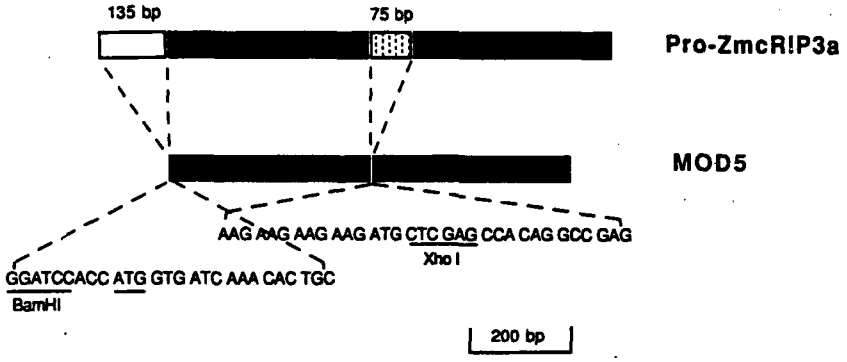


Fig. 1. Schematic representation of Pro-ZmcRIP3a and MOD5 cDNAs. MOD5 was made by removing of 135 bp from the 5′ end and 75 bp from the center of Pro-ZmcRIP3a by a series of polymerase-chain reactions.

from the 5′ end and 75 bp from the center of Pro-ZmcRIP3a by a series of polymerase-chain reactions, generating MOD5. MOD5 protein expressed in *E. coli* was proven to have an RIP activity at similar level to a naturally processed form of the b-32 protein in germinating maize kernels(Hey et al., 1995). cRIP30 is a barley *(Hordeum vulgare* L.) gene encoding a ribosome-inactivating protein which accumulates to high levels only in the starchy endosperm during late seed development (Leah et al., 1991). Expression of the cRIP30 gene in transgenic tobacco resulted in an increased protection of the plants against *Rhizoctonia solani*, evidencing an antifungal activity of the protein in vivo (Logemann et al. (1992). More recently, co-expression of the cRIP30 gene in combination with a barley class II chitinase gene or a barley b-1,3-glucanase gene led to synergistic protective interactions of the co-expressed antifungal proteins in vivo (Jach et al., 1995).

Construction of two expression vectors containing a rice chitinase and RIP genes from other cereals

We constructed two expression vectors containing the rice chitinase gene RCH10 (Zhu and Lamb, 1991) in combination with either the maize MOD5 gene (Bass et al., 1992; Walsh et al., 1991) or the barley cRIP30 (Leah et al., 1991) gene. The vectors

were made in such a way that chitinase and RIP can be expressed in leaf and sheath tissues at the same stage of development of transgenic rice plants.As shown in Fig. 2, one plasmid called pZRC72 contains the coding region of the rice basic chitinase gene

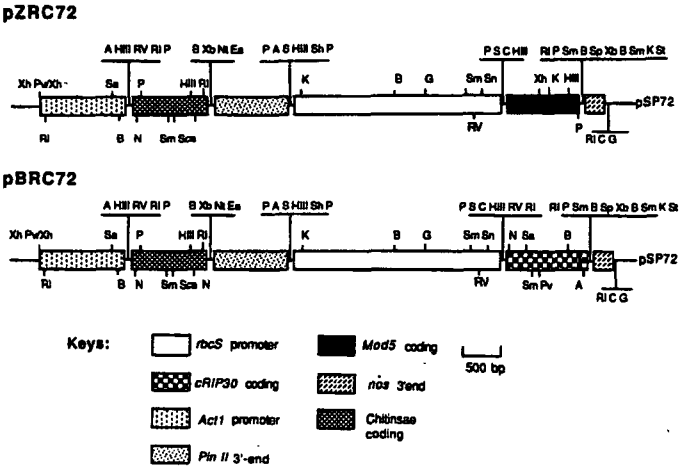


Fig. 2. Schematic representatiom of the plasmids, pZRC72 and pBRC72. pZRC72 consists of the rice Act1 5′ region (1.3 kb) including the promoter, first noncoding exon, and first intron, the coding region of the rice chitinase gene RCH10 (1 kb), and 3′ region (1.0 kb) of the potato proteinase inhibitor II gene plus the rice rbcS promoter (2.8 kb) (Kyozuka et al., 1993), the processed maize RIP gene MOD5 (0.9 kb) and nos 3′ end (0.26 kb). pBRC72 consists of the same cassette as in pZRC72 except that the maize RIP coding region was replaced with the barley cRIP30 cDNA (1.0 kb) (Leah et al., 1991). Restriction sites are abbreviated as follows: A, AccI; B, BamHI; C, ClaI; Ea, EagI; G, BglII; HIII, HindIII; K, KpnI; Nt, NotI; P, PstI; Pv, PvuII; RI, EcoRI; RV, EcoRV; S, SalI; Sa, SacI; Sca, ScaI; Sh, SphI; Sm, SmaI; Sp, SpeI; St, SstI; Xh, XhoI; Xb, XbaI.

RCH10 (Zhu and Lamb, 1991) with rice Act1 5′ region and potato proteinase inhibitor II 3′ end along with the coding sequence of MOD5 (provided by Dr. Boston at North Carolina State University) with rice rbsS promoter and nos 3′ end. The other plasmid called pBRC72 consists of the same cassette as in pZRC72 except that the MOD5 region from maize was replaced with the cRIP30 cDNA from barley (Leah et al., 1991).

Transformation of suspension culture cells and plant regeneration

Rice suspension culture cells were bombarded with tungsten particles coated with the two RIP containing plasmids, pZRC72 and pBRC72 (Fig. 2), and the cells were

allowed to grow for 6 days on KPR solid medium prior to transfer to KPR solid medium containing 7 mg/l glufosinate ammonium. The emergence of resistant cell colonies was observed after 6-7 weeks of continuous culture on selective medium, with subculturing every two weeks. The two plasmids are both effective in conferring herbicide resistance to the calli. No resistant calli were observed in cells bombarded with naked microprojectiles, after subjection to these selection conditions.

Resistant calli were transferred to regeneration medium containing 4 mg/l of glufosinate ammonium. Shoots and roots emerged from resistant calli on the medium within 2-3 weeks and plantlets were subsequently regenerated. Resistant plants continued to grow on selective MS medium with the development of multiple roots and tillers. When the plantlets reached a height of about 15 cm, they were tranferred to soil. Leaves of resistant plants, as well as untransformed plants, were painted with 0.5%(V/V) solution of the PPT-based herbicide Basta. The subsequent growth and development of resistant plant leaves after painting appeared normal whereas untransformed control plant leaves turned yellow and died. A total of 58 independent lines of transformants for two RIP-containing constructs were regenerated and transferred to soil.

Southern-blot analyses of primary (R_0) transgenic plants

Integration of the transferred genes into the rice genome of 23 transformed R_0 plants (16 for pZRC72 and 7 for pBRC72 constructs) was determined by genomic DNA-blot analysis. Total genomic DNA was isolated from leaf tissues of the primary transformants. The DNA from transformants with pZRC72 was digested either with HindIII which excised the 0.6 kb MOD5 fragment or with EcoRV which cut only site within the region of MOD5 expression cassette of the plasmid pZRC72. Gel-blot analyses with HindIII digested DNA from 15 transformants showed hybridized bands when probed with ^{32}P-labeled 0.6 kb HindIII-HindIII fragment of the MOD5 gene. Ten out of 15 Southern-positive lines appear to contain at least one intact copy of the MOD5 gene while 5 lines contain rearranged copies of it (Fig 3). The DNA from transformants with pBRC72 were similarly analyzed. When hybridized with ^{32}P-labeled 1.0 kb EcoRI-EcoRI fragment of the cRIP30 gene, 4 out of 6 transgenic lines appear to contain at least one intact copy of the cRIP30 gene while 2 lines contain the rearranged copies of it (data not shown). Many of the plants analyzed had additional rearranged copies integrated into the genome as shown in Fig. 3. Multiple copies of foreign genes are integrated into rice genome, as determined by digesting DNA either with EcoRV for the MOD5 gene (Fig. 3) or with HindIII for the cRIP30 gene.

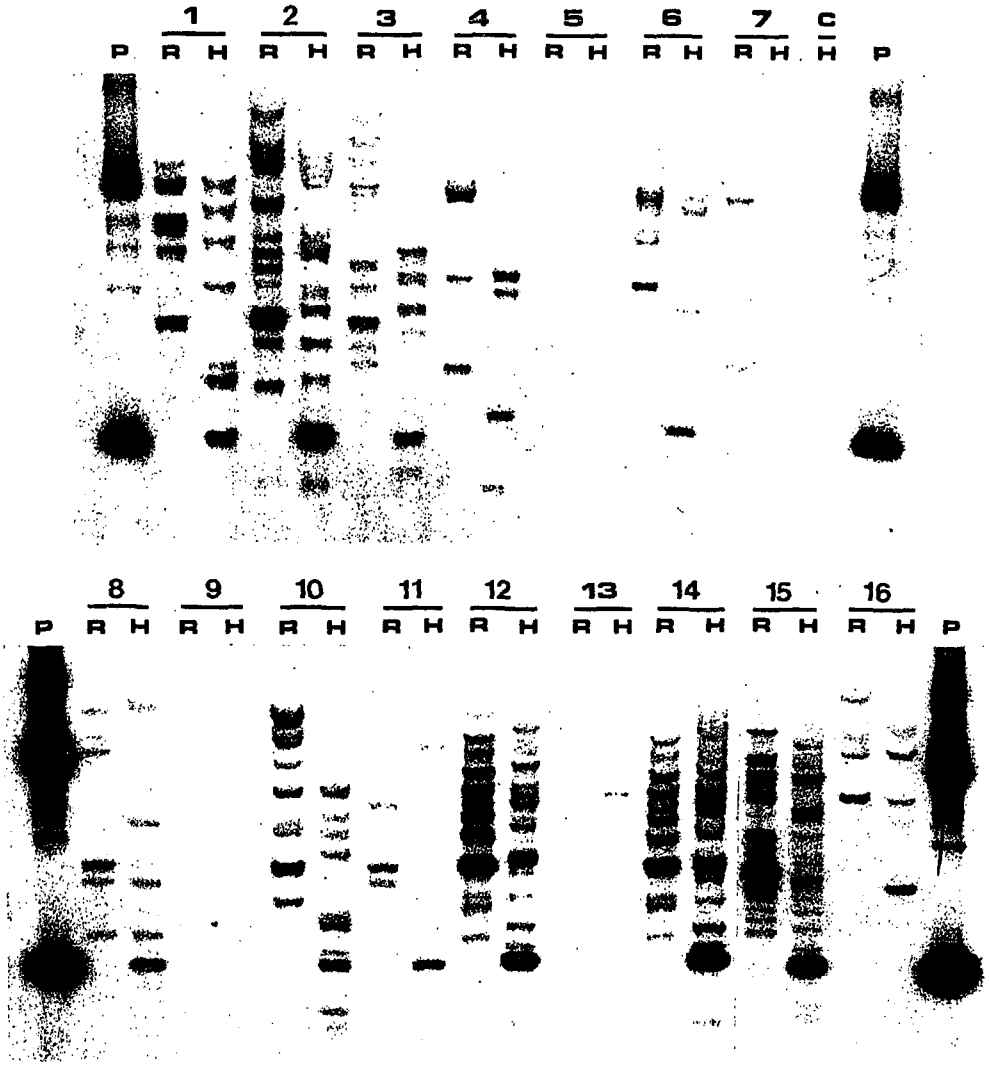
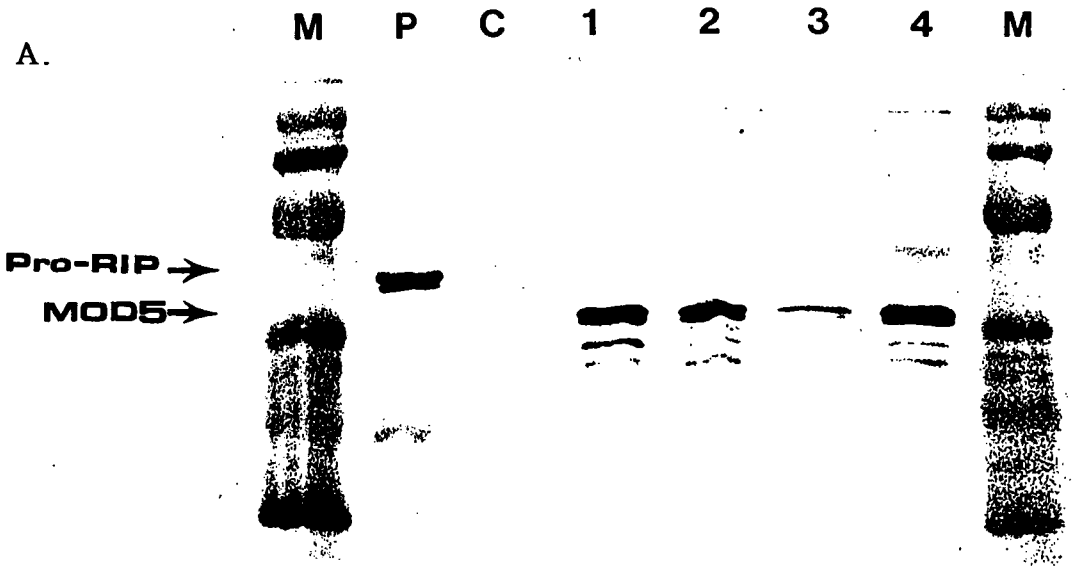


Fig. 3. DNA gel-blot analysis of pZRC72-transformed transgenic rice plants. Genomic DNA isolated from the leaf tissues of T1-T16 transgenic rice plants and from an untransformed control plant (C) was digested with HindIII (H), EcoRV (R) and hybridized with a 32P-labeled HindIII-HindIII digested MOD5 containing restriction fragment (0.6 kb) from pZRC72. P contains HindIII digested pZRC72. Transgenic lines: Lane1, K/ZR2; lane 2, K/ZR1; lane 3, N/ZR8; lane 4, N/ZR7; lane 5, N/ZR6; lane 6, N/ZR5; lane 7, N/ZR2; lane 8, N/ZR13; lane 9, N/ZR12; lane 10, N/ZR11; lane 11, N/ZR10; lane 12, N/ZR9; lane 13, N/ZR4; lane 14, N/ZR3; lane 15, N/ZR15; lane 16, N/ZR14.

Expression of the maize MOD5 and barley cRIP30 genes in R_0 transgenic rice leaves

The presence of the maize MOD5 and barley cRIP30 proteins in the Ro transgenic plants was determined by Immuno-blot analysis of leaf protein extracts, using rabbit antisera raised against preparations of purified maize pro-RIP and barley cRIP30 protein. Seven independent transgenic plants were selected and proteins were extracted from their leaves. The leaf extracts were separated on SDS-polyacrylamide gel, transferred to a PVDF membrane, and the RIPs were detected by an alkaline phosphatase conjugated goat anti-rabbit antibody after the binding of primary antibodies specific to either the maize pro-RIP and barley cRIP30. As shown in Fig. 4A, the expressed MOD5 was clearly detectable in all the transgenic plants analyzed with various levels of expression. By comparing band intensities and migrations with that of known amount of the purified protein, we detected the expected size (29 kDa) of the MOD5 protein and we estimate MOD5 approximately 0.5% of total soluble protein in the leaves of the highly expressing transgenic plants. This value is 10-fold higher than the expression level of the modified cry1A(b) detected in insect-resistant transgenic rice plants (Fujimoto et al., 1993) and is comparable to the expression of barley cRIP30 detected in fungal-resistant transgenic tobacco plants (Logemann et al., 1992; Jach et al., 1995). The expressed cRIP30 protein was also clearly detectable in expected size of 30 kDa in two out of three transgenic plants analyzed with differing levels of expression (Fig. 4B).



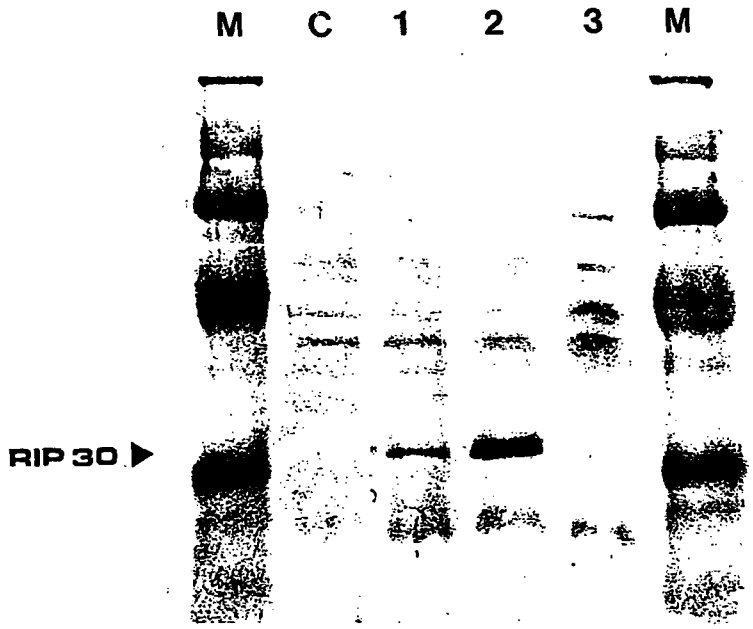


Fig. 4. Immunoblot analysis of 7 primary (Ro) transgenic rice plants. The leaf extract (40 ug of protein per lane for A and B were separated on 12 % polyacrylamide gels containing 0.1 % SDS, transferred to PVDF membrane, RIPs were detected by an alkaline phosphatase conjugated goat anti-rabbit antibody after the binding of an antibody against purified maize pro-RIP for A and purified barley cRIP30 for B. In A, lanes 1, K/ZR1, 2, K/ZR2; 3, N/ZR5; 4, N/ZR8; P, purified maize RIP(60 ng). In B, lanes 1, N/BR1; 2, N/BR3; 3, N/BR6. In A and B, C, untransformed rice nipponbare (40 ug of protein); M, molecular weight marker from Bio-Rad.

Expression of the rice basic chitinase gene RCH10 in R_0 transgenic rice leaves

Expression of the rice chitinase gene RCH10 in leaf tissues of R_0 transgenic plants was examined by investigating levels of chitinase enzyme activity of leaf protein extracts using the native polyacrylamide(PAGE)-overlay gel method (Pan et al., 1991). Five independent transgenic plants were selected and proteins were extracted from their leaves. The leaf extracts were separated on 15% native polyacrylamide gel. After electrophresis, an overlay gel containing glycol chitin as substrate for chitinase was incubated in close contact with the resolving gel. Chitinase isozymes were revealed by UV illumination after staining the overlay gel with fluorescent brightener 28. RCH10 transcripts are known to accumulate to a high level in roots, but only low

levels are found in stem and leaf tissue (Zhu and Lamb, 1991; Zhu et al., 1993). The expressed RCH10 proteins were therefore identified by comparing isozyme patterns of between protein extracts from transformed plant leaves and that from untransformed plant leaves as shown in Fig. 5. There are four major chitinase isozymes detected in

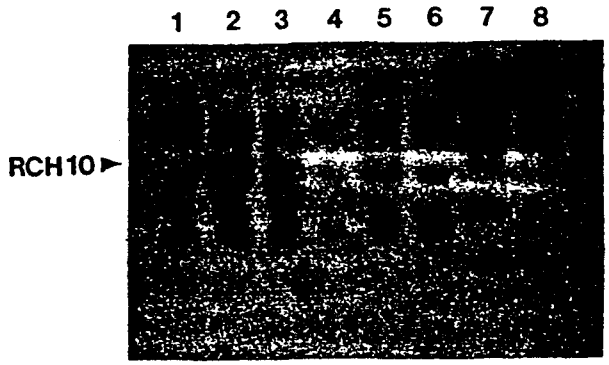


Fig. 5. Detection of RCH10 chitinase activity of 5 primary (Ro) transgenic rice plants. The leaf extracts from transformed (lanes 1-5) and untransformed plants (lane 6) were separated on a 15% native polyacrylamide gel. After electrophresis, an overlay gel containing glycol chitin as substrate for chitinase was incubated in close contact with the resolving gel. Chitinase isozymes were revealed by UV illumination after staining the overlay gel with fluorescent brightener 28. Lanes (40 ug of protein per each lane) 1, N/BR4; 2, N/BR3; 3, N/ZR8; 4; K/ZR2; 5, K/ZR1; 6, untransformed control; 7 and 8, 0.3 and 0.03 units of chitinase from Streptomyces griseus.

protein extracts from untransformed plant leaves (Fig. 5, lane 2) whereas an additional band is clearly detectable in protein extracts from transformed plant leaves (Fig. 5, lanes 3-6) with differing levels of activity.

Analysis of R_1 progeny plants

The primary transformants containing transgenes, pZRC72 and pBRC72, were grown to maturity in the green house. R_1 progenies of the lines, K/ZR2 and N/BR3, were analyzed. Mature seeds collected from each R_0 transformant were germinated to obtain R_1 progeny. Segregation of the bar gene in one-month-old progeny was determined by the localized application of a 0.5%(V/V) Basta solution on young leaves, revealing that segregation ratio are 18:9 and 16:5 in R_1 progenies of K/ZR2 and N/BR3, respectively. Southern blot analysis of 13 R_1 herbicide-resistant plants from the K/ZR2 was performed using two restriction enzymes HindIII and EcoRV as described in analysis of R_0 plants. The Southern blot results revealed the presence of four copies of the MOD5 gene, one appears to be intact and the others are rearranged.

The MOD5 hybridization patterns of R_1 progeny plants were the same as those observed in the R_0 parental plant in terms of gene configuration and copy number of the transgene, indicating that transgene had not undergone any further rearrangements during meiosis. None of four copies of the transgene were segregated from one another in R_1 progenies, suggesting that the four copies were integrated into the same chromosome. In addition, the segregation of the transgene in R_1 progeny plants was in complete agreement with the segregation data obtained from herbicide testing.

The expression of the maize MOD5 gene in the R_1 transgenic plants was determined by Immuno-blot analysis of leaf protein extracts, using rabbit antisera raised against each preparation of purified a (17 kDa) and b (13 kDa) processed fragments of maize pro-RIP. The antisera was provided by Dr. Timothy Hey at DowElanco, who found the processing of the maize pro-RIP of our interest. Sixteen R_1 progenies from the line K/ZR2 were selected and proteins were extracted from their leaves. As shown in Fig. 6, the expressed MOD5 was clearly detectable at similar levels of expression in all the progeny plants analyzed. The levels of expression of MOD5 in the progenies are also simliar to that of their parental line K/ZR2.

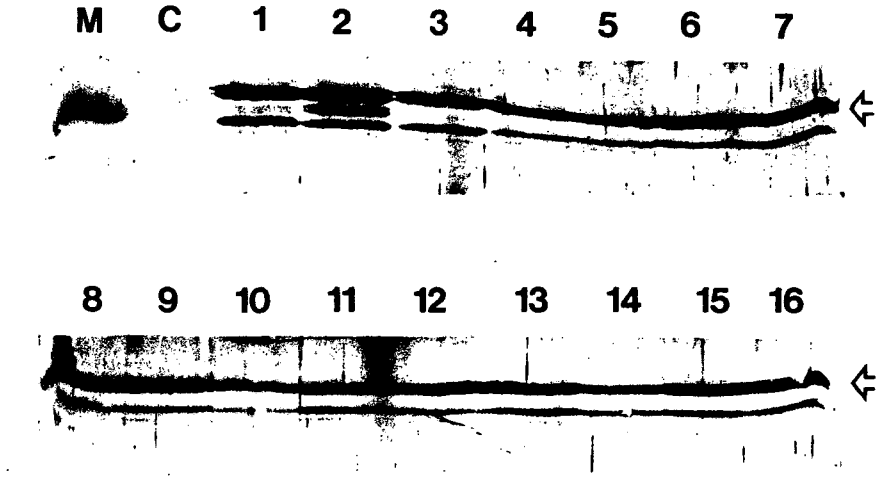


Fig. 6. Immunoblot analysis of 17 R1 progenies of K/ZR2 lines. The leaf extract (40 ug of protein per lane) were separated on 12 % polyacrylamide gels containing 0.1 % SDS, transferred to PVDF membrane, RIPs were detected by an alkaline phosphatase conjugated goat anti-rabbit antibody after the binding of an antibody against purified processed products of Zmcrip3a. Lanes 1-17 are 17 different progenies from the line ZR2. C, untransformed rice (40 ug of protein); M, molecular weight marker from Bio-Rad.

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