

# Transformation of rice for producing agronomically useful transgenic plants and/or analyzing gene expression, including the gibberellin-dependent signaling pathway

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Transformation of rice cells followed by regeneration of fertile plants constitutes a new method for producing agronomically useful transgenic rice plants. We have transformed rice with a plasmid harboring a potato protease inhibitor gene (*PINI*) joined to a wound-inducible *pin2* promoter. The transgenes in plants of the  $R_0$ ,  $R_1$ , and  $R_2$  generations have been analyzed, and the results showed that the transgene is stably inherited. Transgenic plants have also proven to be useful in analyzing the expression of a gene or a modified gene. Transfer of genes into rice cells, followed by determining the amount of the gene products several hours later, constitutes a convenient way to analyze transient gene expression. We have used this transient assay to look for gibberellin (GA)-responsive sequences in GA-induced genes such as the rice  $\alpha$ -amylase gene, *Osamy-c*. In this assay, different lengths of the promoter region were fused to a reporter gene such as the luciferase cDNA. Several plasmids were constructed and transferred into rice aleurone cells by the biolistic method, and the luciferase activity was measured. We have also used the transient assay system to look for intermediates in the GA-dependent signal-transduction pathway by transactivation experiments. These involve transferring two genes on two separate plasmids together into rice cells and observing how the gene product of the first gene affects the expression of the second. By using this approach, we have shown that a ubiquitin-conjugating enzyme gene and a calcium-dependent ATPase gene are putative intermediates in the GA-dependent signal-transduction pathway because either gene can transactivate the expression of a target gene, *Osamy-c*. Identification of other putative intermediates in the GA-signaling pathway is also presented.

The success of our ability to introduce foreign genes or modified genes into rice opens up new opportunities for producing agronomically useful transgenic rice plants and for allowing detailed analyses of gene expression, regulation, and biological function. This success has been made possible by the following developments: first, improvements in cell culture methodologies and regeneration procedures to produce fertile rice plants from either protoplasts or suspension cells; second, development of efficient methods for gene transfer; third, construction of useful plasmids that include strong or regulatable promoters, suitable reporters and selectable markers; and fourth, identification of agronomically useful genes.

A useful plasmid requires a strong or regulatable promoter. An example of a strong constitutive promoter is the rice actin 1 gene (*Act1*) promoter (McElroy et al 1991). An example of a regulatable promoter is a wound-inducible promoter from the potato protease inhibitor gene; when used in conjunction with the *Act1* intron, it gives high-level, wound-inducible gene expression in rice (Xu et al 1993).

There are a number of reporter genes and selectable marker genes that have been used for plant gene transfer experiments. The most useful assayable marker genes include the bacterial  $\beta$ -glucuronidase gene (*Gus*) and the firefly luciferase gene (*Luc*). Three selectable markers have been widely used in rice transformation: the phosphinothricin acetyl transferase gene (*bar*), the hygromycin phosphotransferase gene (*hpt*), and the neomycin phosphotransferase gene (*npt* or *neo*).

Since genes from any source can be introduced into rice using the established gene transfer technology, there are several genes that are potentially useful for rice genetic engineering (for a review, see Toenniessen 1991). However, to date, only a few genes have been transferred into rice and proven to be beneficial, at least in greenhouse tests. Thus, more genes need to be transferred into rice and tested for their usefulness for rice genetic engineering.

## Production of agronomically useful transgenic rice plants

### Production of insect-resistant transgenic rice plants

The loss of rice yield due to direct insect damage is estimated to cost at least several billion dollars a year (Heinrichs et al 1985). By using conventional plant breeding, several useful disease-resistance genes from wild rice have been introduced into cultivated rice. However, the availability of useful rice germplasm is limited, and the introduction of beneficial genes into elite cultivars is time consuming. Moreover, the control of insect pests in rice cultivation by chemically synthesized insecticides often results in environmental pollution and affects food safety. Genetic engineering offers a safe and effective way to deal with these problems. Currently, there are two major types of useful genes that can be transferred into rice for genetic engineering of insect resistance: the insecticidal crystal protein genes from *Bacillus thuringiensis* (*Bt*) and the plant-derived insecticidal proteinase inhibitor genes.

*Bt crystal protein genes.* Several *Bt* genes encoding different crystal proteins have been isolated from bacterial strains, and two of these genes have been introduced into monocot plants such as maize and rice. For example, Fujimoto et al (1993)

introduced a *cryI(A)b* gene into rice protoplasts and regenerated fertile transgenic plants.

*Proteinase inhibitor genes.* Proteinase inhibitors bind and inhibit serine-type proteinases, including trypsin and chymotrypsin. A cowpea trypsin inhibitor gene (*CpTi*) was cloned and introduced into tobacco plants. The transgenic plants became resistant to tobacco insect pests (Hilder et al 1987). Similarly, a potato protease inhibitor gene (*PINII*) was cloned and introduced into tobacco plants (Johnson et al 1989). The transgenic tobacco plants that produced relatively high levels (1% of soluble leaf proteins) of *CpTi* or *PINII* showed resistance to typical tobacco insect pests.

We have constructed a plasmid including the wound-inducible *pin2* promoter, the first intron of rice *Act1* (to increase the level of expression in rice), the *pin2* coding sequence, and the *bar* gene (for selection) driven by the 35S promoter. This plasmid was introduced into rice suspension cells (two japonica varieties: Tainung 67 and Pi 4) by the biolistic method. Of 37 Southern blot-positive lines, 27 were fertile. Southern blot analysis of the second ( $R_1$ ) and third ( $R_2$ ) generations plants from a number of transgenic lines showed that almost all of the  $R_2$  and  $R_1$  plants from a given  $R_0$  plant had the same hybridization pattern, indicating that the *pin2*-containing transgene is stable in transgenic rice plants (Duan et al 1996).

At the same time, the level of *PINII* protein in  $R_0$ ,  $R_1$ , and  $R_2$  plants was determined by a quantitative spectrophotometric assay. The results showed that the *PINII* level in 15  $R_0$  transgenic plants varies between 0.4 and 2.6% of soluble leaf proteins. Ten  $R_1$  plants from each of two  $R_0$  lines were analyzed in detail. For example, in line No. 12, the *PINII* level in the  $R_0$  plant was 0.6%, but it was 1.2% in 4 of the 14  $R_1$  plants. These  $R_1$  plants are likely to be homozygous, and this has been confirmed by analyzing 14  $R_2$  plants from each  $R_1$  line. In all cases, the *PINII* level in the  $R_2$  plants was close to 1.2% (Duan et al 1996). Our conclusion that these  $R_2$  plants are homozygous is further supported by our herbicide resistance test (using Basta) with  $R_3$  plants. All 300  $R_3$  plants from 19  $R_2$  plants derived from plants No. 12-3 and No. 6-8 were resistant to 0.5% Basta (data not shown). As mentioned in an earlier section, different copies of the transgene segregate as a single-copy gene in each line of the transgenic plant. Thus, if these  $R_2$  lines are not homozygous, we would expect 75 of the 300  $R_3$  plants (25%) to be sensitive to Basta. However, our results showed that none of the 300  $R_3$  plants were herbicide-sensitive.

*Small-scale greenhouse test of transgenic rice plants for insect resistance.* The fifth generation ( $R_4$ ) plants of two primary transgenic lines, No. 6 and No. 12, were used for the preliminary insect resistance test. The progeny of the same generation from transformation procedure-derived nontransformed plants were used as controls. Individual plants were grown in enclosed pots kept in the greenhouse. Second-instar larvae of rice pink stem borer were artificially applied to the rice plants at the early heading stage. In our bioassay, a significant difference was observed between transgenic plants and nontransformed control plants in their response to the infestation by rice pink stem borer and development of the whitehead symptom. The number of shoots (tillers) developing the whitehead symptom in transgenic plants was significantly lower than that of nontransformed plants. More than 70% of the tillers in nontransformed

Tainung 67 control plants showed the whitehead symptom (hollow stem and dead panicles), whereas only 16% of the tillers in transgenic plants (no. 6-7-4-1) showed the whitehead symptom. In addition, there was a significant difference between the larvae applied to the transgenic plants and those applied to the control plants in their growth and development during the 5-wk experimental period. Larvae in the transgenic plants showed little weight increase and stayed at the second- or third-instar stage, while larvae in the control plants showed a 3- to 4-fold weight increase and developed to the fourth- or fifth-instar stage. Similarly, 100% of the tillers in the nontransformed Pi 4 control plants and only 17.5% of the tillers in transgenic plants (no. 12-8-4-2) showed whiteheads.

### **Production of transgenic rice tolerant of drought, salt, and temperature**

Environmental stresses, such as drought, increased soil salinity, and extreme temperature, are major factors that limit plant growth and productivity. Recent progress in plant genetic transformation and the availability of potentially useful genes characterized from different sources make it possible to generate stress-tolerant crops using transgenic approaches (Tarczynski et al 1993, Pilon-Smits et al 1995, Xu et al 1996).

There are two general types of molecules that are potentially useful in conferring stress tolerance to plants. The first type includes low-molecular weight compounds such as mannitol and proline. The introduction of the mannitol 1-phosphate dehydrogenase (*MtDH*) gene into tobacco plants led to an increase in the level of this enzyme and accumulation of mannitol in these plants (Tarczynski et al 1993). Transfer of the delta<sup>1</sup>-pyrroline-5-carboxylase synthase (*P5CS*) gene into tobacco led to an increase in this enzyme level and proline accumulation (Kishor et al 1995). In both cases, the transgenic tobacco plants showed increased tolerance to salt stress compared with nontransformed plants.

The second type includes high-molecular weight compounds such as late embryogenesis abundant (LEA) proteins, which highly accumulate in the embryos at the late stage of seed development (Dure III 1993). The expression of LEA genes in vegetative tissues is induced by various environmental stresses, such as drought, salt, and extreme temperature (Shriver and Mundy 1990). However, clear experimental evidence supporting the exact functions of LEA proteins is still lacking.

A group 3 LEA protein, HVA1, was characterized from barley. Expression of the *HVA1* gene is rapidly induced in seedlings by dehydration, salt, and extreme temperature (Hong et al 1992). In our current study, we have taken a transgenic approach to investigating the probable function of the HVA1 protein in stress protection. A plasmid, pBY520, was constructed, which includes the cDNA of the barley *HVA1* located downstream of the rice Act1 promoter. On the same plasmid, the bar coding region is located downstream of the 35S promoter. This plasmid was introduced into rice suspension cells by the biolistic method. Sixty-three independent lines of transgenic plants were regenerated, and 85% of them were fertile. DNA blot hybridization, using the HVA1 cDNA as a probe, showed that 80% of the lines gave the predicted band pattern (Xu et al 1996). Constitutive, high-level expression of the barley *HVA1* gene

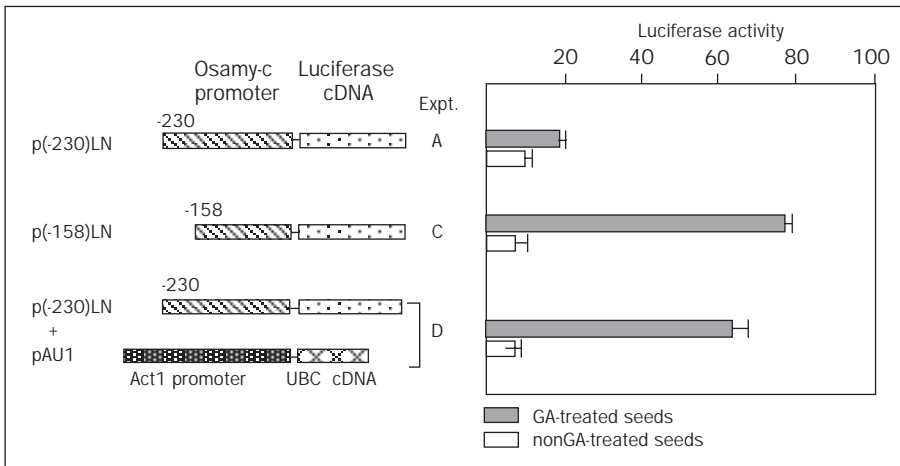
in transgenic rice plants was detected by quantitative immunoblot analysis. We found that accumulation of the barley HVA1 protein in the vegetative tissues of transgenic rice plants conferred increased tolerance to drought and salt stresses; further, the extent of stress tolerance was correlated with the level of HVA1 protein accumulation. Our results not only demonstrate a role of the barley LEA protein in stress protection, they also suggest the potential usefulness of *LEA* genes for genetic engineering of stress tolerance (Xu et al 1996).

### Analysis of gene expression using transient assays

In this section, examples of transient gene expression are presented. In addition, the transient assays used to identify intermediate steps in the gibberellin (GA)-dependent signaling pathway are described.

#### Functional dissection of a rice high-pI $\alpha$ -amylase gene promoter

During germination in cereals, GA is known to induce the transcription of genes that encode hydrolytic enzymes such as  $\alpha$ -amylase, glycosidase, protease, and nuclease. These enzymes mobilize resources that promote the growth of seedlings. We have established a quantitative transient assay based on expression of a luciferase reporter gene in rice aleurone cells, and we have characterized the Osamy-c promoter for GA-dependent regulatory sequences. Using this method, we have shown that the DNA sequence between -158 and -46 is sufficient to confer GA-responsive activation in Osamy-c (see Fig. 1, experiment C). In addition, this sequence is capable of directing GA-dependent expression from a heterologous minimal promoter (data not shown; Tanida et al 1994).



**Fig. 1.** Transient assay for testing the function of the rice UBC cDNA in regulating  $\alpha$ -amylase gene expression.

## Transient assays to identify intermediate steps in the GA-dependent signaling pathway

Until now, studies in different laboratories on GA-induced genes in aleurone cells of various cereals have focused on target genes, such as  $\alpha$ -amylase genes (for a review, see Hooley 1994). However, the identity of the intermediate steps of the GA-dependent signaling pathway in aleurone cells remains unknown. In this section, examples will be presented to show how transient assays contributed to the discovery of two intermediate steps in the GA-dependent signaling pathway.

*A ubiquitin-conjugating enzyme gene as an intermediate in the GA-dependent signaling pathway.* A ubiquitin-conjugating enzyme (*UBC*) gene, induced by GA within 1 hr, was identified in rice (*Oryza sativa*) seeds by the mRNA differential display technique (Liang et al 1993). A full-length *UBC* cDNA clone and a genomic clone have been isolated and sequenced. The deduced amino acid sequence shares significant identity with several known *UBC* sequences, which are probably involved in the pathway responsible for degrading short-lived regulatory proteins. In vivo transient assays using the *UBC* gene promoter, joined to the luciferase cDNA as the reporter gene, showed that the sequence located between positions 231 and 159 upstream of the transcription start site of this promoter is crucial for GA-dependent activation of luciferase cDNA. Finally, transactivation experiments indicated that this *UBC* gene is involved in GA-stimulated  $\alpha$ -amylase gene expression (Chen et al 1995).

*Presence of a repressorlike protein that represses the activity of Osamy-c promoter.* For testing GA response, the plasmid p(-230)LN was used. This plasmid contains a 230-bp sequence from the 5' noncoding region of *Osamy-c* fused to the coding region of the firefly luciferase gene, followed by the Nos 3' terminator sequence (Tanida et al 1994). When plasmid p(-230)LN was introduced into aleurone cells of deembryoed rice half seeds, the luciferase activity was low, and there was only a twofold GA stimulation (Fig. 1, experiment A). The interpretation of this result is that a repressor-like protein binds to the region between positions -230 and -158 of the *Osamy-c* promoter (Kim et al 1992) and strongly represses the promoter activity. This conclusion is consistent with the finding that, in experiment C, deleting the sequence between -230 and -158 resulted in a large increase in the luciferase activity.

*UBC's role in regulating the expression of Osamy-c.* Transactivation experiments were carried out by introducing two plasmids into rice aleurone cells (Fig. 1, experiment D), followed by the same transient assay described above. One of the plasmids, pAU1, contains the actin 1 gene (*Act1*) promoter and the rice *UBC* cDNA; the other one, p(-230)LN, includes the promoter region (between positions -230 and +29) of the *Osamy-c* gene and the coding region of the luciferase gene. By comparing the results shown for the GA-treated half seeds in experiments A and D (Fig. 1), the luciferase activity in experiment D is three times higher than that in experiment A. As a result, there is a sevenfold GA effect in experiment D, instead of the twofold GA effect in experiment A. This result can be interpreted to indicate that the protein product of the rice *UBC* gene (*pAU1*) directly or indirectly degraded the repressorlike protein that binds to the *Osamy-c* promoter region.

*A calcium-dependent ATPase gene as an intermediate in the GA-dependent signaling pathway.* By using a differential display approach, a GA-responsive  $\text{Ca}^{2+}$ -ATPase gene was identified. A genomic clone and a cDNA were isolated and sequenced. To investigate the possible role of this early GA-responsive endoplasmic reticulum (ER)  $\text{Ca}^{2+}$ -ATPase gene in the rice aleurone cell during GA induction, the coding sequence of this gene was fused transcriptionally with the rice Act1 promoter. The resulting plasmid was introduced into rice aleurone cells from nonGA-treated deembryoed half seeds by the biolistic method. mRNAs were isolated 16 or 24 h later and converted to cDNA, which was amplified by polymerase chain reaction (PCR). cDNAs corresponding to Osamy-c mRNA were detected in the lanes (designated 16 and 24) corresponding to mRNAs isolated at 16 and 24 h after introducing the  $\text{Ca}^{2+}$ -ATPase cDNA fusion plasmid into the nonGA-treated samples (Fig. 2). However, no Osamy-c cDNA was found in the control (lane 1, a plasmid consists of Act1 promoter/bar cDNA, was delivered into aleurone cells; 24 h later, mRNAs were isolated, converted to cDNA, and amplified by PCR). The results suggest that rice ER  $\text{Ca}^{2+}$ -ATPase was able to trigger the synthesis of Osamy-c mRNA from the endogenous gene. It appears that the overproduced ER  $\text{Ca}^{2+}$ -ATPase bypassed the GA requirement and induced the transcription of Osamy-c. Thus, the ER  $\text{Ca}^{2+}$ -ATPase gene plays an important role in the GA-dependent signal-transduction pathway in rice aleurone cells.

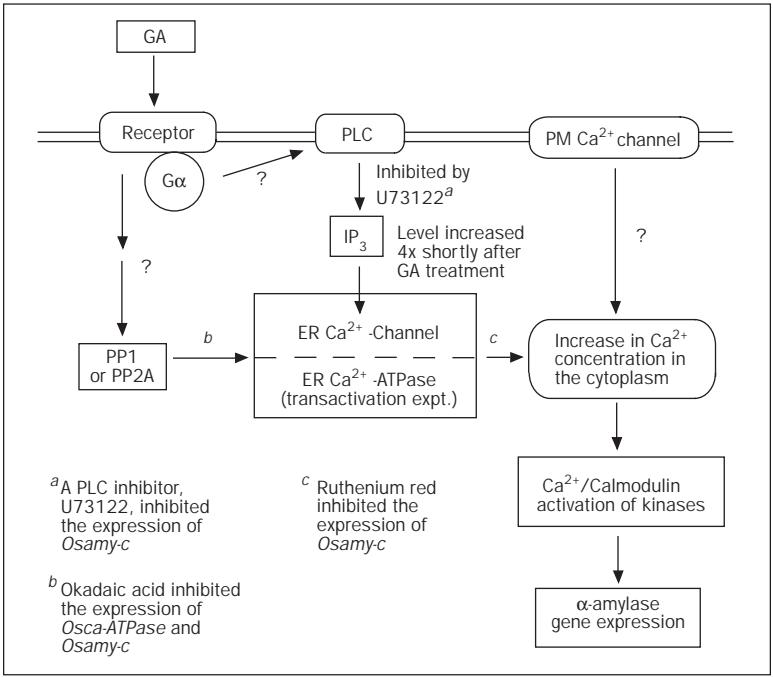
*The relation of  $\text{Ca}^{2+}$  release from ER and the increase of the inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) level in the aleurone cell.* We have provided preliminary evidence to show that  $\text{Ca}^{2+}$  release from ER is critical for the transcription of Osamy-c; moreover, ER  $\text{Ca}^{2+}$ -ATPase is induced by GA and is involved in the GA-dependent signal-transduction pathway. Therefore, it suggests that the regulation of  $\text{Ca}^{2+}$  transport into



**Fig. 2.** A transient assay showed that overexpression of rice ER  $\text{Ca}^{2+}$ -ATPase cDNA in rice aleurone cells can bypass the GA requirement in the synthesis of Osamy-c mRNA. *Oscyto-c*, the expression of the rice cytochrome c gene (a nonGA-responsive gene), as monitored by reverse transcriptase-based PCR, was used as an internal standard for comparing the band density.

the ER is directly linked to the downstream steps of this pathway, which lead to the activation of target genes, such as *Osamy-c*. According to information on other systems,  $\text{Ca}^{2+}$  release from the ER membrane is initiated by inositol 1,4,5-triphosphate ( $\text{IP}_3$ ). After it is produced,  $\text{IP}_3$  can bind to receptors on the ER membrane in just minutes to release  $\text{Ca}^{2+}$  into the cytoplasm (Miyazaki 1995). We examined the possible role of  $\text{IP}_3$  in the rice aleurone cells by measuring the amount of  $\text{IP}_3$  after GA treatment of rice half seeds in a receptor-binding assay. Our results showed that the level of  $\text{IP}_3$  increased more than four times in the seeds after 20-25 min of GA treatment compared with that of nonGA-treated samples. After 40 min, the  $\text{IP}_3$  level dropped back down to near the original level. Therefore, it appears that the increase in  $\text{IP}_3$  was an immediate response to the GA signal.

*Examining the role of the PLC enzyme in the GA-dependent signal-transduction pathway.* The experiment described above showed that the accumulation of  $\text{IP}_3$  was transiently induced by GA in 25 min. Since  $\text{IP}_3$  is produced by the action of the phospholipase C (PLC) enzyme, which is located on the plasma membrane, the possible involvement of the PLC enzyme in the GA-dependent signal-transduction pathway also needs to be examined. A PLC antagonist, U73122 (Powis et al 1992), was used to block the activity of PLC, and the expression level of *Osamy-c* was measured by



**Fig. 3.** A proposed model of the GA-dependent signal-transduction pathway in rice aleurone cells. G $\alpha$  = alpha subunit of G protein; PLC = phospholipase C; IP $_3$  = inositol 1,4,5-triphosphate; PM = plasma membrane; ER = endoplasmic reticulum.

reverse transcriptase-PCR. Our results showed that 4  $\mu\text{M}$  U73122 completely abolished the synthesis of *Osamy-c* mRNA. However, an inactive derivative of U73122, U73344, did not affect the transcription of *Osamy-c*.

*Probable role of protein phosphatase 1 and 2A (PP1 and PP2A) in GA-dependent expression of two GA-induced genes.* Okadaic acid (OKA) is a known inhibitor of PP1 and PP2A in many systems. We found that OKA inhibited GA-dependent germination of rice seeds. In addition, the GA-dependent expression of *Osamy-c* and *OscA-ATPase* in embryo-less half seeds was inhibited by 1  $\mu\text{M}$  OKA. A control experiment showed that OKA did not give a general inhibition of mRNA synthesis in rice seeds. Thus, we conclude that PP1 and/or PP2A are likely to be involved in the GA-dependent signal-transduction pathway in rice aleurone cells (B. Wang, M. Chang, X. Chen, and R. Wu, manuscript in preparation).

The preliminary results described in the above sections and other data (not shown), have led us to propose a plausible model of a GA-dependent signal-transduction pathway in rice aleurone cells based on analogy to animal systems. In our proposed model (Fig. 3), the putative GA-activated receptor (Hooley 1994) triggers the activation of the PLC enzyme on the plasma to produce  $\text{IP}_3$  (Verhey and Lomax 1993).  $\text{IP}_3$  then diffuses to the ER membrane and increases the local concentration of  $\text{Ca}^{2+}$  in the cytoplasm (the  $\text{Ca}^{2+}$  level is coregulated by the ER  $\text{Ca}^{2+}$ -ATPase). Next,  $\text{Ca}^{2+}$ -binding proteins (calmodulin or calmodulinlike proteins) bind  $\text{Ca}^{2+}$  and activate one or several protein kinases (based on information from other well-known systems). Then, presumably a protein kinase phosphorylates a transcription factor which leads to the activation of  $\alpha$ -amylase gene expression.

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## Notes

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*Citation:* [IRRI] International Rice Research Institute. 1996. Rice genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct 1995. Manila (Philippines): IRRI.